Systematics of the genus *Candida*; implications for understanding clinical presentation, mixed infection and antifungal treatment and the influence on strain maintenance and replacement during oral candidiasis in HIV-infected individuals

A thesis presented for the degree of

Doctor of Philosophy

by

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at

Adelaide University

South Australia

8th July, 2002

Declaration

I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any University, and to the best of my knowledge contains no material previously published by another person, except where due reference is made in the text.

This research report may be made available for photocopying and loan.

Michelle Fraser

Acknowledgements

I would like to thank the following;

My supervisors Ross Andrews and Tony Rogers for their encouragement, support and faith.

David Ellis and Tania Pfeiffer for their advice and technical assistance. Thanks David for your input into the development of this research project and your help in understanding fungal taxonomy.

David Wilson, Liz Coates, Michael Stubbs and Richard Logan for collecting the many oral samples used in this research and allowing me access to patient data.

The Dental School, Adelaide University, especially Viv Burgess, Michael Koorndyk, Mandy Keough, Elaine Formenti and Hien Ngo.

Dedications

I dedicate this thesis to my family and friends who have supported me for the duration of this research and who always believed in me. I especially thank Adam, Mum, Dad, Rodney and Beverley for understanding.

Abstract

Oropharyngeal candidiasis is an opportunistic infection associated with immunocompromisation. Despite recent reports of a decline in the prevalence of this oral infection with HAART (highly-active antiretroviral therapy) treatment of HIV infection, it continues to be a significant cause of morbidity in this patient group. There have been numerous studies investigating the epidemiology of *Candida* infection and the taxonomic structure of the genus. This study assesses the systematics (ie., taxonomy, phylogeny and epidemiology) of thirteen *Candida* species of medical importance using type and reference strains plus isolates obtained from 101 HIV-positive individuals and 20 HIV-negative asymptomatic carriers. The techniques used were allozyme electrophoresis at 15-20 independent enzyme loci and sequence comparisons within the 16S, ITS1, ITS2 and 5.8S rRNA regions. The results of this study have confirmed the existence of a number of distinct species but, as previous studies have also reported, questions the validity of the genus *Candida*. This conclusion is made on the basis of the extent of genetic variation observed between *Candida* species and the close relationships between some *Candida* species and species of other fungal genera. Of epidemiological significance, the study supports the theory that oropharyngeal candidiasis is opportunistic with isolates from HIV-positive patients being genetically interspersed with isolates from asymptomatic carriers. Additionally, the strain infecting a patient changes as frequently as weekly, many patients are co-colonised by multiple strains and different strains can be isolated from multiple concurrent lesions. Whilst the results of this study have not resolved many of the epidemiological and taxonomic issues still under debate, they have provided a comprehensive framework that can be used to build our understanding of the complex interactions between strains of *Candida*, antifungal therapy, HIV treatment, the immune response and the oral cavity.

1 INTRODUCTION

Since their description in the five-kingdom system of Whittaker (1969), the taxonomy and phylogenetics of the kingdom *Fungi* have not been unequivocally resolved. The Fungi comprise a broad range of morphological and biochemical variants which has led to a taxonomy where organisms are excluded, rather than included, from taxa. This has made *Fungi* one of the most phenotypically and genetically diverse groups of organisms on this planet. Despite the confusion, their prevalence in infection make *Fungi* the focus of many epidemiological investigations. These investigations provide a foundation for research of the systematics of *Fungi*. As Whitehead (1990) stated "no biological study is worth the paper it is written on unless informed, if not by systematics, then by its subdiscipline taxonomy, while systematics as a whole provides the unifying structure around which all biological exploration must be built.".

Systematics comprises three subdisciplines: **phylogenetics**, or ancestral relationships between taxa; **taxonomy**, or organization of organisms into discrete groups (taxa i.e., Kingdoms to subspecies); and **nomenclature**, or designation of names to the taxa described. The accuracy of a systematic framework is dependent on the organisms included in the investigation. Each taxon should be represented by a number of organisms and the taxa should be representative of the taxonomic range under investigation. This requires the inclusion of representative isolates from closely related taxa as well as outgroups to define the outer taxonomic limits of the organisms under investigation such as from the next most closely related species or genus.

The vast array of organisms within the Kingdom *Fungi* provides an ideal opportunity to construct accurate models on the evolution of higher life forms. Fungi are easier and faster to cultivate than plants and animals, they can be genetically manipulated and, like bacteria, they are relatively simple organisms in which the effects of environment and genetic manipulation on biological characteristics can be determined. Theories on the mechanisms of evolution and gene and protein function can then be applied to the other more complex eukaryotic Kingdoms.

For the purpose of this thesis, the introductory Chapter includes background on the current taxonomy of fungi with particular reference to the genus *Candida*, the most prevalent fungal species in human infection. The genus is described in the context of

biochemical, morphological and genetic variation between and within the currently described medically relevant species, their clinical importance, risk factors for infection, prevalence, and antifungal susceptibilities. Particular attention is paid to the species *C. albicans*, the cause of up to 80% of clinical infections and, consequently, the focus of the majority of *Candida* research.

1.1 The Kingdom Fungi - taxonomy and phylogeny

Genetic evidence, predominantly sequence comparisons of the genomic small subunit ribosomal RNA gene, has suggested that the Kingdom *Fungi* were derived approximately 965 million years ago (Doolittle *et al.* 1996). This occurred soon after animals and plants diverged, with fungi forming a distinct lineage within the eukaryota, which is more closely related to animals than plants (Kuma *et al.* 1995, Doolittle *et al.* 1996) (Figure 1.1 p., 3). However, this branching pattern for the three eukaryotic Kingdoms is not supported by analysis of sequence data using the gene for RNA polymerase II. These results suggest that plants and animals are more closely related to each other and that fungi diverged earlier than either of them (Sidow and Thomas 1994). Loomis and Smith (1987) pointed out that, when comparing sequences with wide variations in the G+C content of DNA, the accuracy of phylogenetic reconstruction is compromised. They suggested that protein trees based on amino acid sequences, which are less variable, provide a more accurate relationship. According to such a tree based on the amino acid sequence of elongation factor-1-alpha, the closer genetic proximity of *Fungi* and *Animalia* is evident (see Hasegawa *et al.* 1993).

The conflicting branching patterns of eukaryotes have not yet been unequivocally resolved, a taxonomic quandary also reflected in lower hierarchical taxa of these Kingdoms. The ability to accurately identify organisms has been particularly problematic for microparasites, which comprise bacteria, viruses, fungi and protozoa (see Anderson and May 1979; Anderson and May 1991). Taxonomists have previously relied on a few morphological and biochemical characters in these organisms for their identification. This is not such an issue for multicellular, more complex organisms with a range of overt identifying characteristics. The approach becomes problematic when dealing with more simple, sometimes unicellular, organisms with limited morphological and biochemical variability,which are usually subjectively interpreted. These conflicts are more pronounced in the Kingdom *Fungi*, where the taxa within it have been defined on the basis

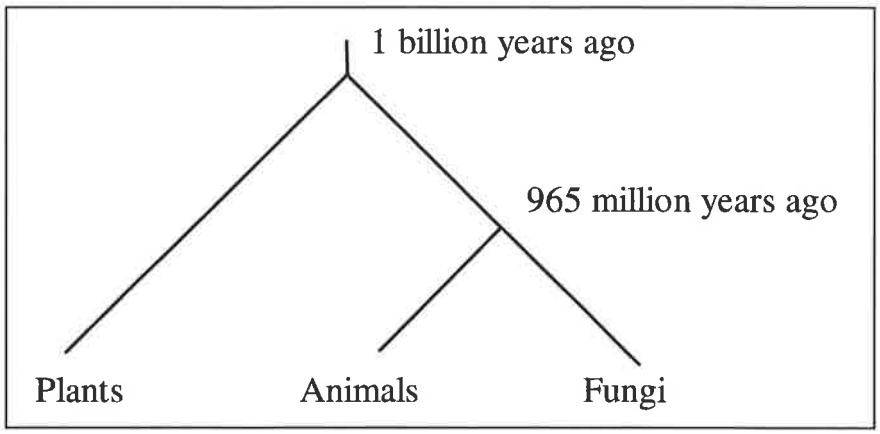


Figure 1.1; Times of divergence of the major eukaryotic lineages estimated using a protein clock (after Doolittle *et al.* 1996)

of overlapping and ambiguous morphological and biochemical characters. These characters, such as appearance, ability to mate, and habitat, are more obvious and discriminatory in animals and plants, which has allowed them to be grouped more accurately in most cases. The discriminatory power of these morphological and biochemical characters is reflected in the high degree of genetic correlation provided for taxonomic divisions within these latter Kingdoms since the introduction of molecular biological techniques.

1.1.1 Definitions

Fungi are morphologically defined as eukaryotic cells that lack chlorophyll and can exist in a unicellular or multicellular form. Moulds are filamentous, whereas yeasts are unicellular. Moulds and yeasts are separated morphologically, but not taxonomically (i.e., both moulds and yeasts can be found within the same taxonomic group). Moulds and yeasts can exist in sexual (sporulating, meiosis) and asexual (conidia or vegetative cells, mitosis) forms. Spore formation is the traditional basis for taxonomic division, with fungi being named according to their morphological appearance and classified into the corresponding phyla *Basidiomycota*, *Ascomycota*, *Chytridiomycota* and *Zygomycota*. Although not all of the corresponding states for each species have been identified, it is assumed that for every asexual state (anamorph) there is also a sexual state (teleomorph). The two states of an organism comprise the holomorph.

Fungi in the teleomorphic state may be heterothallic, that is, existing in more than one mating type where two different types, usually + and -, need to be present for sexual reproduction; or homothallic, where only one mating type exists. Teleomorphs are usually only generated in the laboratory through the mating of compatible strains, which can be influenced by changes in environmental conditions such as nutrient availability, temperature and pH.

Molecular biology has led to the development of new tools to investigate the genetic relationship between morphologically diverse fungi, which has provided evidence of associations between anamorphs and teleomorphs. There have, however, been cases where more than one anamorph has been associated with a single teleomorph, for example *Pseudallescheria boydii* with *Scedosporium* and *Graphium*, where the two anamorphs are called synamorphs. The reverse has also been noted where two teleomorphs are associated with a single anamorph. Although the single anamorph or teleomorph, currently described

in these situations appears succinct according to traditional phenotypic identification characters, it may eventually be divided into two genetically distinct species with each being associated with a different synamorph. This relationship can more accurately be resolved using a combination of morphological, biochemical and genetic characters.

1.1.2 The importance of accurately identifying organisms

A fundamental issue that underpins our understanding of the biology of organisms is our ability to accurately and unequivocally identify and characterize them. The information used to define one organism allows the subsequent identification of others. The name given to an organism brings with it a range of biological information relating to its prevalence, survival, clinical importance and interactions with other organisms. For example, the isolation and diagnosis of a bacterial species from a clinical infection brings with it information on its pathogenicity, potential source of infection, the anticipated infection outcome, appropriate treatment regimes and transmission of the organism. "The accuracy of this deduced information depends on the accuracy of identification, species homogeneity and accuracy in attributing these characters to species; its scope depends upon accumulated case reports of infections caused by the organism." (Magee 1993). Inaccuracy in the systematics of organisms can lead to improperly defined taxa and extreme variation in their defining characters due to the inappropriate grouping of unrelated organisms.

The introduction of molecular biology techniques has provided the opportunity to compare an immense new range of characters with variable diversity and universal applicability. Sequences of some genes are highly conserved and have been implemented for genus and species level investigations, such as the ribosomal RNA genes (Bruns *et al.* 1992). The small subunit ribosomal RNA gene (16S rRNA in prokaryotic cells, 18S rRNA in eukaryotic cells) has become a target for the design of species-diagnostic kits for some microorganisms. Other sequences are quite diverse at the individual level (eg., internal repeat sequences, microsatellites) and have been suggested as particularly useful in determining the epidemiology of infection (eg. Metzgar *et al.* 1998). Problems in using molecular characters for systematics arise when different genes or genetic methods that supposedly resolve genetic relationships at the same taxonomic level yield conflicting results. Specific examples exist for Baobab trees (Baum *et al.* 1998) *Salmonella* (see Christensen and Olsen 1998), garter snakes (Dequeiroz and Lawson 1994), red algae (Liaud *et al.* 1994), Hawaiian crickets (Shaw 1996), bacteria (Huang and Ito 1998) and amniotes (Huelsenbeck and Bull 1996). Kim and Jansen (1994) found that using a combination of the

genes and spacer regions of the nuclear ribosomal RNA region (18S to 25S) of a diverse range of plants provided a phylogenetic relationship that had the least conflict with the phylogenies obtained using each individual DNA segment. This suggests that phylogenies may be more accurately constructed using a number of different sequences with varying rates of evolution, not just a single gene.

It is also being increasingly noted that the results conflict with the currently accepted taxonomic structure based on traditional characters (such as platyhelminths (Balavoine 1997), baobab trees (Baum *et al.* 1998), *Drosophila willistoni* (Gleason *et al.* 1998), hominids (Ruvolo 1994) *Aspergillus* (Geiser *et al.* 1998), frogs (Haas 1997). There are a number of possible reasons for a lack of congruency between phenotypically and genotypically defined taxa (genera and species). Phenotypic characters can be controlled by a different number of genes, from one to a cascade; the genes are not under the same evolutionary constraints as the characters they encode. Redundancy in the genetic code allows variation at the gene level without affecting protein function. The phenotypic characters used may not really be comparable. For example, colony colour may be controlled differently in genetically unrelated organisms but result in the same appearance. Biases may be placed upon more interesting or obvious characters when their phylogenetic and taxonomic utility may not actually be as important (for further discussion see Stackebrandt (1988).

Conversely, conflicting genetic relationships may have been obtained using genes not suitable for systematic comparisons. The sequences may evolve too quickly or too slowly, or there may be segments within the sequence that evolve at different rates. Genetic codes may match by chance and not through common ancestry. Bases may change back to what they were previously (reversion) giving the impression that organisms are more closely related than they actually are. The assumptions underlying specific statistical methods used for sequence comparisons may be invalid for specific data sets.

Inaccuracy in the systematics of organisms may also be attributed to the inappropriate selection of organisms chosen to represent a particular taxon. If an organism has been previously misidentified or has become contaminated with another organism it will severely compromise the accuracy of the results.

Prior to debating the utility of one character versus another, the degree of variation that exists in these characters between and within all of the relevant taxa needs to be determined. This approach relies on an extensive evaluation of the genetic, biochemical and morphological diversity within the taxonomic group of interest and those taxa closely related to it. Inclusion of the next most closely related taxon provides an indication of the outer limit of genetic, biochemical and morphological variation for the taxon of interest (i.e., species, genus, etc.). An accurate systematic framework can be constructed by using the information provided in this manner together with a range of contemporary and traditional techniques. Few such studies have been conducted. For example, Baker *et al.* (1998) found that both morphological and molecular characters were valuable in determining phylogenetic relationships for a range of organisms, with morphological data providing equal or greater support for the phylogenetic tree derived using both sets of characters and that the morphological characters were more consistent. In the case of salamanders, the combined data (morphological and DNA) act synergistically so that good support was obtained for nearly all of the nodes in the resultant phylogenetic tree (Jackman *et al.* 1997). Additionally, concordance between DNA and morphology-based phylogenies for finfoot birds has been provided upon re-examination of the molecular data and incorporating all relevant taxa (Houde 1994).

With the high prevalence and clinical importance of fungi in human infection, there has been a resurgence of interest in determining a more accurate systematic framework. Fungal systematics is particularly riddled with conflict. These investigations have given rise to two schools of taxonomic and epidemiological investigation; one based on the traditionally defined current species and genera, the other based on the genetically defined revised taxa. These conflicts range from populations up to phyla of fungi and so far have mainly focused on the small portion of fungi that cause human disease.

1.1.3 Fungal phyla

The Kingdom *Fungi* is comprised of four loosely-defined phyla; *Ascomycota* (sac fungi, moulds, yeasts eg., *Aspergillus, Candida*), *Basidiomycota* (club fungi, mushrooms, rusts, smuts, jelly fungi eg., *Cryptococcus*), *Chytridiomycota* (water moulds eg., *Allomyces, Saprolegnia*), and *Zygomycota* (bread moulds eg., *Rhizopus, Mucor*). There are also a number of unclassified fungi. Evolutionary relationships (i.e., phylogenetics) of the four phyla based on 18S rRNA gene sequence comparisons suggest that the water moulds are

basal to the other lineages and that basidiomycetes and ascomycetes are sister taxa within *Fungi* (Figure 1.2 p., 9).

With the continued improvement of molecular identification methods, fungal taxonomy remains in a constant state of revision. The extreme genetic, morphological and biochemical variation within the Kingdom *Fungi* makes grouping these organisms into related taxa difficult and has resulted in the description of numerous indistinct taxonomic classes with overlapping defining characters. The consequence of this is that, to date, no single defining character provides unequivocal identification of fungi and therefore, they should not be used in isolation. Additionally, taxonomic divisions below the phylum level are confused, often having been formulated using characters that vary in different environmental conditions. There is also a lack of agreement between the derived evolutionary history (phylogeny) of fungi using traditional morphological and biochemical characters and that obtained using contemporary molecular methods (discussed below in Section 1.2 p., 10). Importantly, this is not a phenomenon limited to fungi (Section 1.1.2 p., 5).

1.1.4 Ascomycota

Members of the Kingdom *Fungi* are ubiquitous in the environment. Most human infection is caused by species within the phyla *Ascomycota*, particularly species of *Candida*, and to a lesser extent *Basidiomycota* (eg., *Cryptococcus neoformans*) and *Zygomycota*. The range of environments from which members of these phyla have been isolated, for example from rivers (Slavikova and Vadkertiova 1997), soil (Vadkertiova and Slavikova 1994), air (Aidoo *et al.* 1995), and poultry carcasses (Viljoen *et al.* 1998), is a reflection of the genetic and biological diversity of the fungi contained within them.

The phylum *Ascomycota* is made up of six morphologically and biochemically distinct classes with overlapping genetic relationships: Archiascomycetes (fission yeast, *Pneumocystis, Taphrina*, etc.), Euascomycetes (black yeasts, powdery mildews, morel, truffle, *Penicillium, Neurospora*, etc.), Hemiascomycetes (Orders Saccharomycetales (budding yeasts), unclassified Hemiascomycetes) mitosporic Ascomycota, unclassified Ascomycota, and unidentified Ascomycota. Members of the phylum *Ascomycota* have been characterized according to traditional characters. Molecular analyses, however, have since indicated that species in different classes of this phylum are genetically identical

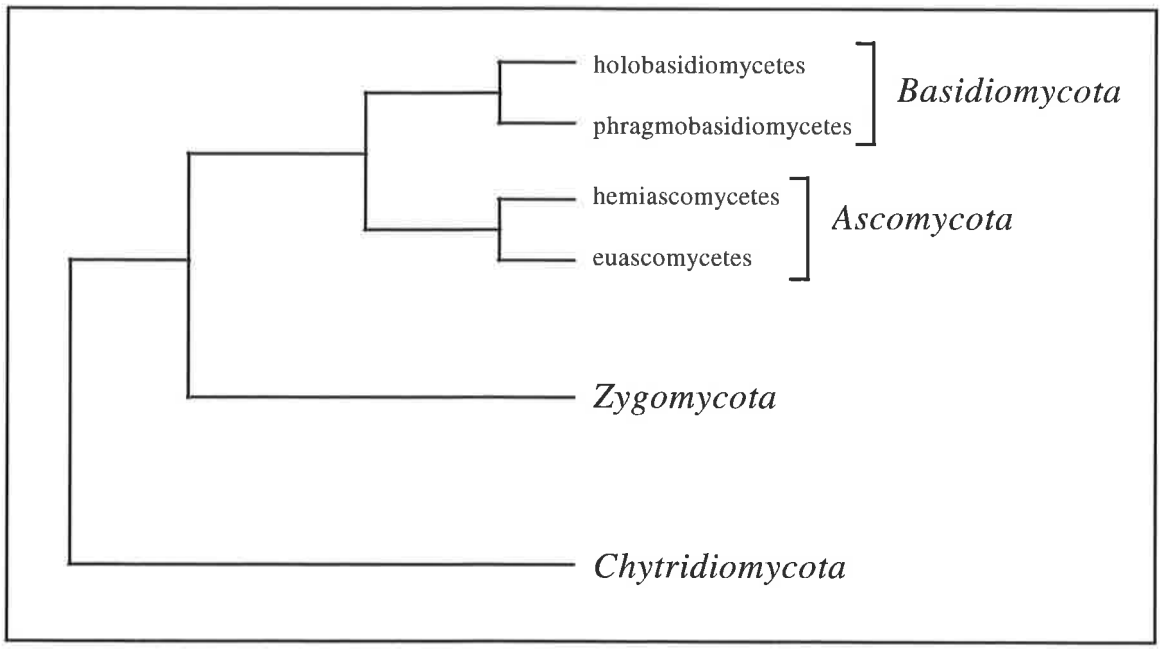


Figure 1.2; The evolutionary origin of phyla and orders within the Kingdom *Fungi*, based on small subunit ribosomal RNA (18S) gene sequence comparisons (after Bruns *et al.* 1992)

morphological variants of each other. These morphological variants comprise anamorphs (asexually reproducing state) and teleomorphs (sexually reproducing state). This has led to the description of new phylogenetic relationships that divide or combine current genera previously differentiated by their morphological and biochemical characters and the description of holomorphic species. Analyses of the sequences of the small subunit ribosomal RNA (18S rRNA) gene has confirmed the genetic relatedness of the *Saccharomyces sensu stricto* complex and retained it in a single genus, but has separated all other *Saccharomyces* species and interspersed them within other ascomycete genera (James *et al.* 1997). Cai *et al.* (1996) have also suggested changes in the phylogenetic relationships within and across traditional taxonomic phyla. The newly identified network of genetic proximity spans all taxa within the phylum *Ascomycota*. The profound effect of this taxonomic redefinition is further highlighted by the recent discovery of new species, detection of the misidentification of isolates belonging to existing species and the description of genetically atypical isolates within species (eg., Anthony *et al.* 1995; Boerlin *et al.* 1995; McCullough *et al.* 1995).

It has been suggested that pathogenicity in fungi has arisen independently on a number of occasions from the extent of morphological and genetic variation of fungi that cause human infection and the range of clinical symptoms they elicit (Bowman *et al.* 1992). Despite the range of yeast species capable of causing human infection, the most predominant species isolated belong to the genus *Candida*, within the phylum *Ascomycetes*.

In a worldwide context, 80% of yeast infections are caused by a single species, *C. albicans* (see Section 1.2.3 p., 14 & Section 1.3 p., 28). It is for this reason that substantial biomedical and basic biological research has focused on this genus. Despite this concerted research effort, the ability to accurately diagnose (ie., identify the causative agent) and treat fungal infections remains confounded by the taxonomic confusion and biomedical controversies, as discussed above.

1.2 The genus Candida (Berkhout)

As mentioned previously (in Section 1.1.2 p., 5), the most important piece of information that can be obtained from an infection is the identity of the causative organism/s. Once this has been determined, clinical outcome can be predicted and an appropriate treatment regime

can be suggested. This becomes extremely difficult when there are no accurate and unequivocal methods by which to identify an organism.

In the case of *Candida*, there are currently two hundred and eleven species (one-hundred and ninety-six species in 1984 (listed in Kreger van Rij 1984) plus 20 others recently renamed or newly described) (http://www.ncbi.nlm.nih.gov/Taxonomy/tax.html last accessed 8/01/02). They were originally grouped together into a single genus because of a lack of taxonomic characters used to identify species of other genera. The genus is closely related to the *Saccharomyces sensu stricto* complex based on cellular characteristics. The species *Saccharomyces cerevisiae* is further described in the materials and methods Section 2.1.2.a (p., 50). Isolates from species within the genus *Candida* are the causative agents of approximately 80% of opportunistic human yeast infections. These infections occur at a variety of external and internal sites, particularly in immunocompromised individuals.

Progress in obtaining an accurate systematics for the genus *Candida* has led to numerous changes in the taxonomy and diagnostic characters commonly used for species identification. However, there are a number of traditional characters for genus and species identification commonly used in a diagnostic context. These morphological and biochemical characters are described below and were derived from the Mycology Online Website, Mycology Unit, Women's and Children's Hospital, Adelaide, SA;

http://www.mycology.adelaide.edu.au/ (last accessed 8/01/02)

1.2.1 Morphology and biochemistry of the genus

Candida cells usually exist in the blastoconidia form (ovoid shape) and reproduce by multilateral budding (i.e., from any position along the cell surface) (Figure 1.3 p., 12). Pseudohyphae (i.e., elongated cells) may be present, rudimentary or absent, some species have true mycelia (i.e., branched cells) and the cells may have swollen terminal vesicles resembling chlamydospores (Odds 1995).

Species of yeast are excluded, rather than included, from the genus *Candida* on the basis of lacking characters that define other ascomycete genera such as the presence of arthroconidia and a pink, orange or red cell pigment. The genus *Candida* can be arbitrarily divided into ten groups based on physiological properties (Kreger van Rij 1984). Due to variability in characters used to define each of these groups, several species may be assigned

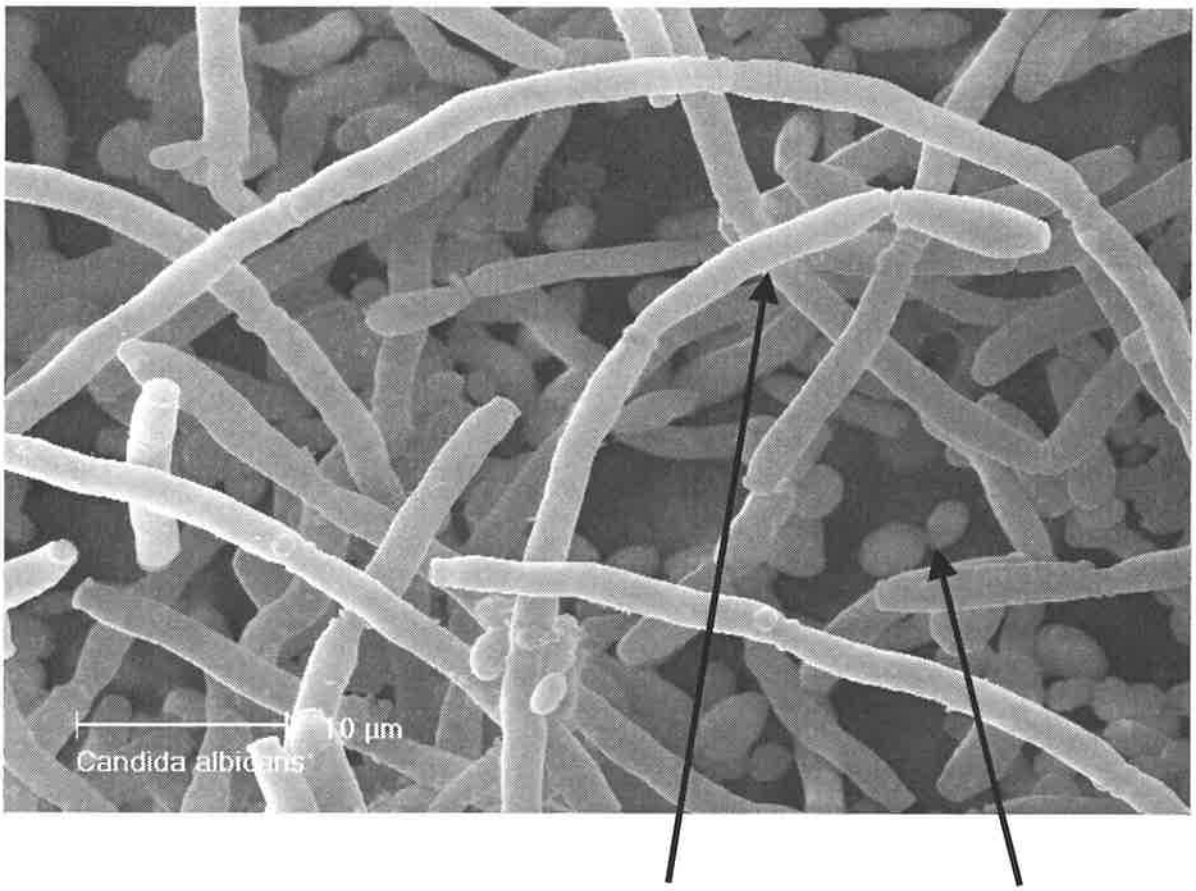


Figure 1.3; Scanning electron microscope image of *C. albicans* CBS 562 grown on Sabouraud's agar (image obtained for this study)

to more than one group. Additionally, the same species may be defined at a number of points in the identification key, making the use of every character in this key vital for the accurate identification of yeasts (Kreger van Rij 1984). None of the groups is mutually exclusive or formed of natural clusters of species (i.e., from the same source, with obvious biological similarities). This implies that the taxonomic structure does not reflect the evolutionary history of the genus.

Genetic analyses have demonstrated that the anamorphs (asexual state) of many *Candida* species are genetically identical to the teleomorph (sexual state) of a number of diverse genera from the phyla *Ascomycota* (*Metshnikowia* to *Citeromyces*) and *Basidiomycota* (*Leucosporidium, Filobasidium*). The anamorph and teleomorph names are interchangeable but, by convention, if the isolate being described is not in the sexual state (i.e., with ascospores or basidiospores), the anamorph name is used. The diversity of the teleomorphic species to which *Candida* species are genetically related is an indication of the genetic diversity within this poorly defined genus.

1.2.2 Genetic characteristics of the genus

The morphological and biochemical variation within the genus *Candida* is also evident in the genetic variation. The genomic DNA base composition within the genus *Candida* ranges from 30 to 66 mol% G+C and the coenzyme Q systems span Q6 to Q10. This is of particular relevance when considering that consistency in these characters is ordinarily used to differentiate bacterial and other fungal genera. Stackebrandt (1988) suggested that this extreme variability is a reflection of the profound age of the genus *Candida* when compared to bacterial genera, which has allowed a longer time for accumulation of mutations. Conversely, Graeser *et al.* (1996) suggest that the variation has arisen from the ability of *Candida* species to exist both as asexual clonal organisms and to recombine in a sexual state. Many authors continue to argue the existence of sexual recombination in the *Candida* life cycle (eg., Tibayrenc 1997, Vilgalys *et al.* 1997, Arnavielhe *et al.* 2000, Tzung *et al.* 2001).

The genetic code of yeasts in the genus *Candida* differs from that of all other organisms in that the codon CUG, which usually encodes leucine, encodes serine (Jukes and Osawa 1996). The reason for this alteration in the genetic code is unclear, but the authors suggest that this is further evidence of the extreme age of the genus.

Investigations of the phylogenetic relationships within the genus *Candida* and related genera, based on small subunit ribosomal RNA sequences, have resulted in conflicting taxonomic data for many of the medically-relevant species and the suggestion that the current taxonomy is incorrect (Barns *et al.* 1991, Hendriks *et al.* 1991). Despite both these studies being based on comparisons of the 18S rRNA gene sequence, the phylogenetic relationships obtained in each differ, probably because of their different selection of isolates and representative species. Nevertheless, the genus *Candida* is not succinct and appears interspersed with other species from different genera. Comparisons of this single gene have supported the taxonomic redefinition of teleomorphic and anamorphic states into holomorphic species due to their genetic identity (eg., Barns *et al.* 1991). This suggestion was also made at a recent conference (International Union of Microbiological Societies, IXth International Congress of Mycology and IXth International Congress of Bacteriology and Applied Microbiology, Sydney, Australia, 1999), based on sequence analyses of the internal transcribed regions of the ribosomal RNA gene repeats.

Using sequence data of the large subunit ribosomal RNA (26S rRNA), Kurtzman and Robnett (1997) investigated the phylogenetic relationships of *Candida* species and suggested that many of the traditionally defined species should be combined (eg., *C. viswanathii* with *C. lodderae* and C. *krusei* with *C. zeylanoides*). From these data, it appears that the large subunit is more conserved than the small subunit and may, in fact, discriminate above the species level.

The clinical importance of many *Candida* species has led to an increased desire to understand how they have arisen, in the hope of determining how particular species became so predominant or how they acquired their resistance to current antifungals. Their clinical importance has also allowed numerous isolates to be collected, thus facilitating large-scale epidemiological investigations. Despite this focus of research, there is still significant controversy about the relationships between these species and also the most appropriate methods for their diagnosis at the species level.

1.2.3 Medically-relevant species

There are thirteen *Candida* species recognized as predominant fungi responsible for a range of oral, vaginal and systemic infections, particularly in immunocompromised individuals, such as HIV-positive patients (Fraser *et al.* 1992, Jordan 1994, Fisher-Hoch and Hutwagner 1995, Hoepelman and Dupont 1996, Pfaller 1996). This thesis focuses upon

these medically relevant species, described below in alphabetical order and summarised in Table 1.1 (p., 16). Although each of these species has been isolated from clinical infection, only the more prevalent species have been extensively reported and investigated. For this reason, little is known about the *Candida* species rarely isolated from clinical infection and therefore their descriptions in this Section are limited. A key to the morphological and biochemical (traditional) identification of the thirteen species is in Appendix 1 (Tables 1 & 2). Identifying characteristics were obtained from the Mycology Online web site written by the Mycology Unit of the Women's and Children's Hospital, Adelaide, South Australia; http://www.mycology.adelaide.edu.au/ (last accessed 8/01/02). The GC content of the genome of each *Candida* species was obtained from the CBS home page (http://www.cbs.knaw.nl last accessed 8/01/02). Colonies of these species appear white to cream, smooth, glabrous and yeast-like on Sabouraud's dextrose agar, a selective medium for the isolation of yeasts and fungi.

The following section (and Table 1.1) provides descriptions of the 13 medically-relevant species included in this study. The descriptions include phylogenetic, phenotypic and epidemiological differences between the species and illustrate the long-term taxonomic confusion surrounding them.

Candida albicans (Robin) Berkhout: The most common infecting species is *Candida albicans*, causing up to 80% of clinical infections (eg., Schiodt *et al.* 1990). The species *C. albicans* has one hundred and ten synonymous names, an indication of the numerous taxonomic and nomenclature revisions that have occurred since its first description.

The phenotypic properties of *Candida albicans* overlap with those of *C. tropicalis* making them hard to distinguish using traditional characters. The most commonly used diagnostic character for *C. albicans* is germ tube formation. However, *C. dubliniensis* is also germ-tube positive, germ tube positive oral isolates of *C. tropicalis* have been described (Nikawa *et al.* 1997) and *C. albicans* isolates are not consistently positive for the production of germ tubes. Nevertheless, those experienced at diagnosing *Candida* species according to the formation of germ tubes can still identify them. Molecular methods continue to identify atypical isolates within *C. albicans* and a re-examination of one set of atypical strains within the species (Sullivan *et al.* 1993) resulted in the first description of the species *C. dubliniensis* (see Sullivan *et al.* 1995).

Table 1.1; Details of the DNA G+C content, identified teleomorph species and antifungal resistance profile of thirteen prevalent medically important *Candida* species

Species	G+C content	Teleomorph	Antifungal resistance
C. albicans	33.9%	None[a]	Fluconazole (acquired)
C. dubliniensis	-[b]	None	Fluconazole (acquired) (Moran *et al.* 1997)
C. famata	35.8-37.7% (type strain 36.8%) (Nishikawa *et al.* 1996)	*Debaryomyces hansenii* (Nishikawa *et al.* 1996, Nishikawa *et al.* 1997)	
C. glabrata	39.6-40.2% (Kreger van Rij 1984)	None	Azoles, particularly fluconazole (innate) (eg., Arias *et al.* 1994, Arias *et al.* 1996, Carrillo Munoz *et al.* 1997; Hoegl *et al.* 1998, Fidel *et al.* 1999)
C. guilliermondii	44.1-44.4%	*Pichia guilliermondii*	Amphotericin B (innate) (Vazquez *et al.* 1995, Vazquez *et al.* 1998)
C. haemulonii	46.1-47.8% (type strain 47.8%)	None	
C. kefyr	41.3%	*Kluyveromyces* spp. (Cai *et al.* 1996)	
C. krusei	39.6 (type strain) -41.5% (Bai and Jia 1996)	*Kluyveromyces lactis* and *Issatchenkia orientalis*	Azole antifungals (innate) (eg., Berrouane *et al.* 1996, Carrillo Munoz *et al.* 1997, Samaranayake 1997)
C. lusitaniae	45.2%	*Clavispora lusitaniae*	Amphotericin B (innate) (Karyotakis and Anaissie 1994, Vazquez *et al.* 1998)
C. norvegensis	35.5-37.5% (Billon-Grant) (Kurtzman).	*Pichia norvegensis*	Fluconazole (innate) (Sandven *et al.* 1997)
C. parapsilosis	40.8%	Probably *Lodderomyces elongisporus* (James *et al.* 1994)	
C. tropicalis	33.1-36.1%	None	Increased resistance to azole antifungals (innate) (Law *et al.* 1996)
C. viswanathii	43.7-46.3% (S.A. Meyer) (F.L. Lee *et al.*)	None	

a; None – teleomorph not identified to date

b; – unknown GC content

Due to its predominance in clinical infection *C. albicans* has become the major focus of yeast biomedical research, and this thesis. This predominance has led to extensive literature on its biology. Despite the volume of research conducted on this species, the taxonomic and systematic controversy surrounding it, as discussed in the previous sections, has precluded an unequivocal resolution of its epidemiology.

Candida dubliniensis: This species is the most recently described within the genus and constitutes a previously described atypical subset of *C. albicans* strains (Sullivan *et al.* 1995). Its morphological and biochemical identity is indistinguishable from that of *C. albicans*, but genetically the two species are quite distinct (Gilfillan *et al.* 1998). Sequencing of the group I intron of the large ribosomal RNA subunit, a phylogenetically informative molecular clock, suggests a close genetic proximity to *C. albicans* (see Boucher *et al.* 1996).

Cell-wall antigens that differentiate *C. dubliniensis* and *C. albicans* (see Bikandi *et al.* 1998a) and recognition of a species-diagnostic repetitive element (Joly *et al.* 1999) are further evidence of their distinction. Differences in cell surface hydrophobicity between the two species have been identified (Jabra-Rizk *et al.* 1999, Hazen *et al.* 2001, Jabra-Rizk *et al.* 2001). This difference has been suggested by the authors as a mechanism for *C. dubliniensis* to adhere to *F. nucleatum*, an anaerobic oral colonising bacterial species, enhancing its ability to asymptomatically colonise the oral cavity and to outcompete *C. albicans* during fluconazole treatment.

C. dubliniensis isolates can also be morphologically differentiated from *C. albicans* by: their appearance on CHROMagar, where they can be described as dark and light green respectively; their inability to grow at 45°C (Kirkpatrick *et al.* 1998); and their absence of intracellular beta-glucosidase activity (Schoofs *et al.* 1997). Nevertheless, the accuracy of these characters used in isolation has been questioned (Tintelnot *et al.* 2000). Recently, a difference in colony appearance and the ability to form chlamydospores on Staib agar was also noted between *C. dubliniensis* and *C. albicans* (see Staib and Morschhauser 1999).

There is a previously-unrecognized, widespread geographical distribution of *C. dubliniensis* among HIV-positive individuals (Sullivan *et al.* 1997). The first cases of *C. dubliniensis* fungaemia have been reported in Europe (Meis *et al.* 1999), North America (Brandt *et al.* 2000) and Australia (Marriott *et al.* 2001). The predominance of this species

in immunocompromised individuals has been noted upon re-examination of cultures collected prior to its first description (eg., Jabra-Rizk *et al.* 2000). *C. dubliniensis* has been isolated from the oral cavity of HIV-positive individuals with obvious candidiasis where it has been suggested that this species is more adhesive to human buccal epithelial cells but less virulent than *C. albicans* with an increased propensity for the oral cavity (Gilfillan *et al.* 1998). The species has also been isolated from sites of asymptomatic carriage and infection in HIV-negative individuals on broad-spectrum antibacterial therapy (eg., Polacheck *et al.* 2000) indicating that colonisation is not limited to HIV-positive individuals.

C. dubliniensis has an increased phenotypic switching frequency when compared to *C. albicans*, but *C. albicans* produces more phospholipase and proteinase, associated with virulence (Hannula *et al.* 2000). Both *C. dubliniensis* and *C. albicans* have an ability to become fluconazole resistant through the increased expression of multidrug transporters, which transport the drug out of the cell's cytoplasm (Moran *et al.* 1998). The sequences of these transport proteins are very similar in the two species (Moran *et al.* 1998) providing further evidence of their close genetic proximity.

In an *in-vitro* experiment, *C. albicans* appeared to outcompete *C. dubliniensis* in both mixed broth and mixed biofilm conditions (Kirkpatrick *et al.* 2000), a situation that may be applicable *in-vivo*, which explains the continued isolation prevalence of the former species. *C. dubliniensis* has an increased phenotypic-switching frequency compared to *C. albicans*, but *C. albicans* produces higher levels of proteinase and phospholipase (Hannula *et al.* 2000), which highlights the complexity of their relationship *in-vivo*.

Candida famata (Harrison) Meyer & Yarrow: This species was formerly known as *Torulopsis candida*. In 1978, the genus *Torulopsis* was incorporated into the genus *Candida* (Yarrow and Meyer 1978) after it was decided that the absence of pseudohyphae in the genus *Torulopsis* was not enough grounds for the distinction of two genera. This observation has subsequently been supported in numerous publications (eg., Odds *et al.* 1997). The nomenclature change is not unequivocally accepted by clinical and research mycologists as is evident from the continued referral to both names in publications (eg., Arikan *et al.* 1998, Krcmery *et al.* 1998, Cresti *et al.* 1999, Becker *et al.* 2000)

Along with the range of genomic G+C contents recorded for the species, karyotyping (i.e., grouping yeasts on the basis of the size and number of chromosomes they have)

provides further evidence of the extensive genetic variation within this species. The number of chromosomes in strains within *C. famata* varies between six and eight and the size of the genome ranges from 12 to 14 mega-base pairs (Versavaud and Hallet 1995).

Candida glabrata (Anderson) Meyer & Yarrow: Like *C. famata*, the species *C. glabrata* was also formerly within the genus *Torulopsis*. *Cryptococcus glabratus* has been recognised as synonymous to *C. glabrata* and is now incorporated into this species. This is a dramatic nomenclature change, since *Cryptococcus* species belong to the phylum *Basidiomycota*. *C. glabrata* is the only haploid *Candida* species commonly isolated from human infection that is incapable of producing pseudohyphae at 37°C (Fidel *et al.* 1999) unless grown on solid nitrogen starvation medium (Csank and Haynes 2000).

In a study by Brandao *et al.* (1995), three strains of *Candida* that were originally described as *C. albicans*, due to their ability to form germ-tubes, were subsequently reidentified as *C. glabrata* on the basis of their karyotype profile. This again raised doubts about the applicability of germ-tube formation for the accurate diagnosis of the *C. albicans* species.

On the basis of 18S rRNA gene sequence comparisons, *C. glabrata* is more closely related to *Saccharomyces cerevisiae* with distant relatedness to *C. kefyr* and *K. marxianus* (see Barns *et al.* 1991). These species formed a distinct subgroup from that comprising most other *Candida* species of clinical significance. Additionally, *C. glabrata* and *Saccharomyces* share their telomeric core sequence (Cohn *et al.* 1998) and the species is frequently misidentified as *Saccharomyces cerevisiae* by fatty acid profile analysis (Crist *et al.* 1996). A primer designed for the "highly-variable" rDNA ITS region of *C. glabrata* was able to cross-react with *Saccharomyces cerevisiae* DNA, but not with that of other *Candida* species (Fujita *et al.* 1995), supporting their close genetic proximity. *C. glabrata* does not have *C. albicans* germ tube antigens in its cell wall, unlike most other *Candida* species (Bikandi *et al.* 1998b).

C. glabrata has a high affinity for the urinary tract (Knoke *et al.* 1997, Nayeri *et al.* 1997) and, after *C. albicans*, is the second most prevalent yeast species in vulvovaginal infections (Abu Elteen *et al.* 1997). The species has increased in prevalence in other *Candida* infections, particularly bloodstream infections acquired during hospitalization (nosocomial infections) (Hospenthal *et al.* 1995, Giamarellou and Antoniadou 1996, Voss *et*

al. 1996, Kaben *et al.* 1997). Its prevalence, along with *C. parapsilosis*, in nosocomial infections in Taiwan has increased dramatically since the introduction of fluconazole (Chen *et al.* 1997). Genetically identical strains of *C. glabrata* have been isolated from different patients, probably attributable to a common source of infection or transmission within the hospital (Vazquez *et al.* 1998). Deep-seated organ infections caused by *C. glabrata* have been suggested as opportunistic and transient, being a consequence of other fungal infections (Vennewald *et al.* 1997, Vennewald *et al.* 1998). In one case where a disseminated infection was caused by *C. glabrata* and *C. albicans*, the *C. glabrata* originated in the patient's oral cavity (Bougnoux *et al.* 1997). Taking into account the increased resistance of this species to fluconazole, it is interesting to note that oral colonization with *C. glabrata* and *C. krusei* increases during fluconazole treatment of a pre-existing *C. albicans* infection in HIV-positive individuals (Drobacheff *et al.* 1996).

Candida guilliermondii (Castellani) Langeron & Guerra: Although *C. guilliermondii* and *C. famata* belong to the same anamorphic genus, their teleomorphs do not. This aberration is not restricted to these two *Candida* species as is evident from descriptions of other *Candida* species in this Section. According to a phylogenetic analysis based on the small and large ribosomal RNA gene sequences, *C. guilliermondii* forms a distinct evolutionary group with *Debaryomyces* species (see Cai *et al.* 1996) and particularly with the *Candida famata/Debaryomyces hansenii* complex (see Nishikawa *et al.* 1997). Due to the close genetic relationship between the eighteen species including *C. guilliermondii*, they are sometimes referred to as the *C. guilliermondii* species complex. This complex comprises six electrophoretic karyotype groups (Bai *et al.* 2000).

Isozyme analyses (i.e., comparisons of different isoforms of an enzyme) and randomly amplified polymorphic DNA (i.e., RAPD) analyses have segregated the species *C. guilliermondii* into two distinct "cryptic" species that were shown to correlate with *C. guilliermondii* and its synonym *Candida fermentati* (see San Millan *et al.* 1997). This conclusion provides evidence that the previous taxonomic union of these two species may have been inaccurate.

Candida haemulonii (van Uden & Kolipinski) Meyer & Yarrow: This species has biochemical similarity to *C. guilliermondii*, making them difficult to distinguish using traditional characters. Isoenzyme and protein profiles have identified two distinct groups

within the species *C. haemulonii*, an observation supported by DNA relatedness estimations and phenotypic characters (Lehmann *et al.* 1993).

Candida kefyr (Beijerinck) van Uden & Buckley: This species is predominantly isolated from neutropenic patients (Lopes *et al.* 1996). Sequence comparisons of the 18S rRNA gene have placed *C. kefyr* distant from other *Candida* species (Barns *et al.* 1991). *C. kefyr* has a number of associated teleomorphs belonging to the genus *Kluyveromyces*, all of which are closely related genetically (Cai *et al.* 1996). These teleomorphs include *Kluyveromyces marxianus* which itself has a number of synonymous species names with their own associated anamorphs (Molnar *et al.* 1996). A recent study of the phylogeny of the 17 species belonging to the *Kluyveromyces* genus using the mitochondrial cytochrome-c oxidase II gene has grouped the genus into four distinct species groups (Belloch *et al.* 2000). The results have questioned the separation of species within *Kluyveromyces* and between the genus and the closely related genera *Saccharomyces, Torulaspora* and *Zygosaccharomyces*. Studies like this have indicated that since the advent of DNA technology, the genetic information that has been uncovered has increased the confusion associated with *C. kefyr* and its associated anamorphs and teleomorphs.

Candida krusei (Castellani) Berkhout: The innate azole resistance of *C. krusei* is attributed to a decreased cellular accumulation of azoles (Venkateswarlu *et al.* 1996). The efficacy of different azoles is directly proportional to the level of intracellular accumulation (Marichal *et al.* 1995). There has been an increased incidence of *C. krusei* infection in leukemic patients in some institutions, especially after bone marrow transplant and fluconazole therapy (Abi Said *et al.* 1997). The species incidence in invasive candidiasis, however, has not been associated with previous exposure to fluconazole (Iwen *et al.* 1995). The increasing clinical predominance of *C. krusei*, its associated treatment difficulties, and the adverse side effects of other drug treatments have driven technology towards developing new antifungals to control *C. krusei* infections. These include voriconazole (eg., Barry and Brown 1996) and 56592 (posaconazole, Zeneca) (Patterson 1999).

Clinical evaluation of the pathogenicity of species of *Candida* in an immunocompromised rat oral mucosal model by Samaranayake *et al.* (1998) has indicated that *C. krusei* is not as pathogenic as *C. albicans*. When compared to *C. albicans*, *C. krusei* is not as adhesive to macrophages (Nessa *et al.* 1997) or human neutrophils (Richardson and Donaldson 1994), making it less readily phagocytosed. *C. krusei* has different adhesive

properties to *C. albicans*, the former having an increased propensity for acrylic surfaces over cell surfaces (Samaranayake *et al.* 1994), suggesting a mechanism for the species prevalence in nosocomial infections.

Sequence analysis of the cytochrome c gene indicated that *C. krusei* is closely related to *Kluyveromyces lactis* (see Freire Picos *et al.* 1995). Conversely, Janbon *et al.* (1997) found, using the same gene, that *K. lactis* was more closely related to *C. albicans*. According to the 18S rRNA gene sequence, *K. lactis* is more closely related to *Saccharomyces cerevisiae* (see Barns *et al.* 1991), which in the Janbon *et al.* (1997) study was more closely related to *C. glabrata*. Also using the 18S rRNA gene, Hendriks *et al.* (1991) found that *C. krusei* formed a distinct evolutionary branch with no close associations with any species of *Candida*.

The conflicting systematics surrounding *C. krusei* illustrates the danger in using a single method or genetic character for these analyses and the confusion that can arise as a consequence. These relationships remain unresolved. Differences in the behaviour of *C. krusei* and other *Candida* species in the host, its virulence and cellular characteristics (eg., cell surface hydrophobicity (Samaranayake *et al.* 1995) have been suggested as confirmatory evidence that this species should be reassigned to a different genus (Samaranayake and Samaranayake 1994). *Issatchenkia orientalis* has been suggested as the teleomorph for *C. krusei*.

Candida lusitaniae van Uden & do Carmo-Sousa: According to 18S rRNA gene sequence comparisons, *Candida lusitaniae* is the earliest evolutionary lineage within the genus *Candida* (see Hendriks *et al.* 1991). Similarity in biochemical profiles between *Candida lusitaniae* and *C. tropicalis* make them difficult to distinguish using only these characters.

Epidemiological investigations have revealed that *Candida lusitaniae* may be transmitted within neonatal intensive care units, causing outbreaks of nosocomial bloodstream infection (Fowler *et al.* 1998). It has also been noted that the same strain can lead to a disseminated infection in hospitalized patients (Merz *et al.* 1992). However, the observation of innate amphotericin B resistance in *C. lusitaniae* seems to be dependent upon the laboratory in which the susceptibility testing is done and the protocol used (Favel *et al.* 1997). The teleomorph for this species is *Clavispora lusitaniae*.

Candida norvegensis (Dietrichson) van Uden & Farinha. ex van Uden & Buckley: This species was a member of the genus *Torulopsis* prior to 1978, when it was redescribed as a *Candida* species (Yarrow and Meyer 1978). *C. norvegensis* is a rare pathogen in human infection, usually being isolated from severely immunocompromised individuals (Nielsen and Stenderup 1996). The teleomorph is *Pichia norvegensis*.

Candida parapsilosis (Ashford) Langeron & Talice: This species is more commonly isolated from hand carriage on health care workers than *C. albicans* (see Diekema *et al.* 1997) and is frequently isolated in cases of onychomycosis (hand or toe infection) (Velez *et al.* 1997). The species is also a common pathogen in neonatal intensive care unit outbreaks of infection (Welbel *et al.* 1996). *C. parapsilosis* has an increased resistance to amphotericin B (Vazquez *et al.* 1998).

According to total chromosome DNA probing, *C. parapsilosis* and *C. albicans* have a high chromosomal similarity and other *Candida* species are less closely related (Liu *et al.* 1994). The same study described conserved regions flanking the ribosomal RNA coding region in all *Candida* species, supporting their inclusion in a single genus. A preliminary study using RAPD analyses identified three distinct subgroups within *C. parapsilosis*; one representing the majority of oral and systemic isolates and two others specific for one of the infection sites (Dassanayake and Samaranayake 2000). This observation, if supported by more extensive analyses, may form the basis for the future description of two cryptic species within *C. parapsilosis*.

Candida tropicalis (Castellani) Berkhout: This species predominates in human respiratory and urinary tracts (Al Hedaithy and Fotedar 1997). Infection caused by this species progresses to systemic infection more often than any other *Candida* species. *C. tropicalis* is the second most predominant cause of hospital-acquired candidaemia (13.5%) after *C. albicans* (56.8%) (Al Soub and Estinoso 1997). The incidence of isolation of *C. tropicalis* from urine is 36%, respiratory tract 22% and vaginal tract 14% although the incidence of vaginal isolation is decreasing (Al Hedaithy and Fotedar 1997).

Candida tropicalis (syn. *Candida vulgaris*) is the type species for the genus *Candida* (Kreger van Rij 1984) and it has fifty-eight synonymous names. *C. paratropicalis* has been reclassified as a synonymous species, based on its morphological and physiological

similarities to the sucrose-negative variant strains of *C. tropicalis*. Some *C. tropicalis* strains are biochemically similar to *C. albicans* and *C. viswanathii* strains, making them difficult to distinguish using these characters.

Candida viswanathii Sandhu & Randhawa: Rarely isolated from human infection; publications to date have focussed on the accurate identification of *C. viswanathii* using contemporary genetic methods, not its clinical manifestations, prevalence or biochemical and morphological characterisation (eg., Maiwald *et al.* 1994, Mannarelli and Kurtzman 1998). It is surprising that although there are two hundred and eleven species within the genus *Candida*, only a limited number are of medical importance. The ability to cause infection requires certain biological characters associated with adhesion and survival in the host. The presence of these characters in a selection of *Candida* species suggests that they share a common ancestor with each other more recently than with other *Candida* species not associated with infection. For this reason, it is anticipated that they will have a closer genetic relationship and may form a subset of the genetic variation within the whole *Candida* genus. This assumption illustrates the importance of population biology, ecology and systematics in our efforts to understand disease processes. In isolation, epidemiological studies at the population level provide important information on strain variation within a fungal/bacterial species. Conversely, an understanding of the systematics of existing species allows accurate assumptions to be made concerning the identity of the population and the evolution of pathogenicity. A thorough understanding of a combination of these issues provides an understanding of the processes involved in previous and ongoing speciation events. This understanding provides insight into how current and emerging virulent organisms may have arisen.

1.2.4 Pathogenicity factors within the genus Candida

Microevolution of the yeast genome is responsible for changes in the susceptibility to antifungals, cellular and colonial morphology, expression of virulence factors, and growth characteristics of a yeast strain (Fries and Casadevall 1998). This microevolution has been suggested as a mechanism by which *Candida* may evade the host immune system. A number of virulence determinants have been identified within the genus, as outlined below (also see review article by Navarro-Garcia *et al.* 2001).

The *Candida* receptors required for adhesion to host cells and subsequent dissemination of infection are induced by haemoglobin (Yan *et al.* 1998). The expression of adhesive proteins for binding to buccal epithelial cells is increased in strains taken from oesophageal sites of infection versus strains from oesophageal sites of heavy colonization without the associated infection. The more adhesive strains also produce more germ tubes and at a faster rate than the less adhesive strains (Wellmer and Bernhardt 1997). *Candida* species have different mechanisms of adhesion to epithelial cells (Bendel *et al.* 1993), but the extent of this adhesion has been correlated with the level of expression of an integrin analogue (Bendel *et al.* 1995). Of clinical significance, non-viable *Candida* cells are more adhesive to buccal epithelial cells than are viable cells (Gorman *et al.* 1996).

The expression of *Candida* proteolytic enzymes has been correlated with tissue colonization in mice (Mendes Giannini *et al.* 1996). There are, however, conflicting reports on the importance of proteolytic enzyme activity to the virulence of *Candida* species. Some reports have associated secreted aspartic proteinases (SAPs) with *C. albicans in-vitro* and *in-vivo* virulence (Borgvonzepelin *et al.* 1998, Monod *et al.* 1998, Schaller *et al.* 1998) whilst others have not linked the presence of these enzymes with *in-vitro* virulence (Szkaradkiewicz *et al.* 1998). The over-expression of SAPs does not appear to increase the virulence of strains (Dubois *et al.* 1998), but subinhibitory concentrations of fluconazole appear to enhance SAP production in fluconazole resistant *C. albicans* isolates due to over-expression of the multidrug resistance efflux pump (Wu *et al.* 2000). Some authors have suggested that the HIV protease inhibitors used in the new antiretroviral HAART therapy are also effective against the SAPs of *C. albicans* (eg., Bektic *et al.* 2001). Different SAPs are produced during systemic and mucosal infections, between early colonisation and following dissemination into deep organs. SAP5 is expressed with the commencement of hyphal formation, SAP6 is present only when *Candida* is growing in the hyphal form and SAP2 is associated with deep organ infection, the authors suggesting that it may participate in protein breakdown for survival (Staib *et al.* 2000).

Phospholipase B has been linked to the ability of *C. albicans* strains to traverse the host cell membranes and cause disseminated infection (Leidich *et al.* 1998) and appears essential for *Candida* virulence (Ghannoum 2000). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), localized in the cell wall, has been associated with the adhesion of *Candida* isolates to fibronectin and laminin, a process important to tissue invasion and the

dissemination of infection (Gozalbo *et al.* 1998). Catalase has also been suggested as important to the virulence of *Candida* species (Wysong *et al.* 1998).

The relevance of the hyphal phase to invasive infection remains controversial (eg., White *et al.* 1995). Thigmotropism, or contact sensing, has been suggested as a mechanism for *Candida* cells to traverse the mucosal membrane gaining access to the bloodstream (eg., Nikawa *et al.* 1997). It has been reported that there are two separate regulatory pathways for the yeast-hyphal transition within *Candida* species and both forms are involved in pathogenesis of the infection (Mitchell 1998). In a rat model for candidaemia (blood infection) caused by lung injury and multiple organ damage, the yeast-hyphal transition was positively associated with the lethality of the infection, but the clinical outcome was not associated with tumour necrosis factor involvement (Matuschak and Lechner 1997). At neutral to slightly acidic environmental pH, the expression of PRA1, a pH regulated secreted glycoprotein, is maximal. Its deletion confers a temperature-dependent defect in hyphal formation (Sentandreu *et al.* 1998), indicating a link between the environment and the ability for *C. albicans* to undergo yeast to hyphal transition.

The phenomenon of colony morphology switching is commonly seen in *Candida* species. It has been suggested that the transition is associated with virulence, indicating alterations in the expression of cell wall adhesive factors, secretion of proteolytic enzymes and the ability to grow in the hyphal form (Odds 1997). The frequency of switching is higher in strains isolated from infection than in strains isolated from asymptomatic colonization. This suggests that switching mediates virulence, facilitates invasion of host tissue, and allows *Candida* to evade the host immune system (Odds 1997).

It has been reported that the expression of virulence factors within the genus *Candida* is controlled by genes which are regulated by the pH of the environment colonized, such as PHR1 (De Bernardis *et al.* 1998). Additionally, CaHsp70, a heat shock protein expressed in response to a rise in environmental temperature, increases the ability of *Candida* to cause systemic infections (Bromuro *et al.* 1998). *Candida* expression of aspartate proteinase has also been demonstrated to elevate with the yeast binding to HIV-1 envelope protein gp160 (Gruber *et al.* 1998), indicating an increase in *Candida* virulence with co-colonisation with the virus.

1.2.5 Mechanisms of antifungal resistance in Candida

There are a number of classes of antifungals, acting in different ways, for the treatment of yeast infection (Appendix 1 Table 3). Some *Candida* species have an innate resistance to particular classes of antifungals, others can acquire resistance or increased tolerance with long-term exposure to them. Whilst not effectively killing *C. albicans*, sublethal doses of antifungals have been shown to decrease the adhesive properties of the species (Ellepola and Samaranayake 1998). *In-vivo*, this treatment regime potentially reduces the strain's ability to colonize the oral mucosa and suppresses its invasive potential but it is also often associated with the acquisition of resistance.

The introduction of fluconazole for the treatment of *Candida* infection in 1992 greatly decreased the incidence of infection for the first few years, with isolates being susceptible to the new drug (Boschman *et al.* 1998). Over time, however, the incidence of infections that are clinically-unresponsive to fluconazole has increased (eg., Lischewski *et al.* 1995, Martino and Girmenia 1996, Diazguerra *et al.* 1998). The acquisition of clinical resistance to antifungals obviously has important implications for the treatment of *Candida* infections. Infections can become unresponsive to treatment due to a superinfection of the individual with an innately resistant *Candida* species or the original infecting strain may acquire a resistance mechanism during treatment (eg., Bart-Delabesse *et al.* 1993, Sangeorzan *et al.* 1994, McCullough and Hume 1995, Metzgar *et al.* 1998). The rate of colonization by species other than *C. albicans* has increased since the introduction of fluconazole for the treatment of candidiasis (eg., Knoke *et al.* 1997, Martins *et al.* 1998). *In vitro* fluconazole resistance of *Candida* species is more predominant in individuals with a low CD4 cell count, prior treatment for oral candidiasis and/or long-term fluconazole treatment for prior infections (Maenza *et al.* 1997). Additionally, the incidence of mixed infections caused by *C. albicans* and other *Candida* species has also increased due to the administration of fluconazole (Metzger and Hofmann 1997).

In some strains of *C. albicans*, resistance to fluconazole has been associated with a decrease in the strain's virulence (Graybill *et al.* 1998) suggesting that resistance may be associated with the survival of the organism and the "shutting down" of the cell. The decrease in virulence has also been noted in a *C. tropicalis* strain that acquired fluconazole resistance upon subculturing in a broth containing the antifungal (Barchiesi *et al.* 2000). The fluconazole resistance in this strain conferred cross-resistance to itraconazole and terbinafine but not amphotericin B.

A recent large-scale investigation confirmed that the *in-vitro* fluconazole susceptibility remains significant with 99% of the *C. albicans* isolates tested, 94% of *C. parapsilosis*, 90% of *C. tropicalis*, 67% of *C. glabrata* and 26% of *C. krusei* being susceptible (Meis *et al.* 2000). However, standardising the method for assessing *in-vitro* antifungal resistance of *Candida* is of particular clinical importance as unstandardised they can give widely variable susceptibility results that may have little or no *in-vivo* relevance (eg., Goutaland and Piens 1996). A recent publication has revealed that cytokines may act synergistically with fluconazole and amphotericin B against *C. albicans* (Mencacci *et al.* 2000) suggesting that the host immune status is of vital importance in combating *Candida* infection.

1.3 Candida infection

Candida species can be carried asymptomatically in a variety of sites in the body. Sometimes individuals may become infected by exogenous or endogenous strains, which cause a range of infections that may contribute to the morbidity of a patient. Immunocompromisation is the most predominant risk factor for human infection. A depressed immune response is common to HIV-positive individuals, cancer patients who have undergone chemotherapy, neonates, bone marrow and organ transplant recipients who are on immunosuppressive anti-rejection treatment and patients who have had multiple courses of antibiotics, intravascular catheters, leg amputation, a neurogenic bladder or low serum albumin levels (eg., Bregenzer et al. 1996, Flanagan and Barnes 1998, Hedderwick et al. 1998, Lopez et al. 1998, Macdonald et al. 1998, Connolly et al. 1999).

1.3.1 The immune response to Candida infection

The *Candida* cell wall is the predominant immunogenic target initiating a cell-mediated immune response (Martinez *et al.* 1998). A humoral immune response is also directed against the protein and glycoprotein components within the cell wall (Martinez *et al.* 1998). The antibodies produced during the humoral immune response coat the outside of the cell and may limit the adhesive properties of the yeast cell and increase their phagocytosis (Martinez *et al.* 1998). The small cell size of *C. glabrata* and *C. guilliermondii*, and their consequential cell wall surface area decrease the ability of these two species to induce

tumour necrosis factor from mouse peritoneal macrophages allowing them to evade phagocytosis (Aybay and Imir 1996).

CD8+ T lymphocytes inhibit the growth of *C. albicans in-vitro* (Mathews *et al.* 1998). A decline in the number of circulating T cell-regulating cytokines in HIV-positive individuals has been associated with their progression to AIDS and onset of oropharyngeal candidiasis (Leigh *et al.* 1998). A decrease in the γ-interferon response in the host immune system also allows establishment of candidiasis (Szkaradkiewicz *et al.* 1998). In the case of *C. albicans* infection, this appears to be due to the species' ability to inhibit IL-12 and therefore limit the effectiveness of a type I immune response (Xiong *et al.* 2000). Baltch *et al.* (2001) demonstrated an increase in monocyte-derived macrophage activity against *C. albicans* in the presence of low concentrations of fluconazole and some cytokines and have suggested using cytokines in an adjunctive therapy.

Infection with HIV increases the *Candida* colony count in asymptomatic carriers and individuals with candidiasis (Teanpaisan and Nittayananta 1998), suggesting that the virus somehow alters the oral environment. The gp120 envelope protein of HIV decreases the antifungal activity of peripheral blood monocytes against *Candida* species (Pietrella *et al.* 1998). *Candida* species also have immunomodulatory properties (eg., Domer 1997), increasing the likelihood of opportunistic infections and further decreasing the survival rate of HIV-positive patients (eg., Chaisson *et al.* 1998).

A decrease in the local immune response but not in cell mediated immunity is more closely associated with oropharyngeal candidiasis (eg., Gottfredsson *et al.* 1999, Leigh *et al.* 2001). This observation negates the association between CD4+ cell count and the propensity for *Candida* infection. Elahi *et al.* (2001) compared clearance of oral candidiasis in immunocompromised mice following subcutaneous or oral immunisation and found that oral immunisation was much more effective than systemic. Their results confirm the importance of the local immune response over the systemic cell mediated immune response and the authors suggest that clearance is predominantly controlled by effector molecules originating from the salivary glands. The authors go on to conclude that "Systemic CD4 T cells are not involved in protection against *Candida* infection at the oral mucosa and that the correlation of reduced, yet largely functional, CD4 T cells to OPC is merely an indicator of a deficiency or dysfunction in independent immune mechanisms at the oral mucosa. Alternatively, the systemic-derived CD4 cells may, in fact, play a role in protection at the

oral mucosa, but, to provide protection, must be maintained at a certain threshold in the peripheral circulation. Hence, as circulating CD4 cell counts decline, there are simply not enough CD4 cells capable of keeping *Candida* "in check" at the mucosal surface... Below the threshold, local immune mechanisms must take over exclusively" .

1.3.2 Epidemiology of infection

The systematics of the genus *Candida* provides insights into the evolutionary origins of its species and the characteristics conserved within them. Epidemiological investigations focused at the population level provide information on genetic changes within species leading to antifungal resistance, alterations in pathogenicity and transmission. Although the latter information appears more clinically relevant, and is often the focus of research, without an understanding of the systematics of an organism, the accurate identification of a population is jeopardised. Inaccuracies in identification at the population level leads to false assumptions being made about the species and potential difficulties in understanding the disease process.

Despite the abundance of epidemiological data published on *Candida*, particularly *C. albicans*, there continues to be no obvious preferred method for the identification of strains within a species. Many of the observations made are in conflict and there has not been agreement on the applicability of different methods for strain discrimination. For example, sequencing of the ITS regions of the ribosomal RNA genes has been suggested in one publication to be discriminatory at the strain level (Lin *et al.* 1995). However, in others it has been used for species identification because it is conserved within a species (eg., Shin *et al.* 1996, Lott *et al.* 1998, Reiss *et al.* 1998).

A comparative study of the efficacy and discriminatory power of morphotyping, serotyping, RAPDs and karyotyping found that the most informative epidemiological approach was the combination of morphotyping and karyotyping which grouped the isolates into clusters (Delcastillo *et al.* 1997). The results showed a concurrent change of morphotype and resistance to azoles but, unfortunately, there was no correlation found between cluster and origin of the strain. Another such study compared morphotyping, antifungal susceptibility and restriction enzyme profiles of isolates from recurrent vaginitis (Maffei *et al.* 1997). The authors suggested that there were changes in the morphology and antifungal resistance within a single genetically stable strain of *C. albicans*. Both these

studies suggest that the most appropriate epidemiological analyses are based on the combination of a number of different methods with varying discriminatory abilities.

Many of the currently published studies are based on observations made using a single technique on a single species examining one clinical presentation in a subset of the human population. Whilst providing invaluable information for those particular circumstances, their overall applicability is restricted. In isolation, a single study provides little information on the general factors associated with the disease processes as a whole. It is important to note that the following Sections reflect the discontinuity of these individual studies and the conflict between them. Nevertheless, the information included is necessary to provide the rationale for the aim and specific objectives of this thesis.

The following sections address currently published epidemiological information for *Candida* infections of the oral cavity, gastrointestinal tract, vagina, urinary tract and bloodstream. These sites of infection are discussed in relation to the risk factors for infection, routes of transmission, clinical symptoms and strain/population specific virulence characters. These characteristics are important not only to our understanding of the epidemiology of infection, but also of the systematics of *Candida* species.

1.3.2.a Oropharyngeal Candida infections

The most predominant mucocutaneous infection site is the oral cavity. Infections may cause general redness and inflammation of the oral cavity, which may spread to the pharynx. Characteristic white lesions may be visible on the buccal mucosa, tongue, gums, palate and pharynx (Ellis 1994). A range of *Candida* species may cause oropharyngeal candidiasis, but it has been noted that the infection does not occur in the absence of *C. albicans* (see Martins *et al.* 1997).

The oral carriage of *Candida* is increased with smoking, azole antifungal use and HIV infection (Schoofs *et al.* 1998). Risk factors for oral infection include smoking and dentures (eg., Kamma and Nakou 1997, Abu Elteen and Abu Alteen 1998) but another study refutes this observation in radiotherapy patients with oral cancer (Ramirez Amador *et al.* 1997). Table 1.2 (p., 32) illustrates the difference in prevalence of *Candida* oral carriage and infection not only between HIV-negative and HIV-positive individuals, but also between studies.

Table 1.2; Examples of the reported prevalence of *Candida* colonisation in carriage and infection of HIV-positive and HIV-negative individuals

Oral Carriage		Oropharyngeal infection	
HIV-negative	HIV-positive	HIV-negative	HIV-positive
11%[a] to 36.8%[b]	67%[a] to 93%[c]	57%[a] to 68%[d]	75%[d] to 90%[e]

The studies were conducted in different geographical locations
a; Thailand (Teanpaisan and Nittayananta 1998)
b; South Africa (Abu Elteen and Abu Alteen 1998)
c; France (Monteil *et al.* 1997)
d; South Africa (Hauman *et al.* 1993)
e; France (Eloy *et al.* 1998)

Most *Candida* infections occur in HIV-positive individuals. It is unclear whether this is due to alterations in the oral environment due to the virus, or a consequence of the immunocompromisation associated with the viral infection. One study suggested that the virus itself has no effect since variation in the viral load did not affect the prevalence of *Candida* in the oral cavity (Bergbrant and Faergemann 1997). Conversely, a second study showed HIV-positive individuals had an increase in oral *Candida* cell count in terms of both carriage and infection (Teanpaisan and Nittayananta 1998). Interestingly, the *C. albicans* strains isolated from HIV-positive individuals prior to their first episode of thrush have been noted as being in a high-frequency switching mode and as more resistant to antifungals (Vargas *et al.* 2000). This observation suggests that the *C. albicans* strains colonising the oral cavity of an HIV-positive individual are already in a more virulent growth and gene expression pattern.

The CD4+ T-cells are used as an indicator of immunosuppression and a predictor of AIDS progression (eg., Chirmule *et al.* 1995). One study reported no correlation between the numbers of *C. albicans* colony-forming units in the oral cavity and individuals CD4+ cell count (Bergbrant and Faergemann 1997). Another found that the rate of carriage increased in HIV-positive individuals versus HIV-negative individuals only when the CD4+ cell count of the HIV-positive patient dropped to below five hundred cells/L (Fong *et al.* 1997). Other studies have found that the oral concentration of *Candida* increased with a decreased patient CD4+ count (eg., Silverman *et al.* 1996, Urso *et al.* 1996, Schuman *et al.* 1998). Patients with a decreased CD4+ count have also been shown to be more likely to have recurrent *Candida* infections rather than a single infection (Reef and Mayer 1995, Redding *et al.* 1997). Sobel *et al.* (2001) recently noted an association between oral colonisation by *Candida* species except *C. albicans* and HIV load, not CD4+ count, in HIV-seropositive women. Also, as was discussed in section 1.3.1 (p., 28), the local immune response plays an important role (Gottfredsson *et al.* 1999, Elahi *et al.* 2001).

The epithelial depth of colonization in HIV-positive individuals is greater than that of cancer patients on chemotherapy due to differences in the causes of their immunocompromisation, that is, HIV-positive individuals have an impaired inflammatory response whilst cancer patients have a decreased polymorphonuclear cell count (Goernig *et al.* 1997). This implies that the host immune response also plays an important role in determining the severity of *Candida* infection and the resultant clinical presentation.

Not only does the rate of colonisation of *C. albicans* differ between HIV-positive and HIV-negative populations but, the karyotypes of the *C. albicans* strain carried by these individuals appear different (Lupetti *et al.* 1995). Additionally, *C. albicans* strains with an innate increased resistance to itraconazole and fluconazole have been associated with HIV-positive individuals (Velegraki 1995, Velegraki *et al.* 1996). The *C. albicans* serotype present in these individuals has been reported as stable over time with no change associated with the onset of oropharyngeal candidiasis (Torssander *et al.* 1996).

The genetic diversity of isolates from HIV-negative individuals, determined using RFLP analysis (i.e., separation of strains according to the profile obtained using whole genomic DNA digested with a restriction enzyme), was much lower than that obtained from HIV-positive individuals, regardless of the presence of clinical infection or carriage (Challacombe *et al.* 1995). Additionally, the predominance of different genotypes varied between HIV-positive and HIV-negative individuals and suggested the existence of a particularly pathogenic genotype in HIV-positive patients with candidiasis. The existence of a subcluster of *C. albicans* strains with increased virulence has been suggested by a number of authors (eg., Schmid *et al.* 1999, Giblin *et al.* 2001, Lott and Effat 2001).

The level of production of intracellular proteinase is greater in strains isolated from HIV-positive patients than strains in HIV-negative individuals (Wu *et al.* 1996). Also it was reported that nystatin, amphotericin B, clotrimazole and miconazole decreased the level of proteinase to a lesser extent in the strains from HIV-positive individuals (Wu *et al.* 1996). A later study by Wu *et al.* (2000) also noted a decrease in intracellular secreted aspartyl proteinase (SAP) and an increase in extracellular SAP in fluconazole-resistant *C. albicans* isolates due to over-expression of the multidrug resistance efflux pump.

Mathaba *et al.* (1995) showed that most HIV-negative individuals had a single (unique) infecting *C. albicans* strain, which persisted during recurrent infections. Also, (Redding *et al.* 1997) revealed that 78% of HIV-positive individuals are infected with a single DNA subtype and 80% of HIV-positive individuals with recurrent infection are infected with multiple DNA subtypes. Healthy children have also been shown to asymptomatically carry more than one oral *C. albicans* strain (Mata *et al.* 2000).

Morphotyping (i.e., grouping strains of yeast according to their morphology on cornmeal agar) and biotyping (i.e., grouping strains according to their biochemical profiles) have

revealed subsets of *C. albicans* that are better adapted for survival in patients with oral leukoplakia and lichen planus infections (Lipperheide *et al.* 1996). This observation again suggests that there are subpopulations within the species with increased survival in particular niches and in disease-associated altered oral environments.

A genetically atypical cluster of germ-tube- and chlamydospore-positive *C. albicans* isolates has been identified from HIV-positive individuals (McCullough *et al.* 1995). These isolates were typed as *C. albicans* according to their biochemical profiles but they had increased levels of intracellular proteinase and adhesion to buccal epithelial cells and a decreased susceptibility to 5-flucytosine, making them more virulent. Anthony *et al.* (1995) reported a predominance of another atypical subset of *C. albicans* strains in HIV-positive individuals. Similarly, results of allozyme electrophoresis (ie., the electrophoretic separation and histochemical staining of metabolic enzyme, discussed in greater detail in Section 2.2.2 p., 54) and REA (restriction enzyme analysis) hybridization patterns with the Ca3 probe (ie., where the genome is restriction digested then probed with a labelled copy of the Ca3 repeat sequence) have discriminated typical *C. albicans* and an atypical group of chlamydospore-forming, germ-tube-positive *C. albicans* (see Boerlin *et al.* 1995). By combining allozyme electrophoresis, REA and Ca3 probe hybridization, each patient's colonizing strain could be discriminated (Boerlin *et al.* 1995). The derived clustering of isolates was not associated with fluconazole susceptibility or clinical symptoms. The typical cluster was predominantly isolated from sites of oropharyngeal candidiasis in HIV-positive individuals and invasive candidiasis in HIV-negative individuals. Isolates from the atypical cluster, which produced no β-glucosidase, were all isolated from asymptomatic HIV-positive intravenous drug users, indicating that they are potentially less pathogenic. A second allozyme electrophoretic study showed no clustering of strains from HIV-positive and HIV-negative individuals who were carriers or who had invasive candidiasis (Boerlin *et al.* 1996). In a later electrophoretic study using the 27A probe, a genetically atypical cluster of *C. albicans* was identified as actually being the newly identified species *C. dubliniensis* (Sullivan *et al.* 1997). Using allozyme electrophoresis, Reynes *et al.* (1996) revealed that in mixed infections, one strain predominates and is present for the duration of the infection.

Candida species are also a common causative agent of denture stomatitis lesions (eg., Abu Elteen and Abu Alteen 1998, Webb *et al.* 1998) and are often associated with sites of oral carcinoma (Nagy *et al.* 1998). These infections may be caused by a combination of *C. albicans* and various bacterial species (Kulak *et al.* 1997). An increase in serum levels

associated with inflammation facilitates biofilm formation on denture acrylic by *C. albicans* and *C. glabrata* (see Nikawa *et al.* 2000). Subtherapeutic concentrations of amphotericin B and nystatin appear to inhibit the adhesion of *Candida* species and decrease the incidence of lesions (Egusa *et al.* 2000). *C. albicans* has also been isolated from apical and marginal periodontitis, a potential reservoir for oropharyngeal infection (Waltimo *et al.* 2000).

The growth of *C. albicans* in the oral cavity appears to be in equilibrium with, and under the control of, the growth of oral bacteria (Basson and Van Wyk 1996). In particular, nitrate reductase-containing bacteria can control the growth of *C. albicans* (see Dougall *et al.* 1995). This observation suggests that oral candidiasis may involve ecological interactions between a range of intrinsic and extrinsic microorganisms.

The affinity of *C. albicans* for the oral cavity is partly attributable to the presence of membrane-associated binding proteins that recognize salivary histatins (Edgerton *et al.* 1998). As the salivary flow rate and the associated submandibular and sublingual saliva histatin concentration and level of secretion decrease, the level of yeast colonization of the oral cavity increases (Jainkittivong *et al.* 1998). The presence and concentration of salivary histatins may also be an important deciding factor in the conversion of asymptomatic carriage to infection. One study showed that the level of histatins in saliva decreased in direct relation to a decrease in the salivary flow rate, a side effect of chemotherapy (Jainkittivong *et al.* 1998). This resulted in an increase in the number of colony forming units of *C. albicans* in the oral cavity of these patients. A decrease in salivary flow rate has also been shown to increase the rate of carriage and infection with *C. albicans* in radiotherapy patients (eg., Ramirez Amador *et al.* 1997, Abraham *et al.* 1998, Rhodus *et al.* 1998). Another salivary component that has been positively linked to the number of *C. albicans* colony forming units is lysozyme (Yeh *et al.* 1997). As the salivary flow rate decreases, the concentration of lysozyme increases, as does the number of colony forming units in the oral cavity.

The oral carriage of *C. albicans* has been found to be significantly associated with individuals who are blood-group O and blood group antigen non-secretors (i.e., their blood-group antigens are not secreted in their saliva) (Ben Aryeh *et al.* 1995). However, another study revealed no correlation between *C. albicans* carriage and either of these predisposing factors in HIV-positive individuals, instead being dependent on the level of immunocompromisation (Fong *et al.* 1997).

Epidemiological investigations have indicated that the same strain of *C. albicans* may be transmitted between HIV-positive husbands and wives and that such strains may develop a decreased susceptibility to fluconazole (Barchiesi *et al.* 1995). A common strain has also been shown to persist over time, with partners being identified as a potential source for reinfection and the possible origin of fluconazole resistant strains prior to the patients' treatment (Dromer *et al.* 1997). The REA (restriction enzyme analysis) method used in their investigation identified an identical clone in two of the patients assessed, suggesting either that there is a widely distributed clone within the HIV-positive population, or that the technique was not discriminatory enough to distinguish closely-related but not identical clones.

Fluconazole resistance is not dependent on the HIV status of the colonized individual (Martins *et al.* 1997). HIV-positive patients at most risk of carrying a fluconazole resistant *C. albicans* strain are those with severe immunosuppression and who have had previous fluconazole treatment, although previous treatment is not necessary (Revankar *et al.* 1996). A clinical response to fluconazole may still occur in an *in-vitro* resistant strain (Revankar *et al.* 1996), but the treatment is usually longer and at higher doses (Revankar *et al.* 1998).

A study by Barchiesi *et al.* (1997) using REA, RAPDs and inter-repeat PCR (i.e., microsatellite amplification), reported that one of five HIV-positive individuals carried three different strains over the five year study period, with the last strain having an increased resistance to fluconazole. The other four patients carried the same strain for the entire study period and that strain developed fluconazole resistance (Barchiesi *et al.* 1997). The acquisition of resistance to fluconazole by a single strain in some patients and superinfection with a resistant strain in others has also been demonstrated using other methods such as karyotyping (Drobacheff *et al.* 1996) and mixed-linker PCR (McCullough and Hume 1995). Takasuka *et al.* (1998) uncovered a vast range of morphotypes, karyotypes and PCR types from a single patient with a fluconazole resistant oral candidiasis over a short time frame.

The extensive use of fluconazole has also led to an increased prevalence of resistant *Candida* species such as *C. krusei* and *C. glabrata* (see Drobacheff *et al.* 1996), particularly in HIV-positive individuals (Schoofs *et al.* 1998). However, it has since been suggested that the presence of other *Candida* species with *C. albicans*, has no bearing on the effectiveness of antifungal treatment for infection or on clinical outcome (Dronda *et al.* 1996).

Patients with oropharyngeal candidiasis who are treated with fluconazole may be cured, with no symptoms or presence of *C. albicans* after treatment; be improved, with no symptoms but still culture positive for *C. albicans*; or have treatment failure with the infecting strain being fluconazole resistant (Barchiesi *et al.* 1998). One study showed that 81% of HIV-positive individuals were still culture positive for *C. albicans* after successful fluconazole treatment (Silverman *et al.* 1996).

A number of studies have identified differences in the fluconazole susceptibility between colonies from a single patient sampling (eg., Dellion *et al.* 1995, Boerlin *et al.* 1996, Barchiesi *et al.* 1998). The accuracy of determining fluconazole susceptibility for a single colony pick is also dependent on the duration of *in vitro* incubation with the drug (Dellion *et al.* 1995).

Using the 27A probe hybridized to REA patterns, it was revealed that the *C. albicans* strain carried by six out of fourteen HIV-positive individuals varied over time (Anthony *et al.* 1995). Additionally, one of ten HIV-negative and four of ten HIV-positive individuals carried more than one strain simultaneously.

Serotype analyses have indicated that the strain infecting HIV-positive individuals appears to be stable over extended lengths of time; and, again, there is no relationship between serotype and pathogenicity of the organism (Torssander *et al.* 1996). This technique, however, may not discriminate at the strain level as other techniques have been able to detect strain variation over time.

REA analysis of the nuclear ribosomal RNA gene and repetitive DNA probing used in combination can distinguish strains from the oral cavity and the vagina of women (Dahl *et al.* 1997), suggesting infection-site preferences in strains. Conversely, another study showed that women may have vaginal colonization with a strain identical to that carried in their oral cavity, a strain that is genetically similar to the oral strain or two distinct strains (Lockhart *et al.* 1996).

The strain of *C. albicans* present in the oral cavity has been found to vary with age of the individual and the geographic location from which they originate (Kleinegger *et al.* 1996). Genetically distinct biotypes of *C. albicans* have been identified in age- and geographically-

distinct infant populations in Tanzania but a correlation with risk factors such as infant's age, gender, breastfeeding or malnutrition, has not been identified (Matee *et al.* 1996). Similar but genetically-distinct biotypes from geographically isolated locations have also been observed in HIV-positive populations (Tsang *et al.* 1995). The results of another study in China have indicated no difference in the biotypes of strains associated with oropharyngeal candidiasis and asymptomatic carriage of *Candida*; however, biotype A1R was only isolated from candidiasis infections (Xu and Samaranayake 1995). This study also described a number of new biotypes in the Chinese population that had not been described previously in Western populations. Teanpaisan *et al.* (2000) identified A1S as the predominant biotype in HIV-positive and HIV-negative individuals with or without clinical infection. Their results also indicated an increased variety of biotypes in the HIV-positive group and that the median amphotericin B MIC of isolates in this group was higher than those of the healthy group. However, there was no association between biotypes and antifungal susceptibility patterns.

Using REA and RAPD profiles, Clemons *et al.* (1997) demonstrated that Singaporean isolates of *C. albicans* are much more genetically diverse than those from Europe or the USA. Additionally, the Southeast Asian isolates were scattered among the clusters from the European and American geographic locations.

1.3.2.b Gastrointestinal candidiasis

The gastrointestinal tract is colonized with yeasts in 20% of individuals (Blinzler *et al.* 1997). *C. albicans*, *C. dubliniensis* and *C. tropicalis* have a high affinity for purified small intestinal mucin and an associated increased virulence in gastrointestinal infection (de Repentigny *et al.* 2000). Fungal infections, predominantly caused by *C. albicans*, are often associated with gastric ulcer and, less frequently, with chronic gastritis. The *C. albicans* infection has been shown to inhibit the healing process in these two diseases (Zwolinska-Wcislo *et al.* 1998).

It has been proposed that chronic gastrointestinal candidiasis may cause immune depression in many chronic fatigue syndrome patients making it one of the causal factors of the syndrome (Cater 1995) prompting an increased medical interest in this particular site of infection. There remains controversy about the existence of this infection and its over-reporting without supportive culture diagnosis (Knoke 1996). However, the incidence of carriage remains unknown and much of the aetiology of the infection is still unclear.

A decreased intestinal mucosal immunity caused by HIV infection, a decreased CD4+ count and a decreased mucosal IgA have been suggested as the major predisposing factor for *Candida* associated diarrhoea (Kakai *et al.* 1995).

1.3.2.c Vaginal candidiasis

Asymptomatic vaginal carriage of *Candida* occurs in approximately 7.3% of the healthy female population (Perera and Clayton 1994) and the species *C. albicans* colonizes 24% of women with some symptoms of vaginal infection (Eckert *et al.* 1998). Risk factors for *Candida* vaginal colonization have been reported to include frequent sexual intercourse, the use of condoms, recent antibiotic use and past gonococcal infection (eg., Eckert *et al.* 1998). There has also been a strong link made between increased oestrogen levels and the presence of *Candida* infection (Dennerstein 1998). There is no evidence that immunosuppression increases the risk of vaginal candidiasis in women (White *et al.* 1997). However, HIV-positive women have a higher incidence of the infection (Schuman *et al.* 1998) but only when their CD4+ count drops below two hundred cells/mm^3 (Duerr *et al.* 1997).

C. albicans remains the predominant causative species of vaginal candidiasis but *C. glabrata* and *C. tropicalis* are also prevalent (eg., Kaya and Kiraz 1994, Al Hedaithy and Fotedar 1997, Abu Elteen *et al.* 1997). As with oral infections, recurrent *Candida* vaginal infections may be caused by a number of different strains of *C. albicans* whose predominance changes over time, or by the same strain that may undergo minor genetic alterations during successive infections resulting from antifungal drug treatment (Schroppel *et al.* 1994). The source of sequential infections with the same infecting strain has been traced to the oral cavity of the women's male sexual partner (Lockhart *et al.* 1996). The male's oral cavity has also been identified as a source for replacement strains during successive infection causing a mixed infection at first, then becoming the dominant colonizing strain (Schroppel *et al.* 1994). Another epidemiological study showed that in some patients with recurrent vaginitis, there is a common genotype isolated from vaginal, faecal and oral samples of the infected woman and from seminal, faecal and oral samples of their male partner (Mendling *et al.* 2000). Using various molecular techniques, recurrent vaginal infection has been attributed to three different scenarios. Namely, maintenance of the original infecting strain over time, minor genetic variation in the original infecting strain and strain replacement (eg., Schroppel *et al.* 1994, Lockhart *et al.* 1996, Seoighe *et al.* 2000).

There has been no evidence of increased antifungal resistance in strains of *C. albicans* isolated from recurrent vaginal infection despite long-term antifungal treatment (Lynch *et al.* 1996). It has, however, been reported that there may be selection for *C. glabrata* strains with antifungal treatment, causing chronic or recurrent infection (D. Ellis, pers comm.).

Candida colonization has been reported to increase with pregnancy, an increased sexual activity, the use of hormonal contraception and diabetes mellitus (Margariti *et al.* 1997). Other studies have found no correlation with diabetes mellitus (Hunjak *et al.* 1997a) or the complications associated with diabetes (Hunjak *et al.* 1997b). In one study, a decrease in *Candida* colonization was noted post-menopause (Hillier and Lau 1997), whereas another found no association (Hunjak *et al.* 1997b).

Atypical *C. albicans* isolates have been identified from vaginal infections. These strains were unable to use glucosamine or N-acetylglucosamine as their sole carbon source, they were germ-tube positive and chlamydospore negative, but were still identified as *C. albicans* using RAPD profiles (Tietz *et al.* 1995).

1.3.2.d Candiduria

Candiduria, or *Candida* colonization of the urinary tract, is prevalent in 1-5% of hospitalized individuals (Munoz *et al.* 1998). Often the candiduria is associated with candidaemia (disseminated infection) (Phillips and Karlowicz 1997), in which the kidney is one of the organs involved (Munoz *et al.* 1998), and may involve the formation of renal fungal balls in the pelvis (Phillips and Karlowicz 1997). Associations have also been made with diabetes mellitus and urinary tract abnormalities (Kauffman *et al.* 2000). *C. tropicalis* and *C. glabrata* are frequently isolated from cases of candiduria (eg., Al Hedaithy and Fotedar 1997, Knoke *et al.* 1997). Between 1994 and 1998, there was an increase in the minimum inhibitory concentration (MIC) of fluconazole and the incidence of fluconazole resistance in *C. albicans* and *C. tropicalis* isolated from candiduria (Baran *et al.* 2000).

Urinary catheterisation or nephrostomy are primary risk factors for urinary tract *Candida* infection (eg., Oravcova *et al.* 1996). However, other risk factors, such as concomitant fungal infection at another site and diabetes mellitus have also been positively correlated with this infection (eg., Oravcova *et al.* 1996). Additionally, patients on antibiotics, with

indwelling catheters and diabetes mellitus are at increased risk of recurrent candiduria (Khatib *et al.* 1998).

Treatment of the patient with broad spectrum antibacterials or more than one being used concurrently, and the use of corticosteroids, quinolones, and immunosuppressive agents have all been identified as potential risk factors for urinary tract *Candida* infection (eg., Durupinar *et al.* 1996).

In one study, all but one case of recurrent infection was caused by persistence of the original infecting strain (Khatib *et al.* 1998). This result suggests reinfection from a common source or re-emergence of the symptoms associated with the infection.

1.3.2.e Systemic and disseminated *Candida* infections

Nosocomial fungal infections, usually involving infections of surgical wounds or the bloodstream, are caused by *Candida* species in 78% of cases (Grohskopf and Andriole 1996). These candidaemias are often associated with high mortality (Lundberg *et al.* 1998). The incidence of nosocomial candidaemia has continued to increase, even after the introduction of fluconazole. Unlike other types of infection, there has been an increase in the fluconazole and itraconazole susceptibility of *C. albicans* isolates obtained from these infections (Deleroziere *et al.* 2000). Some authors have reported an increase in the prevalence of *Candida* nosocomial infections since the introduction of fluconazole in the early 1990's (eg., Chen *et al.* 1997, Chiu *et al.* 1997), whilst others have reported a significant decrease during the same time (eg., Debusk *et al.* 1994).

The prevalence of nosocomial infections caused by species other than *C. albicans* has also increased. Recently, it was reported that, in the Slovak Republic, the incidence of species other than *C. albicans* isolation from fungaemia increased from 0% in 1989 to 46.3% of *Candida* species in 1998 (Krcmery and Kovacicova 2000). *C. albicans* remains the most frequently isolated *Candida* species in bloodstream infections (Pfaller *et al.* 2000). The second most prevalent species, *C. glabrata*, has been associated with increased mortality and was more commonly isolated in young females in a survey of Israeli hospitals (Rennert *et al.* 2000).

Underlying gastrointestinal disease or abdominal surgery is a predominant risk factor for peritonitis (Stratov *et al.* 1998, Hennequin 2000). The hospitalized patients most at risk are

those previously immunocompromised through HIV infection, cancer, bacterial sepsis, burns and post-operative complications (eg., Grohskopf and Andriole 1996, Dean and Burchard 1998). Additional risk factors for these individuals include the presence of a central venous catheter, prior antibiotic use and neutropenia, or low white blood cell count (Grohskopf and Andriole 1996). An increased risk that the infection will become fatal occurs when a patient is elderly, when a patient is infected with *C. parapsilosis*, when they have hypotension, if their catheter is not removed, or with no antifungal treatment (Nucci *et al.* 1998).

In the study by Shin *et al.* (2001), persistent or recurrent fungaemia due to *C. parapsilosis* was predominantly caused by a different strain at each infection. Luu *et al.* (2001) identified an immense range of genetic diversity between bloodstream isolates and clustering with oral isolates. These authors suggest that invasive potential is more dependent on host immune status than the genotype of the infecting strain. Poikonen *et al.* (2001) found multiple *C. albicans* strains in 11 bloodstream infections, also suggesting that the infection is opportunistic.

Malignancies, prematurity and congenital abnormalities are risk factors for neonatal candidiasis (Chiu *et al.* 1997). Leukaemic and neutropenic patients are more commonly infected with *C. tropicalis*, whilst patients receiving fluconazole are more likely to be infected with *C. glabrata* , suggesting that the infecting *Candida* species may be influenced by the patient's immune system (Hung *et al.* 1996). Burns patients are more likely to get *Candida* sepsis if they have large burns (14-98% of their body surface), a central venous line and respiratory problems requiring ventilator support (Fridkin and Jarvis 1996).

It has been shown that *Candida* may be carried on the hands of healthcare workers in an intensive care unit, thus providing an important source of infection for patients within the ward (eg., Flanagan and Barnes 1998, Huang *et al.* 1998). The prevalence of carriage of *C. albicans* on the hands of healthcare workers has been estimated at 17% (Voss *et al.* 1995), and of *C. parapsilosis* at 26% (Diekema *et al.* 1997). However, not all systemic infections are of nosocomial origin. One study reported that the *C. glabrata* isolated from a mixed disseminated infection with *C. albicans*, originated in the patient's oral cavity (Bougnoux *et al.* 1997).

1.4 Aims

This thesis will address some of the current conflicts of mycological research in relation to the human pathogenic fungal genus *Candida* and, in particular, the most predominant species *C. albicans*. Determining the clinical importance of different populations/strains of *C. albicans*, that is, whether there are clusters of strains that cause systemic infections, that have acquired antifungal resistance, that are more pathogenic, etc., has obvious clinical implications in the future diagnosis and treatment of *C. albicans* infections. The biological model produced using this species forms the basis for an evaluation of the genus *Candida* that can then be superimposed onto other fungal pathogens. This type of evaluation requires the use of methods for the accurate identification of theorganism to the species level, a highly discriminatory method for strain differentiation with in the species and the ability of these methods to cluster isolates into a genetic framework of relatedness. This facilitates the recognition of clinically significant genetically related groups of isolates. The biological characteristics of clinical importance must also be accurately identified. For example, the importance of *C. albicans* infections in HIV-positive individuals, the acquisition of antifungal resistance by strains of *C. albicans*, the clinical symptoms of infection and the immunocompromisation of the infected individual. Unfortunately, as with characters currently available for species identification, many of the predisposing factors and *C. albicans* strain-specific characteristics remain controversial.

To reiterate, our knowledge of the disease processes of *Candida* will be greatly enhanced by having a comprehensive understanding of the systematics, and hence, the epidemiology of this important fungal pathogen in humans. Moreover, it is important to keep in mind that *Candida* is a parasitic infection of humans, hence being influenced directly and indirectly by the host as well as other organisms co-inhabiting the sites of colonisation and infection. This is particularly complex in such sites as the oral cavity, in which there are dynamic interactions between numerous co-inhabiting organisms.

1.4.1 Specific Aim

The aim of this thesis is

To examine the systematics of the genus Candida using a combination of traditional and contemporary methodologies. These methods will also be assessed to determine their diagnostic potential to unequivocally identify and characterise species and strains of this medically and dentally important yeast genus.

1.4.2 Rationale

Why use a combination of traditional and contemporary characters to accurately identify organisms? No single character should be used to identify any genus or species prior to a comprehensive assessment of its utility in the context of an overview of the relationships obtained using all of the available information from a range of techniques. This approach stems from the discipline of systematics. A more simplified diagnostic approach can be designed once a range of different characters has been assessed concurrently and an overview of the variability within the taxa to be identified has been determined.

Each technique has its advantages and disadvantages. DNA based methods have the advantage of being universally comparable and single characters or genes can be compared at any one time, but the accuracy of the obtained phylogeny relies on the accuracy of the gene chosen to represent the molecular clock. It is not likely that the resultant genetic relatedness based on a single gene of two obviously morphologically and biochemically diverse organisms will be accepted readily. Herein lies the problem associated with the reclassification of fungi into genetically related anamorphs and teleomorphs when, according to traditional taxonomy, they are derived from biochemically and morphologically distinct genera or even phyla. The teleomorphs described so far for species within the single genus *Candida* span these taxonomic divisions.

An accurate indication of true taxonomic and phylogenetic relationships also relies on a systematic evaluation of numerous reference and clinical isolates. These isolates represent the scope of known biological diversity. Results from such a study may uncover contentious issues regarding the validity of taxa and the diagnostic characters used to define them. Without investigations of the systematics of clinically important organisms, diagnostic techniques, treatment regimes, disease prognoses, epidemiological information, and transmission mechanisms are based on limited data sets, perhaps consisting of

clinically-irrelevant reference isolates. Such issues are particularly relevant when investigating and treating potentially pathogenic organisms in human disease. Once the correct systematics of the genus, and the species within it, has been determined, an accurate epidemiological investigation can be conducted.

1.4.3 Specific objectives

The specific aim of this thesis will be addressed by achieving the following specific objectives;

(a) to evaluate and optimise the method of allozyme electrophoresis (syn. multilocus enzyme electrophoresis) to study yeast genetics and then to apply this method;

(b) to determine the taxonomy of the genus *Candida* and related genera and

(c) to assess the epidemiological and diagnostic utility of allozyme electrophoresis for *C. albicans* and related species. Then appropriate isolates will be selected on the basis of the derived genetic framework

(d) to determine the phylogenetic and taxonomic utility of potential molecular clocks and

(e) to determine the epidemiological utility of highly variable molecular sequences.

The following Chapters encompass the general materials and methods used throughout this thesis; minor method modifications, results and interpretations for each experimental component; and a general overview discussion. The discussion addresses the practical diagnostic utility of the methodologies assessed and the impact the results will have on current and future approaches to studying the systematics of fungi, particularly *C. albicans*.

2 GENERAL MATERIALS AND METHODS

Traditional and contemporary methods were used to identify and characterise isolates of *Candida* and related genera in order to address the specific objectives outlined in Chapter 1. Chapter 2 encompasses descriptions and protocols for the specific methods used throughout the thesis. Appendix 2 provides the chemical formulae and full details of the isolates used throughout this thesis.

The type and reference strains described in this thesis were originally derived from single colonies of each sample prior to their deposition in the culture collections. It was therefore assumed that the cultures obtained from reference collections were pure cultures and their purity upon receipt was only checked using cellular morphology. In order to address the issue of mixed colonization in clinically derived samples obtained during the current study, multiple well-isolated colonies were selected and subcultured independently. The cell lysates of these single colonies were then analysed as a pooled sample. This prevented the genetic selection of one of the types present in a mixed sample during culturing prior to its analysis in the current study.

Each individual colony used in this thesis was initially identified using traditional biochemical and morphological characters as described in section 2.1.3 (p., 51). The type and reference strain identities were assumed to be correct upon supply of the strain from the reference collection. The clinical samples were initially identified as yeasts from their colonial and cellular morphologies then assessed for their ability to form germ-tubes, which identified them as either *C. albicans* or another species of *Candida*. Each sample was then subjected to allozyme electrophoresis (section 2.2.2 p., 54-58) to determine its genetic relatedness to the type and reference strains. This method has proven successful for this type of research in bacteria (eg., Selander *et al.* 1990), and has resulted in the recognition of bacterial cryptic species and species complexes (as discussed in the Introduction). Particular samples were then selected on the basis of the allozyme electrophoresis results (presented in Chapter 3, p., 65) for molecular analysis. The DNA targets for this analysis included the 18S rRNA gene and the two highly variable flanking regions of the 5.8S rRNA gene, ITS1 and ITS2. The 18S rRNA gene has been well recognized as a potential molecular clock due to its high conservation (eg., Hendriks *et al.* 1991), the ITS regions are more variable and have been suggested as discriminatory below the species level (eg., Lin *et al.* 1995), and the 5.8S rRNA gene is used as a conserved anchoring point for their accurate

sequence alignment. The approach used therefore encompasses traditional and contemporary methods with a range of levels of discriminatory power, each result contributing different information and allowing a preliminary evaluation of current methods used to determine the identity and epidemiology of *Candida*.

2.1 Sample collection, growth and preparation

2.1.1 Type and reference strains of Candida and related genera

The type and reference isolates used in this thesis and their original environmental or clinical sources are given in Table 2.1 (p., 49). These isolates comprise the thirteen medically-relevant species of *Candida* (Section 1.2.3 p., 14); a representative of *Saccharomyces cerevisiae* which represents a sister taxa to *Candida* (see Cai *et al.* 1996, James *et al.* 1997); and two basidiomycete species *Cryptococcus neoformans* and *Trichosporon beigelii*, providing a distant fungal taxonomic relationship,. Thelatter 3 species and their clinical relevance are described in section 2.1.2 (p., 48). The *Candida* reference and type strains used in the pilot studies were obtained from the Mycology Unit of the Women's and Children's Hospital (WCH), North Adelaide, South Australia. Subsequently, these and additional reference strains were purchased from the American Type Culture Collection, Manassas, VA, USA (ATCC strains) and the Centraalbureau voor Schimmelcultures, Baarn, Netherlands (CBS strains). It is important to re-iterate at this point that type strains are the only true representative of a species and all other isolates, including reference strains, are identified according to their similarity with these strains. The appropriateness of designated reference strains relies on the accuracy of the method initially used to identify it.

2.1.2 Non-Candida fungal species of clinical relevance

There are a number of opportunistic fungal pathogens that do not belong to the genus *Candida*, but that are of increasing clinical importance in human infection. Following is a description of the identifying characteristics and clinical relevance of the non-*Candida* species used in this study, as detailed in the "WCH Mycology Online" web site (http://www.mycology.adelaide.edu.au/ last accessed 8/01/02). The defining characteristics

Table 2.1; Details of the reference and type strains analysed in this thesis

Isolate	Species	Site of isolation
ATCC 90028	*C. albicans*	blood, Iowa
CBS 562^{T} [a]	*C. albicans*	skin (interdigital mycosis), Uruguay
17484	*C. albicans*	Human
ATCC 90029	*C. albicans*	blood, Iowa
CBS 834^{T}	*Kluyveromyces marxianus (C. kefyr)* [b]	kefyr grains, Netherlands
ATCC 46764	*C. kefyr*	clinical isolate, Texas
15526	*C. dubliniensis*	Human
CBS 7987^{T}	*C. dubliniensis*	oral cavity, HIV-positive, Ireland
CBS 4024^{T}	*C. viswanathii*	meningitis, cerebrospinal fluid, India
ATCC 22981	*C. viswanathii*	cerebrospinal fluid, India
CBS 566^{T}	*Pichia guilliermondii (C. guilliermondii)*	Sputum
ATCC 6260	*C. guilliermondii*	Bronchomycosis
CBS 604^{T}	*C. parapsilosis*	case of sprue, Puerto Rico
ATCC 22019	*C. parapsilosis*	case of sprue, Puerto Rico
ATCC 90018	*C. parapsilosis*	blood, Virginia
CBS 6564^{T}	*Pichia norvegensis (C. norvegensis)*	pregnant woman's vagina, London
CBS 138^{T}	*C. glabrata (Torulopsis glabrata)*	human faeces
ATCC 90030	*C. glabrata (Torulopsis glabrata)*	blood, Iowa
CBS 6936^{T}	*Clavispora lusitaniae (C. lusitaniae)*	citrus essence, Israel
ATCC 42720	*C. lusitaniae*	blood, myelogenous leukaemia patient, California
CBS 94^{T}	*C. tropicalis*	Bronchomycosis
ATCC 13803	*C. tropicalis*	
CBS 573^{T}	*Issatchenkia orientalis (C. krusei)*	sputum, bronchitic convict, Sri Lanka
ATCC 6258	*C. krusei*	sputum, bronchitic convict, Sri Lanka
CBS 5149^{T}	*C. haemulonii*	gut of *Haemulon sciurus* (fish), Florida
CBS 940^{T}	*C. famata*	air, Japan
ATCC 2601	*Saccharomyces cerevisiae*	
CBS 6289^{T}	*Cryptococcus neoformans* var. *gattii* [c]	spinal fluid of man, Zaire
ATCC 90113	*Cr. neoformans* var. *gattii*	cerebrospinal fluid, Pennsylvania
CBS 132^{T}	*Cr. neoformans var. neoformans* [c]	fermenting fruit juice
ATCC 90112	*Cr. neoformans var. neoformans*	cerebrospinal fluid, Pennsylvania
CBS 2466^{T}	*Trichosporon beigelii*	

[a] ^{T} = type strain of the species

[b] Species names in brackets are the synonymous or teleomorph species names of the isolates supplied.

[c] *Cr. neoformans* var. *gattii* and *Cr. neoformans* var. *neoformans* are the two recognized serovars of the species.

[d] The following pairs of strains originated from the same strain; CBS 4024 and ATCC 22981, CBS 566 and ATCC 6260, CBS 604 and ATCC 22019, CBS 573 and ATCC 6258.

described have been used by the Mycology Unit at the WCH for the identification of isolates in this thesis.

2.1.2.a *Saccharomyces cerevisiae* Meyen ex Hansen

Saccharomyces cerevisiae is an ascomycete and is commonly used in bread, cheese, beer and wine production. This species is rarely isolated from human infection but it has been suggested that some strains within the species are better adapted than others to cause infection (McCullough *et al.* 1998). According to phylogenetic information, is the fungal species most closely related to the genus *Candida*. Colonies of *S. cerevisiae* have the same appearance as *Candida* on Sabouraud's dextrose agar; that is they are white to cream, smooth, glabrous and yeast-like. Microscopic morphology shows large, globose to ellipsoidal budding yeast-like cells or blastoconidia. This cells usually exists as budding blastospores but can form pseudohyphae. No associated teleomorph has been identified for the species.

Genetic variation within the genus *Saccharomyces* is evident from telomeric repeat sequence comparisons (Cohn *et al.* 1998). However, these sequences also possess a conserved core sequence with genetic identity to *Candida glabrata*, which supports the close phylogenetic proximity of the genera *Candida* and *Saccharomyces*.

2.1.2.b *Cryptococcus neoformans* (Sanfelice) Vuillemin

Unlike the ascomycete genera discussed previously, *Cryptococcus neoformans* belongs to the phylum *Basidiomycetes* because it produces basidiospores. The teleomorph for this species is *Filobasidiella neoformans*. The species *Cr. neoformans* has been divided into two well-defined serogroups *Cr. neoformans* var. *gattii* and *Cr. neoformans* var. *neoformans*. Associated with this, the teleomorph has also been separated into two varieties, which have subsequently been confirmed as distinct mating types. The two serovars are also distinguishable biochemically, with *Cryptococcus neoformans* var. *gattii* turning Canavanine-glycine-bromothymol from clear to blue (CGB) agar blue while *Cr. neoformans* var. *neoformans* unable to. A number of molecular techniques that are able to discriminate between the two serovars of this species have also been described (eg., Meyer *et al.* 1993, Brandt *et al.* 1995, Sandhu *et al.* 1995).

Cr. neoformans is most commonly isolated from respiratory tract and nervous system infections. Non-immunocompromised individuals are more often infected with *Cryptococcus neoformans* var. *gattii*, whilst *Cryptococcus neoformans* var. *neoformans* is more frequently isolated from immunocompromised individuals, particularly those who are HIV-positive. The latter serovar is also the most common causative organism of fungal meningitis.

Cryptococcus neoformans var. *gattii* has been associated with *Eucalyptus* species, particularly *E. tereticornis*, which has been identified as the natural habitat of this cryptococcal serovar (ref.). On the other hand, the source of infection for *Cryptococcus neoformans* var. *neoformans* has been identified as avian excreta and particularly weathered pigeon droppings (ref.).

2.1.2.c *Trichosporon beigelii* (Kuchenmeister & Rabenhorst) Vuillemin

Trichosporon beigelii is also a basidiomycete species. *Trichosporon cutaneum* Ota is a synonym of this species. *Trichosporon beigelii* has colonies that appear white or yellowish to deep cream in colour, smooth, wrinkled, velvety and dull in appearance with a mycelial fringe on Sabouraud's dextrose agar. This species forms abundant pseudohyphae and some true hyphae segmenting into arthroconidia. The genomic G+C content of the species ranges from 60.7% to 63.5%.

This species is normally associated with skin and is a common environmental isolate. It usually causes disseminated opportunistic infections in immunocompromised individuals, particularly those who have cancer or are HIV-positive. The infections often cause lesions in organs.

2.1.3 Candida clinical sample collection and culturing

For the purposes of this thesis, one hundred HIV-positive patients and forty HIV-negative individuals were sampled by methods described below. Clinical samples were collected for assessing; the prevalence of different *Candida* species in HIV-positive and HIV-negative individuals with and without obvious candidal lesions; the rate of mixed colonization; and the genetic variability within and between the species of *Candida*, particularly *C. albicans*, isolated from these individuals. The full details of the origins of all

clinical strains used in this thesis are contained within Appendix 2 (Table 1) and the details of the particular strains used for each component of the current study are summarized in each of the relevant Sections of the Results Chapter. The following is a description of the sampling strategy employed for their collection, cultivation and preparation.

Clinical *Candida* samples from the oral cavities of HIV-positive individuals with and without obvious candidiasis infection were obtained using an oral rinse protocol. Some of these patients were participating in a Fungilin® lozenge trial (see http://www.thebody.com/pwa/ampho.html for a description of various Amphotericin B delivery systems and dosages). The subjects were asked to mouthrinse for 30 seconds with 10mL of sterile distilled water, which was then collected in a sterile universal container. This technique is acknowledged to be an appropriate method for the recovery of *C. albicans* from the mouth (McCullough and Hume 1995). Also, any obvious candidal lesions were sampled separately using a cotton swab. All of the collected samples were handled microbiologically, as described below. For sampling from HIV-negative individuals, 0.5ml of stimulated, undiluted saliva was used instead of the oral rinse sample. Since it has been estimated that 40% of healthy individuals asymptomatically carry *Candida* and the numbers of colony forming units is predicted to be lower, a more concentrated saliva sample was required from HIV-negative individuals. The prevalence of carriage of *Candida* in this population was also determined during this phase of the experimentation.

All samples were plated onto selective Sabouraud's dextrose agar plates containing chloramphenicol and gentamycin, purchased from Medvet Science Pty. Ltd. (Adelaide, South Australia). Six well-separated colonies from the culture positive samples from HIV-negative asymptomatic carriers were chosen at random. Preliminary species identification of colonies involved determining the ability of the sample to form germ-tubes which identified the sample as *C. albicans* or another *Candida* species. The six colonies were then re-plated separately onto Sabouraud's dextrose agar for confluency and grown at room temperature for two to three days, after which they were harvested in 2mL of sterile distilled water. Clinical samples from HIV-positive individuals were purified in the same way at the IMVS diagnostic unit and positive cultures were forwarded to the Mycology Unit of the WCH for the germ-tube test. Six colonies were chosen to determine the incidence of mixed colonization and to allow effective statistical analyses. Once the clinical samples had been preliminarily identified and recultured on Sabouraud's dextrose agar, they were forwarded from the WCH along with limited patient details. The clinical isolates were also harvested

in 2mL of autoclaved distilled water and all cultures were stored at room temperature. The purity of isolates was confirmed during manipulations using Gram staining, cell morphology, and growth on selective media.

Additional clinical isolates collected prior to the commencement of this project were also provided from a culture collection of the Mycology Unit at the WCH. These isolates were obtained from various sites of infection as stated in Appendix 2 (Table 1). Originally, single well-separated colonies had been sent to the WCH where their species identity was determined and they were subsequently stored in glycerol. For the purposes of this thesis, particular strains were chosen for their biological relevance, taken from this storage, grown to confluency of Sabouraud's agar slopes for inclusion in the study. These isolates were also stored in 2ml of autoclaved distilled water until required for experimentation.

2.1.4 Sonication and cell lysate preparation for allozyme electrophoresis

Samples were taken from water storage and regrown on Sabouraud's dextrose agar plates for twenty-four to forty-eight hours at room temperature. Their purity and identity were confirmed using Gram staining. Confluent cell growth was then harvested in approximately 1 ml of 100 mM Tris HCl pH 7.4, and the cells were pelleted using centrifugation. An appropriate volume of lysis buffer (5-50µl; dependent on the cellular volume collected after centrifugation), consisting of 0.1% β-mercaptoethanol and 100µgml^{-1} NADP in 100 ml H_2O, was added and the cells were disrupted by ultrasonication. The cell debris was pelleted using centrifugation and the resultant cell lysates were collected in capillary tubes in approximately 2µl aliquots and stored at -20°C until electrophoresed.

2.1.5 DNA preparation

The cellular debris pellets obtained during the preparation of lysates for allozyme electrophoresis (after sonication and removal of the lysate) were also stored at -20°C. These cell pellets were subsequently used as a source of DNA for the molecular component of this thesis. Either the cell pellet itself was used in the PCR, or the DNA in the cell pellet was partially purified using Chelex beads (Appendix 2, Table 2).

2.2 *Techniques*

Traditional and contemporary techniques were used in the research component of this thesis. The traditional methods included those currently used in mycological diagnostics such as determination of the biochemical profile and cellular and colony morphology. Although there are a number of alternative contemporary methods available for species and strain discrimination, the objectives of the present study did not allow assessment of the utility and applicability of every technique. However, this thesis has focused on the techniques of allozyme electrophoresis and sequence comparisons of various segments of the ribosomal RNA gene region with differing levels of genetic conservation. The justification for the choice of these techniques have been stated throughout this thesis (ie., allozyme electrophoresis Sections 2.2.2 p., 54, 3.1 p., 65 and 3.2 p., 93; 18S rRNA sequence comparisons Sections 1.1 p., 2 and 3.5 p., 200; ITS 1 and 2 sequence comparisons Sections 1.3.2 p., 30 and 3.6 p., 225).

Each of the following methods is a standardized protocol. Any minor modifications are addressed in the relevant subsections of Chapter 3 (p., 65).

2.2.1 Traditional identification techniques

Each isolate obtained was identified to the species level according to traditional characters as described above. Isolates were initially exposed to 0.5ml of horse serum containing 0.8% glucose, for 2hrs at 37°C, to determine their propensity to form Germ tubes, a diagnostic character for *C. albicans*. In the present study, if an isolate was germ tube positive it was presumed to be *C. albicans* but if it was germ tube negative the isolate was further typed using the API 20C biochemical assimilation methods. Although the utility and diagnostic value of the germ-tube test has been questioned (Section 1.2.3 p., 14), it is still widely used as a diagnostic tool. The preliminary identities of isolates that were obtained were used as a basis for further identification analyses in this thesis.

2.2.2 Allozyme electrophoresis

Allozyme electrophoresis is the differential migration of non-denatured proteins, particularly enzymes, across a support medium under the influence of an electrical charge (Andrews and Chilton 1999). A difference in the net charge of proteins, their size and/or shape influences the point of their migration. The charge of the protein is also influenced by

the pH of the buffer used for the electrophoresis (ie., the running buffer), in a similar manner to isoelectric focussing. The differences in net charge are attributable to differences in the secondary (amino acid) structure of the enzymes, which are a reflection of the primary (codon) structure of the protein. The separated enzymes are visualized on gels using histochemical stains that rely on the activity of the specific enzyme they detect. Differences in the banding patterns of enzymes therefore indicate differences in the genomic sequence of the enzymes. It should be noted however that the reverse is not an assumption; that is, identity in the migration position is not necessarily indicative of identity in genomic sequence. Redundancies in the genetic code and similarities in the charges of different amino acids will not be detected (Selander and Whittam 1983, Graur 1986).

The metabolic enzymes investigated using allozyme electrophoresis are independent (ie., not genetically-linked) and spread across the genome. The number of loci at which two isolates differ from each other is used to infer the genetic relatedness of the isolates ie., the more alleles two isolates differ at, the more distantly related they are to each other. A recent review by Andrews and Chilton (1999) further discusses the interpretation of allozyme electrophoresis results. Since each enzyme represents an independent genetic character, it is more important to ensure that an adequate number of loci are assessed (ten to fifteen according to Selander and Whittam 1983) than a large number of isolates assessed at few loci.

2.2.2.a Electrophoresis and histochemical staining methods

The cell lysates for each sample were loaded onto cellulose acetate ('Cellogel', Chemetron, Milan) and electrophoresed at 200 V for 1¼ hrs at 4°C. Enzymes were histochemically stained according to the methods of Richardson *et al.* (1986) and Selander *et al.* (1986) except for some minor modifications noted in Chapter 3 (p., 65). After screening thirty-seven enzyme loci using a small selection of isolates (Appendix 2, Table 3), a panel of twenty enzymes was selected for use in the majority of allozyme electrophoretic analyses. This panel of enzymes had sufficient staining intensity, resolution and diagnostic potential. The optimisation of this technique is further explained in Section 3.1 (p., 65). The enzyme identities, EC numbers and the most appropriate running buffer for their electrophoresis are detailed below in Table 2.2 (p., 56).

Table 2.2; Enzyme loci that were histochemically stained, their E.C. numbers and the running buffer used in the present study. The running time for all loci was 1¼ hrs

Enzyme locus	E.C. number	Running buffer[a]
alcohol dehydrogenase (ADH)	1.1.1.1	TM
aldolase (ALD)	4.1.2.13	TM
enolase (ENOL)	4.2.1.11	Phos
esterase (EST)	3.1.1.1	TM
fructose diphosphatase (FDP)	3.1.3.11	Phos
fumarase (FUM)	4.2.1.2	TM
glutamate dehydrogenase (GDH)	1.4.1.3	Phos
glucose-6-phosphate dehydrogenase (G6PD)	1.1.1.49	Phos
glucose phosphate isomerase (GPI)	5.3.1.9	Phos
hexokinase (HK)[b]	2.7.1.1	TM
inosine dehydrogenase (IDH)	1.1.1.42	Phos
nucleoside diphosphate kinase (NDPK)	2.7.4.6	Phos
nucleoside phosphorylase (NP)	2.4.2.1	Phos
leucine-alanine peptidase (PepA)	3.4.11 or .13	CP
leucine-glycine-glycine peptidase (PepB)	3.4.11 or .13	CP
phosphoglycerate mutase (PGAM)	2.7.5.3	Phos
6-phosphoglucose dehydrogenase (6PGD)	1.1.1.44	Phos
phosphoglucomutase (PGM)	2.7.5.1	Phos
pyruvate kinase (PK)	2.7.1.40	Phos

[a] Running buffers are annotated as follows; TM is 0.05 M Tris maleate buffer pH 7.8, Phos is 0.02 M phosphate buffer pH 7.0 and CP is 0.01M citrate phosphate buffer pH 6.4.
[b] Two HK loci were evident for most species in the present study.

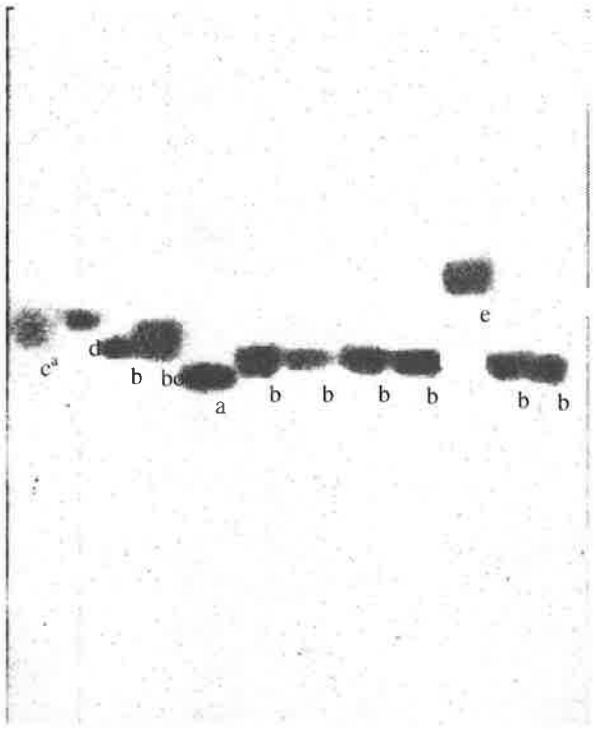
2.2.2.b Analytical methods

The interpretation of the banding patterns obtained can be a predominant source of inaccuracy when using allozyme electrophoresis. Prior knowledge of the quaternary structure of the enzyme and the purity of the culture are vital to avoid misinterpretation. Additional factors that need to be taken into account include the presence of multiple loci and multiple alleles for the enzyme, imperfect resolution of the banding pattern, warping on the gel, differential staining intensity across the gel and post-translational modification of the enzyme (Andrews and Chilton 1999). The banding patterns of species and genera will also differ according to the zygosity of the organisms (Figure 2.1 p., 58). Additionally, differences in the number of bands observed between isolates may indicate contamination of a sample.

The banding patterns may be interpreted using zymodemes (ie., the phenotype of banding patterns of all enzyme examined are compared between isolates) or allozymes (ie., genotypic interpretation for each locus, where different alleles are assigned to different allozymes) (see Andrews and Chilton 1999 for further discussion). For the purposes of the current study, allozymes were used and scored in order of increasing anodal migration. The resultant allelic profiles were statistically analysed using pairwise comparisons of the unweighted pair group method using arithmetic averages (UPGMA) (Sneath and Sokal 1978). This gave rise to a matrix of the percentage of fixed differences between isolates, or the number of enzyme loci at which two strains shared no alleles in common. The data from the matrix was then combined and diagrammatically represented in the form of a phenogram.

There are a number of distance measures that can be used to estimate the genetic difference between isolates, including fixed differences, Nei's D values and Roger's distance. For this study, the most appropriate method was fixed differences because the basic assumption it makes is that each isolate is a distinct population that is genetically and evolutionarily isolated. Conversely, the other two distance measures allow the inclusion of multiple isolates into a population and then a comparison of populations. A recent review by Andrews and Chilton (1999) further addresses this issue and the application of allozyme electrophoresis for genetic studies.

Figure 2.1; Typical enzyme activity banding patterns observed in the current study using allozyme electrophoresis and their allelic interpretation



a; Cell lysates were loaded at the bottom (cathodal) end of this gel. Each band from left to right represents a different sample. The dark staining bands are the electrophoretic position that the glucose-6-phosphate enzyme has migrated to, which was subsequently detected using histochemical staining. The allelic designations then assigned to each of these samples are denoted beneath each band. The fourth sample from the left has two bands of activity for this enzyme and is therefore assumed to be either a mixed culture or heterozygous for this locus.

2.2.3 Molecular biological methods

The molecular biology component of the current study comprised methods aimed at determining the genetic relationships between and within *Candida* species and related genera, and strain variation within a single patient in a mixed culture or over time with infection treatment.

2.2.3.a 18S ribosomal RNA sequencing

The primers used for sequencing the 18S rRNA gene in the current study were designed from all of the complete sequences for this gene available on Genbank for the genus *Candida*, particularly the species and related genera used in this thesis (Section 3.5.2.b, Table 3.45, p., 202). Genbank sequences were aligned, both manually and using the Malign program, and areas of conserved homology between the sequences were assessed for their suitability as primer binding sites using DNASIS (version 7.0, Hitachi Software Engineering Company Ltd, 1991). The final primer design for amplifying the entire 18S rRNA gene incorporated six overlapping primers, three 5' and three 3', as depicted in Figure 2.2 below (p., 61). Each PCR product overlaps with the next forming a continuous sequence of the entire gene. The alignment of Genbank sequences produced a consensus sequence and the sizes of the PCR products estimated from this consensus sequence are; M18S1-M18S6 491bp, M18S5-M18S3 550bp, M18S4-M18S2 683bp. The sequences of each primer and the sequence base number that they bind to, are contained within Table 2.3 (p., 62). Primers were purchased from BRESAtec, (Thebarton, South Australia) at the 40nmol scale in a reverse phase purified form. The primers were diluted to a concentration of 1mg/ml with milliQ water. PCR amplifications were conducted using a Perkin Elmer GeneAmp PCR System 24000 according to the protocol in Table 2.4 (p., 62).

2.2.3.b 5.8S ribosomal RNA and flanking region sequencing

As was the case for the 18S rRNA gene sequencing, cell pellets of appropriately selected isolates were used as DNA sources for the amplification and sequencing of the 5.8S rRNA gene and the internal transcribed sequences (ITS) flanking this gene within the ribosomal RNA coding region. The primer binding sites were located within the 18S and 26S rRNA genes on either side of the ITS regions (Figure 2.3 p., 63). The rRNA subunit genes are

highly conserved for functional reasons and therefore make ideal stable binding sites. The primer sequences used to amplify these regions were as used by White *et al.*(1990), namely;

ITS4 - 5' TCC TCC GCT TAT TGA TAT GC 3' – forward primer

located at the 3' end of the 18S rRNA gene

ITS5 - 5' GGA AGT AAA AGT CGT AAC AAG G 3' – reverse primer

located at the 5' end of the 26S rRNA gene

The PCR mix and protocol were as used for the amplification of the 18S rRNA gene, contained within Table 2.4 (p., 62).

2.2.3.c Sequencing methods

The successful amplification of PCR products was confirmed using agarose gel electrophoresis (on 0.8% agarose TAE gels) following standard procedures (Sambrook *et al.* 1989). Positive PCR products were purified and the primer removed using the BRESAclean DNA purification kit (Bresatech Pty Ltd, Thebarton, South Australia) according to manufacturers instructions. The presence of clean PCR products was again confirmed using agarose gels. Once the products were cleaned, a sequencing PCR reaction was performed using an Applied Biosystems Prism Ready Dye Terminator sequencing kit, the protocol for which was common to all PCR products in the current study and is contained within Appendix 2 (Table 4). PCR products for sequencing were then ethanol precipitated (Appendix 2, Table 5) and the sequence of the products was determined using an Applied Biosystems automated DNA sequencer model 373A and, later, model 377.

The sequences generated were aligned using the SeqEd package from Applied Biosystems. The forward and inversion of the reverse sequence were compared to obtain a consensus 5' to 3' sequence of the gene of interest. The derived sequences were then compared with published sequences to confirm the accuracy of the gene amplification.

2.2.3.d Analysis programs

The sequences obtained for the same gene from different isolates were aligned using ClustalW (Thompson *et al.* 1994) using the default program settings. This analysis produced a phenogram using UPGMA (Sneath and Sokal 1978). The sequences obtained for the complete 18S rRNA gene were also phylogenetically assessed using a range of computer packages namely, PHYLIP version 3.5c (J. Felsenstein), PAUP* version 4.0.0d49 for DOS (D.L. Swofford) and MEGA version 1.01 (S. Kumar, K. Tamura, and M. Nei).

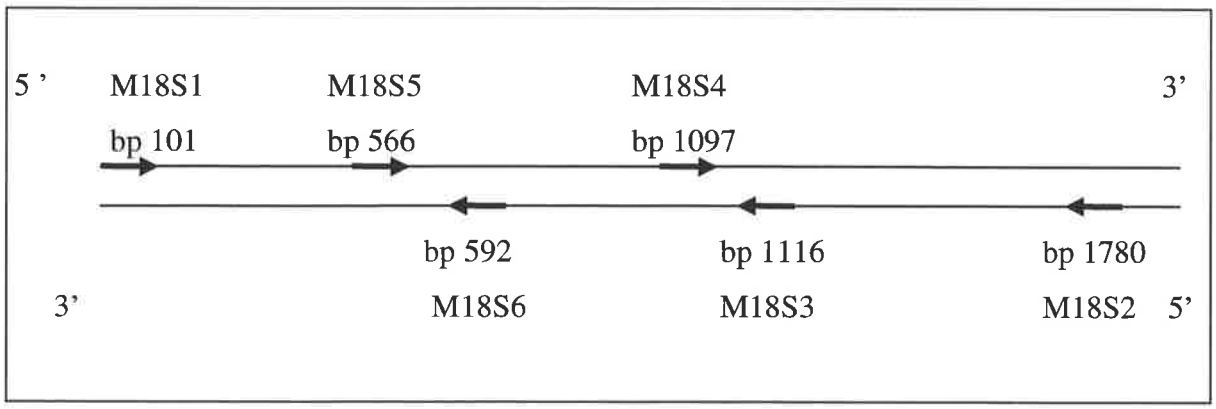


Figure 2.2; Primer binding positions along the length of the 18S rRNA gene of *Candida* and related genera used to amplify the entire gene

Table 2.3; Primer sequences for amplification of the 18S rRNA gene from *Candida* and related genera, the base they bind to according to previously published Genbank sequences and the direction they amplify

M18S1 - 5' TTA AAT CAG TTA TCG TTT ATT TG 3' bp 101 of sequence, forward
M18S2 - 5' CTT CCG CAG GTT CAC CTA CG 3' bp 1780 of sequence, reverse
M18S3 - 5' CAG CCT TGC GAC CAT ACT CC 3' bp 1116 of sequence, reverse
M18S4 - 5' GGA GTA TGG TCG CAA GGC TG 3' bp 1097 of sequence, forward
M18S5 - 5' AGC AGC CGC CRT AAT TCC AG 3' bp 566 of sequence, forward
M18S6 - 5' TTT GGA GCT GGA ATT ACC GC 3' bp 592 of sequence, reverse

Table 2.4; PCR mix and protocol for the amplification of the 18S ribosomal RNA gene from *Candida* species and related genera

PCR mix (per sample)	PCR protocol
31.5µl H_2O	95°C for 5 minutes
5µl Amplitaq Gold buffer II	40 cycles of 94°C for 30 seconds
4µl Amplitaq Gold $MgCl_2$	55°C for 30 seconds
4µl of dNTPs	72°C for 1 minute
2µl of each primer	72°C for 8 minutes
0.5µl Amplitaq Gold	hold at 4°C
1µl DNA sample	

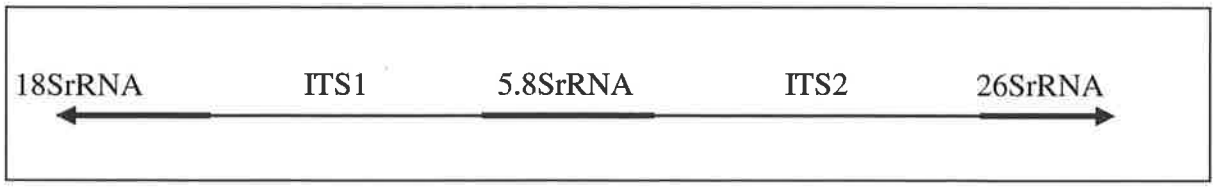


Figure 2.3; Order of the coding regions for the ribosomal RNA subunits, 18S, 5.8S and 26S, and the transcribed flanking regions ITS1 and ITS2

A number of different analytical methods were used because each program has different assumptions and may be based on different models of evolution. The methods used included distance measures, where the evolutionary distance between isolates is estimated, and character-based methods, where each nucleotide is considered a different character under its own selective pressures. A consensus phylogenetic relationship was derived from bootstrap analyses using 500 replicates and CONtree was used to construct a diagrammatic representation or phenogram of the relationships. This approach was utilized, as it is more likely to minimize the individual biases of the different analysis techniques and result in a more accurate phylogenetic relationship. The partial 18S rRNA, ITS1, 5.8S and ITS2 sequences were phylogenetically assessed using PHYLIP version 3.5c (J. Felsenstein). The relationships were obtained using maximum likelihood analyses. Bootstrap values for all of the phylogenetic consensus trees were obtained using seqBOOT (J. Felsenstein) based on five hundred replications.

3 RESULTS AND INTERPRETATIONS

The following subsections address the specific objectives of this thesis (Section 1.4.3 p., 46) and include justification for, and any modifications of the methods described in Chapter 2 (p., 47). Specific interpretations are included in each subsection and are discussed in general terms in Chapter 4 (p., 254).

3.1 Evaluation and optimisation of allozyme electrophoresis for taxonomy and epidemiology

Allozyme electrophoresis has previously been applied to study the epidemiology of *C. albicans* (eg., Boerlin *et al.* 1996, Reynes *et al.* 1996). This technique has been described as more discriminatory than DNA-DNA sequence complementarity estimations in studying the intraspecific variation among yeasts (eg., Boerlin *et al.* 1995, Le Guennec *et al.* 1995, Arnavielhe *et al.* 1997). Allozyme electrophoresis has also recently been used for epidemiological investigations (eg., Mata *et al.* 2000, Arnavielhe *et al.* 2000). A study by Pujol *et al.* (1997) found that allozyme electrophoresis, RAPDs and repetitive DNA fingerprinting were equally discriminatory for clustering *C. albicans*, resolving down to the point of determining microevolution within a single strain. However, according to the literature, allozyme electrophoresis has not previously been used to investigate the systematics of the genus *Candida*. In this thesis, allozyme electrophoresis was applied to establish an accurate genetic framework for the genus, with particular reference to the species *C. albicans*. This study involves using isolates from the range of known genetic diversity to investigate the taxonomy of the genus *Candida* and the epidemiology of infection. It is proposed that such a framework will facilitate the unequivocal identification and characterization of "strains and species" of this medically and dentally important yeast genus. The subsections of this Chapter comprise optimisation of allozyme electrophoresis, assessment of the reproducibility of the technique and taxonomic and epidemiological investigations within the genus *Candida*. The appendix for this Chapter (Appendix 3) contains matrices of pairwise comparisons of allelic profiles for each isolate, used to construct the phenograms. Portions of these matrices, where multiple isolates from a single patient are directly compared, are also presented in this Chapter.

Table 3.1; Page numbers for the isolate descriptions, phenograms and interpretations for each allozyme electrophoresis component of this thesis

Study	Isolate description	Phenogram	Interpretation
Preliminary	Table 3.2, 70	Figure 3.1, 72	74
Pilot 1	Table 3.4, 78	Figure 3.3, 80	82
Pilot 2	Table 3.7, 85	Figure 3.4, 87	88
General interpretation			90
Taxonomy	Table 3.10, 96	Figure 3.5, 98	99
Clinical *Candida* genus	Table 3.12, 104-5	Figure 3.6, 107	108
General interpretation			108
Study 1	Table 3.14, 113-4	Figure 3.7, 117	118
Study 2	Table 3.16, 120-1	Figure 3.8, 123	124
Study 3	Table 3.18, 125	Figure 3.9, 127	129
Study 4	Table 3.21, 131-2	Figure 3.10, 135	137
Study 5	Table 3.24, 140	Figure 3.11, 142	144
Study 6	Table 3.27, 146-7	Figure 3.12, 150	152
Study 7	Table 3.30, 156-7	Figure 3.13, 160	162
Study 8	Table 3.33, 165-6	Figure 3.14, 169	171
Study 9	Table 3.36, 174-5	Figure 3.15, 178	181
Study 10	Table 3.39, 185-6	Figure 3.16, 189	191
Study 11	Table 3.42, 194	Figure 3.17, 196	197
General interpretation			197

3.1.1 Pilot screen

3.1.1.a Background

Prior to analysing vast numbers of reference, type and clinical isolates, a panel of the most appropriate enzyme loci for the majority of allozyme electrophoresis analyses needs to be identified. This was performed using a small panel of representative samples to screen a large number of enzyme loci from different metabolic pathways. This electrophoretic screen ensures that large numbers of isolates are assessed for only the informative enzyme loci.

The **aim** was thus;

- *to determine the diagnostic value of allozyme electrophoresis within the species C. albicans and a selection of related species; and*
- *to identify an appropriate panel of enzyme loci and optimise their electrophoresis and staining conditions.*

3.1.1.b Materials and methods

A pilot screen of thirty-seven potentially diagnostic enzyme loci was conducted (Appendix 2, Table 3) using 12 clinical isolates. The isolates used for the initial screen are detailed in Table 3.2 (p., 70). The electrophoresis and staining conditions were varied where necessary (ie., running buffer, electrophoresis times and stain components) to improve the enzyme staining intensity and resolution (Appendix 2, Table 3).

Once enzyme loci with sufficient staining intensity, resolution and separation had been selected, the pilot screen incorporated an assessment of a number of sample preparation variables. The variables assessed were the storage method of isolates (glycerol versus water), growth conditions for the cells (incubation temperature, culturing media) and method for the lysis of the cells (freeze-thawing versus sonication versus both). Three isolates, representing the range of genetic diversity observed in the first part of the pilot screen, were selected to examine the effect of these parameters (Section 3.1.1.c p., 68). The results obtained using variants of each parameter were compared to those already obtained in the primary screen of enzyme loci.

3.1.1.c Results

All twelve isolates in Table 3.2 (p., 70) were used to screen the thirty-seven loci outlined in Appendix 2 Table 3. Twenty of these loci (Table 2.2 of the Material and Methods Chapter, p., 56) were identified as discriminatory, with sufficient staining intensity and resolution to be reliably and accurately scored. These enzyme loci have the potential to be diagnostic within the genus *Candida* and related genera.

The allelic profiles obtained for the twelve isolates used in the pilot study at the panel of twenty loci selected for continued use in this thesis are in Table 3.3 below (p., 71). The matrix of their percentage fixed differences is in Appendix 3 (Table 1) and the phenogram that was constructed is in Figure 3.1 (p., 72). The twelve isolates differed at 21% to 100% of the twenty loci scored. Five distinct clusters of isolates could be discriminated with fixed differences at greater than 54.2% of the loci. The first cluster contained isolates 12716, 17130, 18502, 17640 and 18715; the second cluster contained isolates 16272, 18527, 16517 and 18735. The remaining three clusters contained single isolates 14130, 17484 and 15526, which differed from the first two clusters at 71.9%, 74.7% and 86.4%, respectively.

Every isolate had two bands of activity for hexokinase (HK) (Figure 3.2 p., 73). The position of these bands did not correspond in each isolate ie., isolates shared none, one or two band positions. This result indicates either the presence of two copies of the enzyme in the *Candida* genome or a second enzyme that uses the stain components of this enzyme equally as efficiently (see Andrews and Chilton 1999 for a more detailed discussion). Since both enzyme activities could be accurately discriminated, they were both scored and used in the ensuing analyses. All isolates assessed possessed single bands at the remaining enzyme loci examined implying that they have single copies of the 19 other enzyme loci.

Three isolates, 15526, 18502 and 18735, were then selected for assessing other environmental variables that may influence staining intensity of the enzymes and to optimise the electrophoretic conditions and histochemical staining reaction for some loci. These particular samples were chosen because they represented the range of genetic variation observed in the pilot screen above and exhibited strong enzyme staining activities. As with the HK enzyme stain, the malate dehydrogenase (MDH) stain revealed two distinct bands. The separation of the two loci identified for MDH and HK was optimal with 1¼ hours of electrophoresis on citrate phosphate and tris maleate running buffers, respectively, with the samples loaded at the cathodal end of the gel. Unfortunately, the staining

resolution of MDH was not deemed sufficient for it to be accurately scored, so it was eliminated from the subsequent analyses.

Many of the peptidase stains produced multiple bands due to the ability of peptidases to metabolize a number of different peptides to varying degrees. Using the same three isolates utilized in the MDH and HK screens, the peptidases A to D were stained separately and then together on a single gel. This analysis confirmed that the band appearing directly after staining corresponded to the specific peptidase being investigated. Other peptidase activity that appeared later was attributed to alternative peptidase enzyme activity.

The same three isolates were used to assess the enzyme activity obtained when *Candida* cells were lysed using freeze-thawing, sonicating and a combination of both. Each of these methods provided the same staining intensity for the enzymes assessed. Due to the labour intensity of freeze-thawing and combining both methods, it was decided that sonicating samples was sufficient for future electrophoretic analyses.

The three isolates 15526, 18502 and 18735 were also used for the selection of the most appropriate running buffers for the enzymes chosen for the larger analyses as outlined in Appendix 2, Table 3. The final running conditions selected are in Table 2.2 of the previous Chapter (p., 56).

Table 3.2; Details of the twelve *Candida* strains used for the pilot allozyme electrophoresis screen

Isolate Code	Sex[a]	HIV status[b]	Site of Isolation	Candidiasis Infection[c]	Antifungal Resistance[d]	Patient's Therapy[d]	Other Notes
12716	M	+	Oral Swab	+	Fluconazole, moderate 5-flurocytosine		Mixed infection with *C. krusei*, resistant to fluconazole, Itraconazole and 5-flurocytosine
17130	F	+	Oesophageal Biopsy	+	Fluconazole, Itraconazole	Fluconazole	
18502	M	+	Oral	+	-	Itraconazole trial	Low CD4 count
17640	M	+	Oral	+	-		
18715	M	+	Oral Buccal Mucosa Swab	+	Fluconazole	Fluconazole	
16272	M	?	Systemic; Blood	?	-		
18527	M	?	Pancreas	?	-		Multiple abscess and on liver taken at laparotomy; from *C. albicans*, enterococci and multiple resistant *P. aeruginosa*
16517	M	?	Systemic; Blood	?	-	Penicillin	
18735	F	?	Systemic; Blood	?	-		
14130	M	+	Oral	+	-	Fluconazole	Outpatient. Reidentified at WCH as *Trichosporon beigelii*
15526	M	?	Oral	+	-	Ketoconazole	Germ-tube negative, recurrent infection
17484	M	+	Throat Swab	+	Fluconazole, Itraconazole, 5-flurocytosine	Ketoconazole	

a; Sex of the patient

b; HIV status of the individual that the *Candida* sample was obtained from + indicates HIV-positive, - indicates HIV-negative and ? indicates status unknown.

c; Candidiasis infection + for infection at sites sample was taken, gastric, systemic and nervous indicate infections within areas fluid was obtained from. Again, ? indicates status unknown.

d; Antifungal resistance and patient therapy; antifungals listed where positive for treatment or resistance, - for no resistance, blank where treatment or resistance data unavailable or undetermined.

The dividing single lines designate separate clusters of isolates that differ at over 50% of loci.

Annotations are standard for all following isolate details tables in the Results Chapter.

Table 3.3; Allelic profiles for the twelve samples used in the pilot screen at a panel of sixteen metabolic enzyme loci determined using allozyme electrophoresis

	Enzyme Locus															
Isolate Code[a]	ENOL[b]	EST	FUM	GDH	GOT	G6PD	GPI	HK1[c]	HK2[c]	ME	PepA	PepB1	PepB2	PGAM	PGM	PK
12716	b[d]	d	b	b	-[e]	b	a	b	a	b	c	b	a	c	-	b
17130	d	-	b	c	b	b	b	b	a	b	c	b	a	c	-	b
18502	b	c	b	d	b	b	b	a	a	c	c	b	-	c	c	c
17640	b	a	-	d	b	b	b	b	a	a	c	b	a	c	-	c
18715	b	d	a	d	b	b	b	b	a	c	c	b	a	c	-	b
16272	b	b	b	d	b	c	b	b	a	c	c	c	b	c	a	d
18527	b	b	b	d	b	c	b	d	a	d	c	b	b	c	-	e
16517	c	-	-	e	-	e	b	d	a	e	c	c	b	c	a	c
18735	c	b	-	-	b	d	b	c	a	e	c	c	b	c	c	c
14130	e	a	-	a	b	f	-	e	a	-	c	a	c	b	b	c
15526	a	a	a	-	a	a	a	b	b	d	b	c	b	b	b	a
17484	a	d	-	-	-	g	c	e	a	b	a	b	a	a	c	b

a; Isolate code description is within Table 3.2 p., 70

b; Loci abbreviations are contained within Table 2.2 p., 56 of the Materials and Methods

c; HK1 and HK2 represent the two distinct hexokinase loci evident in *Candida*, scored in order of their appearance for each strain after histochemical staining ie., the first locus to appear is HK1, the second is HK2.

d; Alleles are designated alphabetically in order of increasing anodal migration ie., allele "a" has migrated the least distance.

e; - designates insufficient staining intensity or resolution for accurate scoring of that enzyme locus

The dividing lines designate separate clusters differing at over 50% of loci. Annotations are standard for all following isolate allelic profile tables in the Results Chapter.

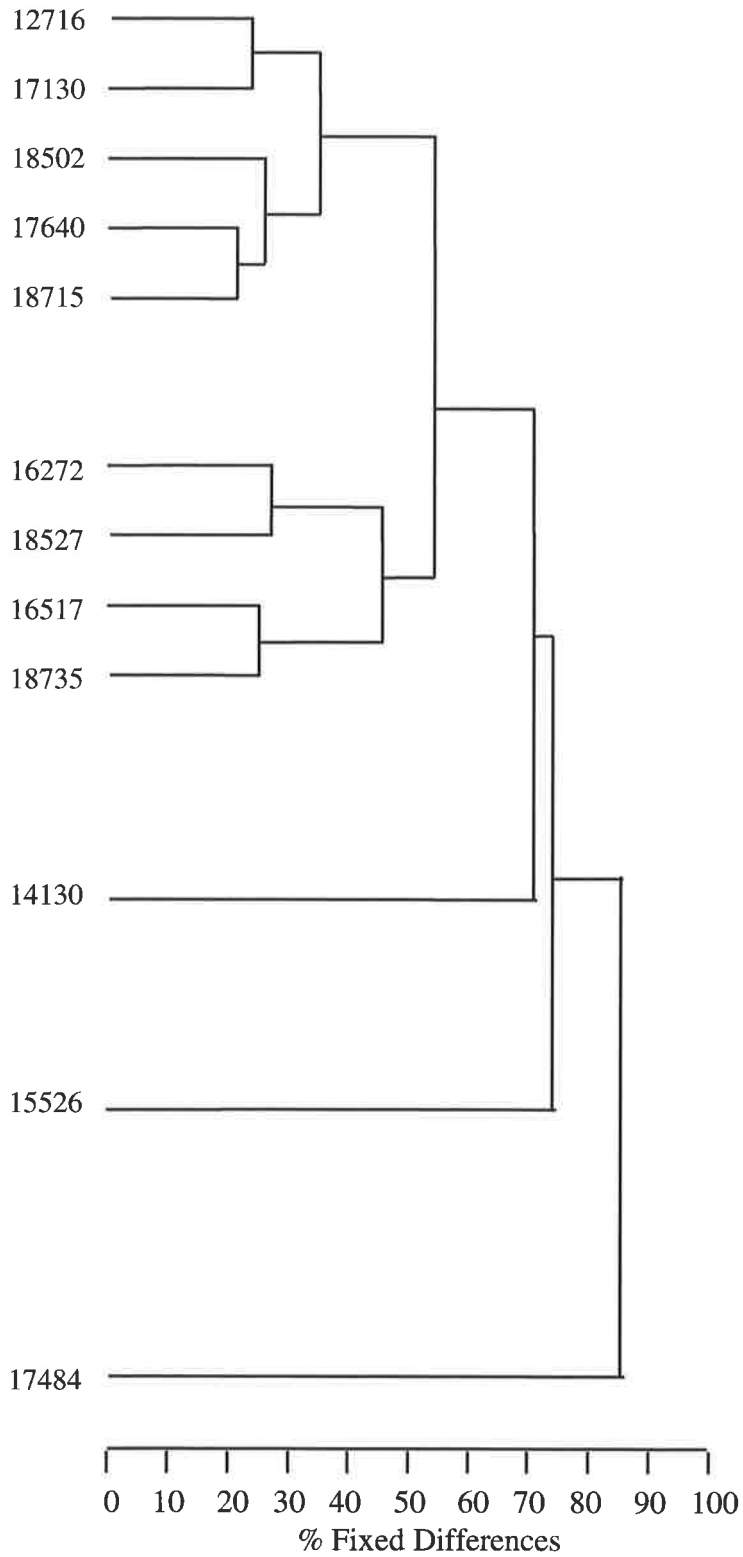


Figure 3.1 **Phenogram depicting the genetic relationships between twelve clinical isolates of *Candida* used in the Pilot screen, as determined from an allozyme electrophoresis analysis using sixteen enzyme loci.**

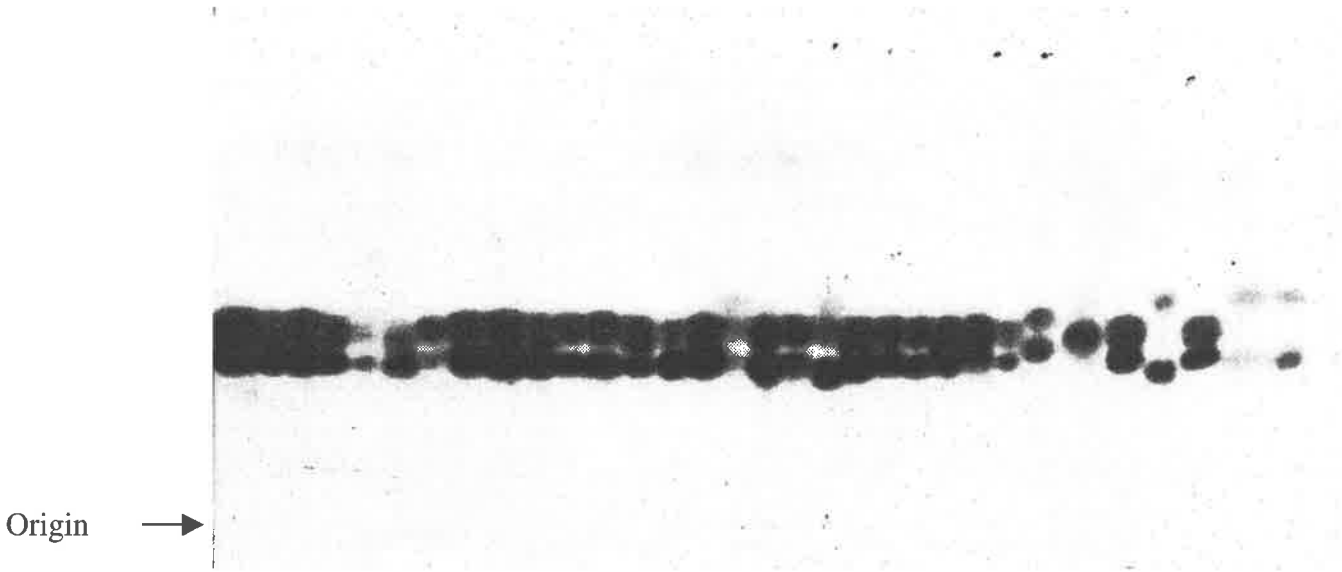


Figure 3.2; Example gel showing the double banding pattern of hexokinase for *Candida* species; the presence of two distinct bands for this enzyme indicate that there are potentially two expressed copies of the gene

3.1.1.d Interpretation

This component of the project identified that twenty of the thirty-seven loci assessed provided sufficient staining intensity, resolution and discriminatory power to be potentially diagnostic. This panel of enzymes was subsequently used for the rest of this study. After assessing storage and growth parameters, the following optimal conditions were determined and used for the remaining analyses. The samples were all stored in water at room temperature. When required for analyses, they were grown on Sabouraud's dextrose agar at room temperature for 48 to 72 hours. The cells were harvested in Tris HCl pH 7.8, lysis buffer was added and the cells were lysed using ultrasonication (as outlined in the Materials and Methods, Chapter 2 p., 47). The lysates were stored at -20°C until they were required for electrophoresis. The electrophoretic conditions and staining reactions were all optimised and the same protocol was employed for all subsequent analyses (detailed in the Materials and Methods Chapter 2 Table 2.2 p., 56).

The results from this preliminary screen suggested that the delineation of *Candida* from other genera might be possible using allozyme electrophoresis. Interestingly, the oral isolates assessed clustered separately from those obtained from systemic infections. This preliminary observation suggests that there may be genetic differences between *Candida* strains isolated from different sites of infection.

3.1.2 Pilot Study 1

3.1.2.a Background

Once an appropriate panel of enzyme loci has been chosen and the protocol has been optimised, the diagnostic-potential of the method needs to be assessed. This requires analysis of a broader range of samples, encompassing the range of biological diversity to be assessed in the study proper. This range should include:

a) a representative of the most closely related species to the one under investigation,
b) a genetically diverse species within the same genus,
c) a representative from another closely related, but genetically distinct genus and,
d) a number of isolates from within the species to be predominantly investigated that represent the range of genetic, clinical, biochemical and morphological variation detected to date.

The overall **aim** was

- *to assess the diagnostic potential of allozyme electrophoresis for the discrimination of species of Candida and related genera, and for strains of C. albicans.*

3.1.2.b Materials and methods

Twenty-eight isolates of differing clinical origins were assessed in this preliminary study (Table 3.4 p., 78). They include eighteen clinical isolates of *C. albicans*, two reference strains of *C. albicans* (ATCC90028 and ATCC90029), one reference strain of *C. glabrata* (ATCC90030) and three reference strains of *Cryptococcus neoformans* (ATCC90112, ATCC90113 and ATCC90118). The purity of isolates was confirmed during manipulations using Gram staining, cell morphology and growth on selective media. All isolates were assessed using the twenty enzyme loci established in the pilot study screen (Section 3.1.1 p., 67, and Table 2.2 of Chapter 2 p., 56).

Clinical isolates 17A and 17B were collected from one patient during consecutive dental appointments for the present study. In order to determine the presence of mixed infection in this patient, six individual colonies were collected and analysed separately. This number of colonies was arbitrarily chosen to be statistically relevant. The same number of colonies was collected for each clinical isolate obtained during the project. However, all other isolates used in this particular study were originally derived from a single colony pick.

In a previous study, using RFLP mixed-linker PCR analysis and probing with the 27A repeat sequence, McCullough *et al.* (1995) found that in patients 1 and 2, the sequential *C. albicans* isolates were genetically identical clones, whilst in patient 5 the isolates were genetically unrelated. These same isolates were also compared in this study (isolates M1A, M1B, M2A, M2B, M5A and M5B in Table 3.4, p., 78 kindly provided by Mrs. S. Hume and Dr. M. McCullough, Mycology Laboratory, Fairfield Hospital, Victoria, Australia).

C. albicans isolates P1 and A2 were appreciatively obtained from Dr C.M. Allen (College of Dentistry, The Ohio State University) who had previously reported that they induce lesions in 0% and 60% of oral cavities in a rat model, respectively (Allen and Beck 1987). These strains were included for a preliminary investigation of the existence of a genetic basis for virulence ie., whether different genetic clusters of isolates exhibited different pathogenicities.

3.1.2.c Results

The allelic profiles for each isolate are in Table 3.5 below (p., 79) and the pairwise comparisons between each of the isolates are contained within the matrix (Appendix 3, Table 2). These pairwise comparisons form the basis for the construction of the phenogram for this analysis (Figure 3.3 p., 80).

All isolates had a unique allelic profile with fixed differences at 5 to 91.5% of the enzyme loci. Six clusters of isolates can be identified with fixed differences between them occurring at over 50% of loci. The first contains the majority of clinical isolates and the three reference strains for *C. albicans*, indicating that these isolates belong to this species. The fixed differences between the *C. albicans* isolates in this cluster ranged from 5% to 49.2% of loci. In this cluster, the six individual colonies from patient samples 17A and 17B were genetically-distinct, although closely related strains. The variation observed suggests that this patient was colonized with a number of distinct strains.

Isolate pairs M1A and M1B and M2A and M2B, which were indistinguishable using the 27A probing method (McCullough *et al.* 1995), differed at 6 and 19% of the loci, respectively. This cluster also contains the *C. glabrata* reference strain ATCC 90030, which differs from the *C. albicans* reference strain ATCC 90028 at only 5% of the loci. This suggests that either *C. albicans* and *C. glabrata* are genetically indistinguishable using allozyme electrophoresis or that one of the reference strains has been contaminated. These observations require further investigation using additional *C. glabrata* clinical isolates and confirmation of the species identity of the two strains.

The most closely related cluster to the first contains a single clinical isolate, 18527. It is a pancreas infection isolate. The next cluster differs from the first two at 66.3% of loci. It also contains a single isolate, 5B, which was distinguishable from isolate 5A using the 27A probe method (McCullough *et al.* 1995) and also in the current study.

The fourth cluster contains isolates ATCC 90112 and ATCC 90113, reference strains for the *Cr. neoformans* serotypes *neoformans* and *gattii*, respectively, which differ from each other at 37% of loci. They, in turn, differ from *C. parapsilosis* reference isolate ATCC 90118 at 51.5% of the loci. These three isolates differ from the previously described clusters at 75.6% of loci. Isolates 15526 and 14130 exist in unique clusters that differ from

all other isolates at 87% and 91.5%, respectively. They were subsequently identified using assimilation profiles as *C. dubliniensis* and *T. beigelii.*

Table 3.4; Details of the twenty-eight reference and clinical strains used to investigate the diagnostic potential of allozyme electrophoresis

Isolate Code	Sex	HIV status	Site of Isolation	Candidiasis Infection	Antifungal Resistance	Patient's Therapy	Other Notes
17B.1 to 6	M	+	Oral Rinse	-			
M1A	M	+	Oral Rinse	+			
M1B	M	+	Oral Rinse	+			Genetically identical to 1A
17A.1 to 6	M	+	?	?			
17640	M	+	Oral	+	-		
ATCC 90028			Blood	systemic			*C. albicans* reference strain
ATCC 90030			Blood	systemic			*C. glabrata* reference strain
P1			Patient with acute pseudomembranous candidiasis				Lesions induced in 60% of rats
17130	F	+	Oesophageal Biopsy	+	Fluconazole, Itraconazole	Fluconazole	
18735	F	?	Systemic; Blood	?	-		
16517	M	?	Systemic; Blood	?	-	Penicillin	
16272	M	?	Systemic; Blood	?	-		
18502	M	+	Oral	+	-	Itraconazole trial	Low CD4 count
18715	M	+	Oral Buccal Mucosa Swab	+	Fluconazole	Fluconazole	
M2A	M	+	Oral Rinse	+			
M2B	M	+	Oral Rinse	+			Genetically identical to 2A
17484	M	+	Throat Swab	+	Fluconazole, Itraconazole, 5-flurocytosine	Ketoconazole	
12716	M	+	Oral Swab	+	Fluconazole, moderate 5-flurocytosine		Mixed infection with *C. krusei*
ATCC 90029			Blood	systemic			*C. albicans* reference strain
A2			Patient with chronic atrophic candidiasis				Lesions absent in rat.
M5A	M	+	Oral Rinse	+			
18527	M	?	Pancreas	?	-		Multiple abscess and on liver taken at laparotomy; from *C. albicans*, enterococci and multiple resistant *P. aeruginosa*
M5B	M	+	Oral Rinse	+			Genetically different to 5A Two colonial morphologies.
ATCC 90112			Cerebrospinal fluid	nervous			*Cr. neoformans* reference strain
ATCC 90113			Cerebrospinal fluid	nervous			*Cr. neoformans* reference strain
ATCC 90118			Sputum	gastric			*Cr. neoformans* reference strain
15526	M	?	Oral	+	-	Ketoconazole	Germ-tube negative, recurrent infection
14130	M	+	Oral	+	-	Fluconazole	Outpatient. Reidentified WCH on 14/12/95 as *Trichosporon beigelii*

The dividing lines designate separate clusters differing at over 50% of loci. Annotation as in Table 3.2 (p., 70)

Table 3.5; Allelic profiles of thirty-eight isolates analysed in pilot study 1 at twenty-two enzyme loci

	Enzyme Locus																					
Isolate Code	ADH	AK	ALD	AP	ENOL	EST	FDP	FUM	GOT	G6PD	GPI	HK1	HK2	IDH	NDPK	NP	PepA	PepB	PepD	PGAM	PGM	PK
17B.1	e	c	c	-	e	a	a	d	b	b	b	d	d	d	d	a	b	f	d	-	d	d
17B.3	e	c	c	-	e	a	a	d	b	b	c	d	d	d	d	a	b	f	d	f	d	d
17B.4	e	c	c	d	e	a	a	d	b	b	c	d	d	d	d	a	b	f	d	d	d	d
17B.2	e	c	c	-	e	a	a	d	b	b	b	d	d	d	-	a	b	f	d	f	d	f
17B.5	e	c	c	-	e	a	a	d	b	b	c	d	d	d	e	a	b	f	d	-	d	c
17B.6	e	c	c	f	e	a	a	d	b	b	c	d	d	d	e	a	b	f	d	f	d	d
M1A	e	-	c	-	e	a	-	d	b	b	a	d	d	d	d	a	b	f	-	f	-	d
M1B	e	-	b	-	e	a	-	d	b	b	a	d	d	d	-	a	b	f	-	f	-	d
17A.3	e	c	c	d	e	a	b	d	b	b	c	d	d	d	d	a	b	f	e	f	d	d
17A.4	e	a	c	d	e	a	b	d	b	b	c	d	d	d	d	a	b	f	e	f	d	d
17A.1	e	c	c	d	e	a	b	d	b	b	b	d	d	d	e	a	b	f	e	f	d	d
17A.2	e	c	c	c	e	a	b	d	b	b	c	d	d	d	e	a	b	f	e	f	d	d
17A.5	e	-	c	d	e	a	c	d	b	b	c	d	d	d	-	a	b	f	e	f	d	c
17A.6	e	-	c	d	e	a	c	d	b	b	c	d	d	d	e	a	b	f	e	f	d	d
17640	e	c	b	c	e	-	-	d	b	b	b	d	d	d	e	a	b	f	-	h	-	d
ATCC 90028	e	e	c	c	e	a	c	d	b	b	b	d	d	d	d	a	b	f	d	f	d	d
ATCC 90030	e	-	c	c	e	-	c	d	b	b	b	d	d	d	-	a	b	f	d	g	d	d
P1	e	c	a	a	e	a	c	d	b	b	c	d	d	d	d	a	b	f	-	g	d	d
17130	-	c	c	b	f	a	c	d	b	b	a	d	d	d	d	a	b	f	d	i	d	e
18735	-	b	c	a	e	a	c	d	b	c	b	c	d	d	c	a	b	g	d	f	d	c
16517	e	b	c	b	e	a	c	d	b	c	c	d	e	d	c	a	b	g	f	f	d	d
16272	e	a	c	c	e	a	c	d	b	b	c	d	e	d	c	a	b	g	c	f	d	e
18502	e	b	b	c	e	a	c	d	b	b	b	c	c	d	e	a	b	f	f	h	d	d
18715	e	b	b	c	e	a	c	d	b	b	b	c	c	d	e	a	b	f	f	j	c	c
M2A	e	b	b	d	e	a	c	d	b	b	b	d	c	d	-	a	b	f	d	f	e	d
M2B	-	-	b	d	e	a	-	d	b	b	b	c	c	d	c	a	b	f	-	e	-	c
17484	-	-	b	d	e	-	c	d	b	b	c	c	c	d	e	a	b	f	b	j	d	e
12716	d	b	a	c	e	a	c	d	b	b	c	d	d	d	d	a	b	f	a	i	c	e
ATCC 90029	e	-	ce[a]	b	ce	a	c	b	b	b	b	d	e	d	d	a	b	e	e	g	d	h
A2	d	-	c	b	e	a	c	d	b	c	a	d	f	d	c	a	b	e	-	g	-	g
M5A	c	a	b	c	f	a	c	d	c	b	b	e	d	d	c	a	b	f	cg	-	d	e
18527	d	a	a	c	e	a	c	d	b	c	c	c	e	d	-	a	b	d	d	f	f	g
M5B	e	d	a	e	f	b	b	c	b	b	b	e	c	c	e	c	b	f	d	g	d	e
ATCC 90112	-	-	e	c	a	a	b	d	-	a	c	a	b	b	-	a	a	a	-	ab	d	f
ATCC 90113	-	-	e	-	d	-	b	a	b	a	b	b	a	-	-	a	a	-	-	a	d	f
ATCC 90118	-	-	e	-	b	-	-	a	d	a	b	d	d	b	-	a	a	b	-	-	-	h
15526	a	b	c	a	d	-	d	c	a	a	a	e	f	d	b	b	-	g	-	g	b	a
14130	a	cd	d	f	g	-	e	b	c	d	d	f	h	a	f	-	b	c	b	-	b	f

a; Two alleles at the same locus indicate either an isolate that is heterozygous for that enzyme or it is a contaminated sample
The dividing lines designate separate clusters differing at over 50% of loci. The remaining annotations are as in Table 3.3 (p., 71)

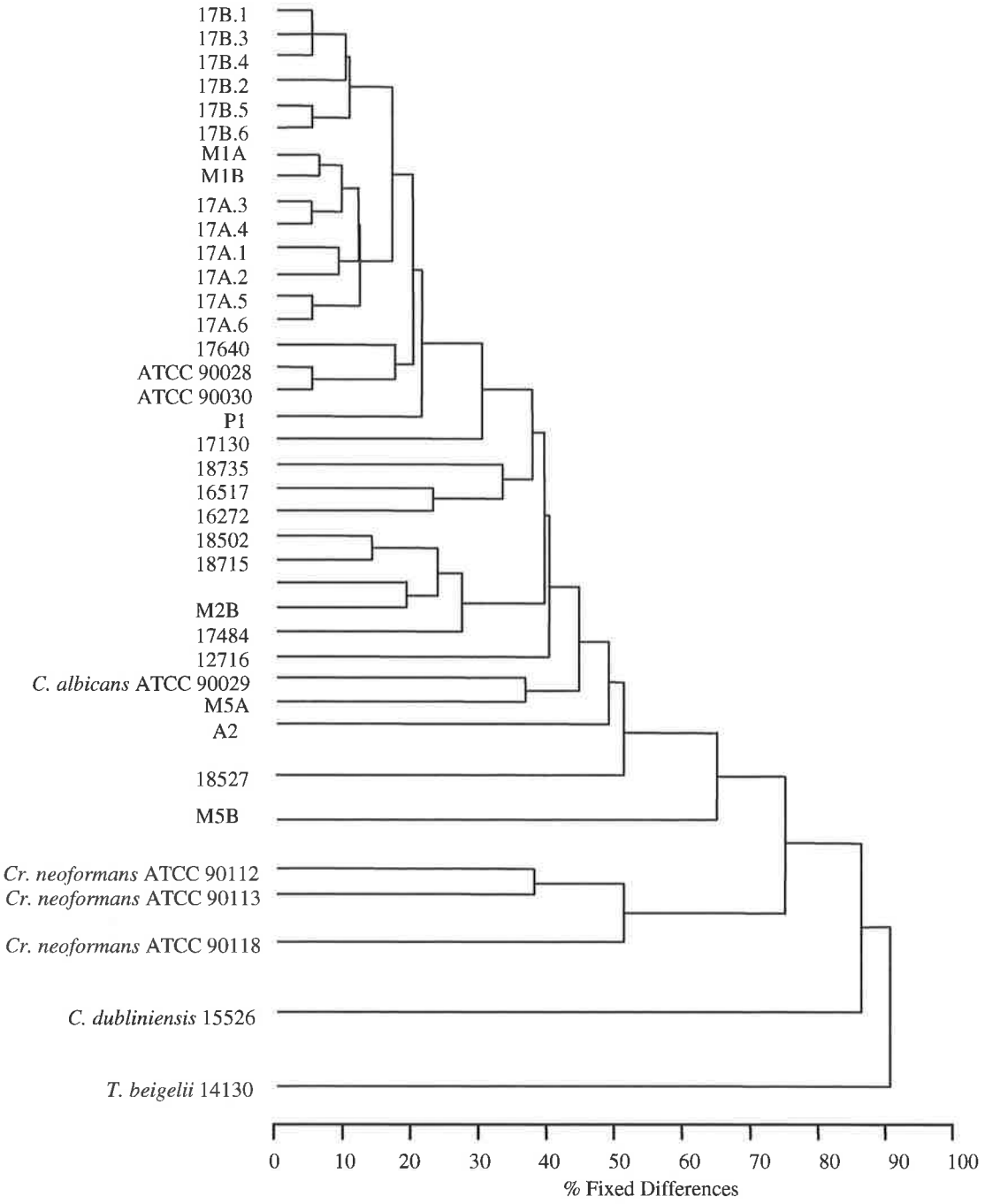


Figure 3.3; Phenogram depicting the genetic relationships between thirty-eight isolates of *Candida* and related genera used in pilot study 1, as determined from an allozyme electrophoresis analysis using twenty-two enzyme loci

Table 3.6; Matrices of the percentage of fixed genetic difference between individual colonies from two clinical isolates from patient 17 in pilot study 1

Isolate	17A.1	17A.2	17A.3	17A.4	17A.5
17A.2	9				
17A.3	9	9			
17A.4	14	14	5		
17A.5	15	15	10	10	
17A.6	10	10	10	10	5

Isolate	17B.1	17B.2	17B.3	17B.4	17B.5
17B.2	5				
17B.3	5	10			
17B.4	5	15	5		
17B.5	15	11	10	10	
17B.6	10	10	5	14	5

3.1.2.d Interpretation

Single colonies from isolate 17A differed at 5 to 15% of the loci assessed, as did colonies from isolate 17B. This genetic difference represents strain variation within this patient's sample. Strains of isolate 17A differed from those of isolate 17B at 10 to 25% of the loci assessed. Both samples were oral rinses but isolate 17A was taken when the patient had oral candidiasis while 17B was taken in the absence of clinical infection. The close genetic proximity of isolates from infection and asymptomatic carriage implies that oral candidiasis is an opportunistic infection.

Each cluster of isolates in this analysis potentially represents a different species of *Candida* or a different but related genus. It is important to note that the separation of isolates into distinct genetic groups herein was not influenced by the measure of genetic distance used in the analyses (ie., fixed differences or Nei's D values).

Allozyme electrophoresis appears more discriminatory for detecting genetic variation than the use of the 27A probing method because it was able to discriminate three pairs of strains (M1A, M1B, M2A, M2B, M5A and M5B), which had previously been recognised as genetically identical using the probing technique (McCullough *et al.* 1995).

Isolate 15526, originally described as a *C. albicans* isolate, was retyped as *C. dubliniensis* after the species original description in 1995 (Sullivan *et al.* 1995) and following the results presented in this section. Also originally typed as *C. albicans*, isolate 14130 was retyped as *T. beigelii* following identification of its close genetic association with this species in the present data. Isolate 14130 may have become contaminated after its original clinical diagnosis.

The twenty-two enzyme loci analysed herein form the basis for a large-scale epidemiological study of *Candida* species infecting HIV-positive patients using allozyme electrophoresis. An important point pertaining to subsequent analyses is that each clinical sample collected during this entire study is represented by up to six individual colonies (as with isolates 17A and 17B in pilot study 1).

3.1.3 Pilot Study 2

3.1.3.a Background

This analysis constitutes the second preliminary study of clinical isolates. It was used to confirm the reproducibility of allozyme electrophoresis and incorporates the investigation of additional clinical isolates. These additional clinical isolates are more biologically diverse than those used in pilot study 1 and may therefore also exhibit more notable genetic variation.

The **aim** was then;

- *to confirm the discriminatory power of the allozyme electrophoresis technique using a wider variety of clinical strains with distinct morphological, biochemical and/or clinical characteristics.*

3.1.3.b Materials and Methods

The isolates analysed are detailed in Table 3.7 (p., 85) and the enzyme loci scored are listed in Table 3.8 (p., 86). They include reference strains for *C. albicans*, *C. glabrata* and *Cr. neoformans*, also clinical isolates from sixteen patients, three single colony picks and thirteen multiple colony isolates. Additionally, four isolates from HIV-negative asymptomatic carriers were included. Three of the clinical isolates have varying antifungal sensitivity; one was from a postradiotherapy head and neck cancer patient who was an asymptomatic carrier and whose colony isolates were very adhesive on Sabouraud's dextrose agar. Six of the clinical isolates were identified as *Candida* isolates but not *C. albicans*, one of these was identified as *C. glabrata* while the other five could only be defined to the genus *Candida* using preliminary assimilation studies. The isolates were assessed at twenty enzyme loci using allozyme electrophoresis. The six individual colonies from each clinical sample were grown independently. The lysates were combined in equal proportions and analysed as a single sample on the gel. Mixed or heterozygous samples could be identified by the presence of more than one band at a single enzyme locus where the majority of other samples analysed consisted of a single band. If this was observed, the sample was excluded from the analyses because the cause of the multiple banding pattern was not pursued further in this thesis. This protocol was followed in all ensuing studies in this thesis. To determine the cause of multiple banding, each individual colony needs to be

analysed as a distinct isolate to assess the zygosity or mixed culture characteristic of the sample.

3.1.3.c Results

Double banding and enzyme allele differences between pilot study 1 and this analysis for the reference isolates suggested they were contaminated. For this reason, they were eliminated from the analysis. Clinical isolates 40I, 54A, 62A, 68A and 70A and asymptomatic carrier isolates S21 and S23 all contained multiple banding patterns at one to seven enzyme loci. For the remaining isolates, fixed differences occurred at 0 to 84.6% of the twenty loci assessed.

Four clusters of isolates can be distinguished in Figure 3.4 (p., 87). The first contains the majority of clinical isolates with fixed differences within in ranging from 5 to 44.9% of loci. Previous results and identity of these isolates in pilot study 1 suggests that this is a '*C. albicans* cluster'. Isolate 68B formed a unique cluster that differed from the first cluster at 51.2% of loci. The third cluster differed from the first two at 71.4% of loci and since it contained isolate 15526, later identified as *C. dubliniensis*, it is presumed that this cluster comprises the species. Isolates 40G.1 to 6 formed two distinct groups, differing from each other at 10% of loci. This isolate was suspected as mixed after subculturing, due to the different colony morphologies detected on Sabouraud's agar. The current results suggest that this patient had been colonized by two different strains of *C. dubliniensis*. Isolate 34A constituted a distinct cluster, which differed from all other isolates at 84.6% of loci. This isolate belongs to a third species.

Table 3.7; Details of the twenty-four reference and clinical samples analysed in pilot study 2 using allozyme electrophoresis

Isolate Code	Sex	HIV status	Site of Isolation	Candidiasis Infection	Antifungal Resistance	Patient's Therapy	Other Notes
C1	M	-	Undiluted Saliva	-			
17484	M	+	Throat Swab	+	Fluconazole, Itraconazole, 5-flurocytosine	Ketoconazole	
56A.1 to 6	M	+	Oral Rinse	-			Post radiotherapy patient, not HIV-positive. 56A.2 and .4 growth on agar slopes very adhesive
18715	M	+	Oral Buccal Mucosa Swab	+	Fluconazole	Fluconazole	
54K.1 to 6	M	+	Oral Swab	Oral pseudomembranous candidiasis			Amphotericin B trial
91A.1 to 6	M	+	Oral Rinse	?			*Candida* species, germ tube + but possibly mixed with other yeasts
68B.1 to 6	M	+	Oral Rinse	?			not *Candida albicans*, unidentified *Candida* species
40G.1 to 6	M	+	Oral Rinse	?			*Candida* species (not *C. albicans*), germ tube +, looks mixed
30A.1 to 6	M	+	Oral Rinse	+	Fluconazole	On 50mg of fluconazole a day	Previous isolate fluconazole sensitive
15526	M	?	Oral	+	-	Ketoconazole	Germ-tube negative, recurrent infection
34B.1 to 6	M	+	Oral Rinse	?			Growth on agar slopes very adhesive and granular
S8	M	-	Undiluted Saliva	-			
34A.1 to 6	M	+	Oral Rinse	?			Growth on agar slopes very adhesive and granular
ATCC 90113			Cerebrospinal fluid, Pennsylvania	systemic			*Cr. neoformans* type strain
ATCC 90028			Blood, Iowa	systemic			*C. albicans* reference strain
ATCC 90029			Blood, Iowa	systemic			*C. albicans* reference strain
ATCC 90030			Blood, Iowa	systemic			*C. glabrata* reference strain
S21	F	-	Undiluted Saliva	-			Not *C. albicans*
S23	F	-	Undiluted Saliva	-			
40I.1 to 6	M	+	Oral Rinse	?			*Candida* species
54A.1 to 6	M	+	Oral Rinse	?			54A.1 growth on agar slopes very adhesive
62A.1 to 6	M	+	Mouth swab	?			HIV Ab+, immunosuppressed, clinical HSV of penile shaft
68A.1 to 6	M	+	Oral Rinse	?			not *Candida albicans*, unidentified *Candida* species
70A.1 to 6	M	+	Oral Rinse	?			*Candida glabrata*

The double dividing line separates isolates whose genetic relationships were analysed from those that appeared mixed/heterozygous due to multiple bands at a single enzyme locus and that were subsequently eliminated from the analysis. Annotations as in Tables 3.2 (p., 70) and 3.4 (p., 78).

Table 3.8; Allelic profiles for the twenty-nine individual isolates analysed in pilot study 2 at twenty enzyme loci

	Enzyme Locus																			
Isolate Code	ADH	ALD	ENOL	EST	FDP	FUM	GDH	G6PD	GPI	HK1	HK2	IDH	MPI	NP	PepA	PepB	PGAM	6PGD	PGM	PK
C1	d	a	-	c	b	-	c	b	b	b	f	c	c	a	e	-	d	c	b	c
17484	d	a	j	c	-	d	c	c	b	c	f	c	c	b	f	b	c	c	b	b
56A.1 to 6	d	a	h	c	b	e	d	c	b	a	e	c	c	b	f	b	d	c	b	b
18715	d	a	h	c	b	e	d	c	b	a	e	c	d	b	f	b	d	c	b	b
54K.1 to 6	c	a	h	c	b	e	d	c	b	a	e	c	a	b	f	b	c	c	b	b
91A.1 to 6	c	a	i	c	b	e	c	c	b	a	d	c	e	b	f	b	d	d	c	b
68B.1 to 6	c	a	g	c	a	f	d	d	c	b	e	c	d	b	f	c	d	c	b	b
40G.2	c	a	e	a	b	d	b	b	a	b	f	c	b	d	d	b	b	d	a	a
40G.3	c	a	e	a	b	d	b	b	a	b	f	c	b	d	d	b	b	d	a	a
40G.4	c	a	e	a	b	d	b	b	a	b	f	c	b	d	d	b	b	d	a	a
40G.6	c	a	e	a	b	d	b	b	a	b	f	c	b	d	d	b	b	d	a	a
40G.1	c	a	e	a	b	d	b	b	b	b	f	c	b	c	d	b	b	d	a	a
40G.5	c	a	e	a	b	d	b	b	b	b	f	c	b	c	d	b	b	d	a	a
30A.1 to 6	d	a	e	a	b	d	b	b	b	b	g	c	d	c	d	b	b	c	a	a
15526	d	a	d	a	c	d	b	b	b	b	f	c	b	d	d	b	c	c	a	a
34B.1 to 6	b	a	e	a	c	d	b	b	b	b	f	c	e	d	d	b	c	c	a	c
S8	d	a	-	a	c	-	b	a	b	b	g	c	c	c	e	e	b	c	a	a
34A.1 to 6	b	a	j	d	d	g	e	b	c	a	b	b	c	e	g	c	e	a	a	c
ATCC 90113	b	b	c	c	b	ab	a	b	a	a	a	a	c	c	a	b	a	b	b	c
ATCC 90028	a	b	c	c	b	ab	a	b	a	a	h	a	c	b	a	b	a	b	b	c
ATCC 90029	b	b	c	c	b	ab	a	b	a	a	h	a	e	c	a	b	a	b	b	c
ATCC 90030	bd	a	ch	c	b	af	a	bc	b	a	e	ac	c	c	af	b	ad	b	b	bc
S21	b	-	b	c	b	ab	a	b	a	a	h	a	c	c	b	b	a	b	b	c
S23	d	a	h	c	b	c	c	c	b	a	f	c	a	a	f	ab	d	c	b	c
40I.1 to 6	c	a	f	a	b	d	b	b	b	b	f	c	b	d	d	bd	b	d	a	a
54A.1 to 6	d	a	h	ac	b	c	bd	bc	b	a	e	c	a	bc	df	be	b	c	b	ab
62A.1 to 6	d	a	h	c	b	e	d	cd	b	b	e	c	e	b	f	b	d	c	b	c
68A.1 to 6	b	a	ah	c	b	f	d	bd	bd	b	cf	c	f	b	f	b	a	c	b	b
70A.1 to 6	a	c	c	a	-	d	b	b	b	b	f	d	b	d	cd	d	c	a	a	c

Annotations as in Tables 3.3, 3.5 and 3.7 (p., 71, 79 and 85)

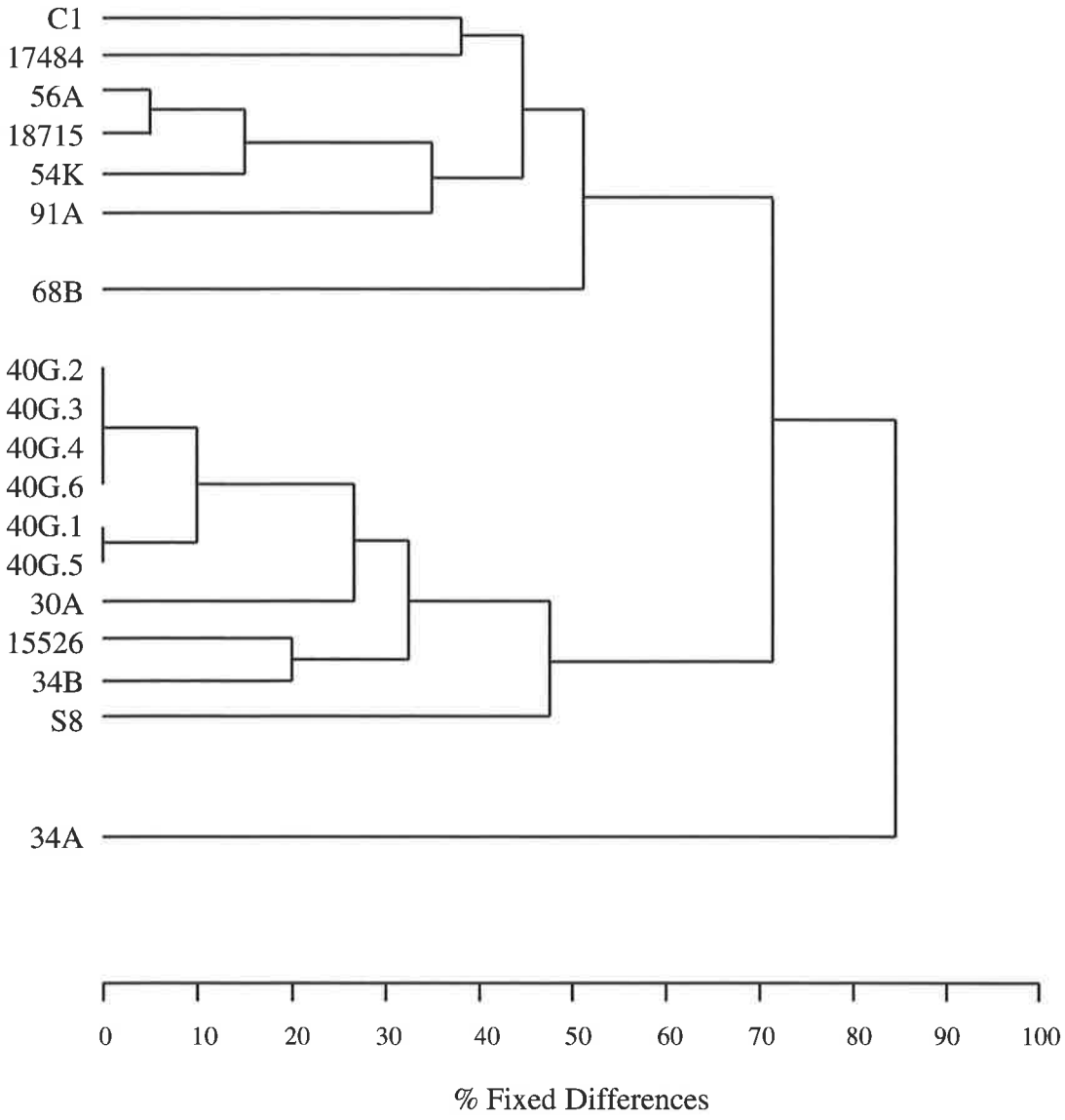


Figure 3.4; Phenogram depicting the genetic relationships between eighteen isolates of *Candida* and related genera used in Pilot Study 2, as determined from an allozyme electrophoresis analysis using twenty enzyme loci

3.1.3.d Interpretation

The 4 ATCC reference strains used in this analysis were retyped using traditional methods and it was discovered that they were contaminated with other yeast species. The original glycerol stocks were also contaminated so a new and more extensive range of type and reference strains was obtained.

The majority of isolates assessed belonged to two clusters, which, according to previous results (Sections 3.1.1 and 3.1.2 p., 67 and 74), probably comprised *C. albicans* and *C. dubliniensis*. The allelic profiles for the clinical isolates examined remained unchanged between different electrophoretic studies. Allelic profiles for the reference strains were not consistent between electrophoretic studies. This can be explained, however, by these isolates having been contaminated during subculturing (Section 3.1.4 p., 89).

The isolates examined electrophoretically herein, belong to four distinct species of *Candida*, namely *C. albicans*, *C. dubliniensis* and two undetermined species. Isolate 68B may still belong to the species *C. albicans* and represent the boundary of genetic variation detectable within the species, but isolate 34A definitely constitutes a distinct *Candida* species. Later characterisation using traditional morphological and biochemical techniques identified this isolate as *C. tropicalis*.

Clinical isolate 56A yielded colonies that were particularly adhesive to agar. These colonies were not genetically distinct to other less adhesive colonies, uas defined by allozyme electrophoresis. Therefore, it can be implied that this particular adhesive characteristic is not restricted to a specific genetic group of isolates and perhaps may not represent a genetically inherited virulence factor.

Isolates of *C.albicans* that were clinically-resistant to fluconazole did not form a separate cluster distinct from non-fluconazole resistant *C. albicans*. These strains instead were found interspersed throughout the *C. albicans* species cluster.

The electrophoretic results obtained suggest that both isolates 40G and 15526 belong to the recently described species *C. dubliniensis*. This implies that the germ tube characteristic is variable within this species since isolate 40G was germ tube positive while isolate 15526

was germ tube negative. Additionally, patient 40 was simultaneously colonised by two distinct strains.

Isolates obtained from HIV-negative individuals that were asymptomatic *Candida* carriers were scattered among isolates obtained from clinical infection in HIV-positive individuals. This result suggests that *Candida* infection is opportunistic and that transmission from asymptomatic carriers provides a potential reservoir for the infection of immunocompromised individuals.

3.1.4 Detection of reference strain contamination

3.1.4.a Background

For allozyme electrophoresis to be an effective diagnostic or epidemiological tool, it is important that there are no changes in the allelic profile of isolates between electrophoretic runs. Therefore, it was of concern that variability in the allozyme banding position of five of the reference strains (ATCC strains 90028, 90029, 90030, 90112 and 90113) was detected between the pilot screen and pilot studies 1 and 2. It was hypothesized that the genetic variation may have arisen in response to growth or storage conditions, considering the dimorphic nature of members of this genus, or contamination of the reference strains.

The **aim** was;

- *to determine the cause of variation in the allelic profile of reference strains between electrophoretic runs.*

3.1.4.b Materials and Methods

The same twenty-two loci as were used in Section 3.1.2 (Table 3.5 p., 79) were used to screen five of the reference strains used in these preliminary analyses. The reference strains were grown in different media (Sabouraud's agar, blood agar), at different temperatures (room temperature, 25°C, 37°C), aerobic vs. anaerobic, strains that were glycerol stored vs. water stored, and then compared directly on a single allozyme gel to determine whether the variation was growth phase specific.

3.1.4.c Results

Although the results are not shown, no allelic variation was observed with different culture or storage conditions. The reference isolates were then sent to the WCH for species identity checks. The results of four of the five ATCC reference strains indicated that they were contaminated with a number of other species (90028, 90029 and 90112 appeared to be *C. parapsilosis*, 90030 was identified as *C. tropicalis* and 90113 remained *Cr. neoformans*) (Table 3.9 p., 92).

3.1.4.d Interpretation

The variation in electrophoretic banding patterns were not due to morphological variation associated with strain growth conditions. Subsequent reidentification of the reference strains using conventional methods indicated that they were contaminated with other *Candida* species. The genetic relationships between these five reference isolates and the clinical isolates in the preliminary electrophoretic analyses were therefore not accurate or appropriate. However, no such problems are applicable to the relationships between the clinical isolates. It was decided at this point to incorporate strains representing thirteen medically and dentally relevant *Candida* species discussed earlier in this thesis (Chapter 1.2.3, p., 14). The relevant type and reference strains were ordered from the Centraalbureau voor Schimmelcultures, Baarn, Netherlands (CBS strains) and the American Type Culture Collection, Manassas, VA, USA (ATCC strains) and are identified in the following section (Chapter 3.2, p., 93).

3.1.5 General Interpretation of preliminary investigations

One of the main aims of this Chapter was to optimise the conditions of the allozyme electrophoretic technique for use in the taxonomic and epidemiological studies of *Candida*. The results have indicated that each isolate possesses a unique electrophoretic profile, *Candida* species clustered into separate lineages and genetic relationships between isolates and clusters of isolates can be determined on the basis of allozyme electrophoretic analyses. Contamination of reference strains was detected because their allelic profiles were not stable between electrophoretic runs. There was no effect on enzyme banding position with varied storage, growth or lysate preparation protocols.

The findings of this Chapter suggest that epidemiological investigations on *Candida* species are possible using allozyme electrophoresis. Determination of the species identity

of isolates on the basis of this technique has been demonstrated. Mixed or heterozygous sample detection has also been indicated. Overall, allozyme electrophoresis is an effective, suitable and reliable method for the identification and characterisation of *Candida* species and the epidemiological investigation of the disease process.

Table 3.9; Details of the contaminant identities of reference strains used in the pilot allozyme electrophoresis analyses

Isolate	Original Identity[a]	Contaminant[b]
ATCC 90028	*C. albicans*	*C. parapsilosis*
ATCC 90029	*C. albicans*	*C. parapsilosis*
ATCC 90030	*C. glabrata*	*C. tropicalis*
ATCC 90112	*Cr. neoformans* var. *neoformans*	*C. parapsilosis*
ATCC 90113	*Cr. neoformans* var. *gattii*	*Cr. neoformans*

a; Identity of the supplied strain from ATCC
b; Current identity of the contaminated sample

3.2 *Taxonomic analyses using allozyme electrophoresis*

3.2.1 Type and reference strain relationships

Prior to investigating the epidemiology of *Candida* colonisation using clinical isolates, genetic relationships within the genus *Candida* and related genera should be determined. As mentioned in the introduction (Section 1.2.3 p., 14), there are thirteen *Candida* species of predominant clinical importance. The framework of their genetic relationships will allow subsequent identification of clinical isolates and will provide a basis for determining the significance of the genetic relationships between clinical isolates that are examined.

3.2.1.a Background

As stated by Tibayrenc (1996), "many microbial species exhibit considerable genetic diversity. It is reasonable to expect this genetic variability to have a profound impact on the biological properties of microorganisms, especially those properties that are medically relevant". This implies that isolates that are closely related genetically also share common biological properties, some of which may be involved in the disease process. To this end, it would be anticipated that the *Candida* species isolated from human infections will be closely related both phenotypically and genetically.

The two **aims** of this study were;

- *to use allozyme electrophoresis to establish a range of genetic markers to distinguish each of the thirteen most commonly isolated Candida species and*
- *to determine the genetic relationships between these thirteen species and three related genera.*

3.2.1.b Materials and methods

Thirty-five samples representing thirteen medically important *Candida* species were included in this investigation (Table 3.10 p., 96). The clinical relevance of these species has been discussed previously in this thesis (Section 1.2.3 p., 14). For most species, the CBS type strain and an ATCC reference strain were included. Additionally, the choice of strains allowed an investigation of the genetic relationship between anamorphs and teleomorphs of *Candida* species. In the cases of *C. albicans* and *C. dubliniensis*, additional clinical isolates were included since *C. albicans* is the most predominant species in clinical infection and *C. dubliniensis* is newly described and not as well characterised. Three outgroups, or genetically distinct species, were also included; namely, the ascomycetes *Trichosporon beigelii* and *Cryptococcus neoformans* and the most closely related basidiomycete, *Saccharomyces cerevisiae* as described in Section 2.1.2 (p., 48). Their inclusion allows definition of the outer limits of genetic variability that exist within the genus *Candida* and the upper limit of the discriminatory power of the allozyme electrophoresis, that is, the utility of the technique in taxonomic investigations and phylogenetic reconstructions.

3.2.1.c Results

Each isolate was allelically scored at the twenty enzyme loci, which were established in Section 3.1 (p., 65). The allelic profiles obtained are presented in Table 3.11 (p., 97). The genetic relationships, based on the matrix of pairwise comparisons (Appendix 3, Table 3), are diagrammatically represented in a phenogram (Figure 3.5 p., 98).

The results showed that each species of *Candida* could be easily distinguished from one another based on fixed genetic differences of over 60%. This is equivalent to the level used to discriminate among bacterial species (eg., Selander *et al.* 1990). In addition, the results show that anamorphs and teleomorphs for each species always clustered together.

There are five discrete clusters of species that differ at over 80% of loci, the level previously used to discriminate among genera of bacteria (eg., Selander *et al.* 1990). Genetic cluster 1 comprises *C. albicans*, *C. kefyr*, *S. cerevisiae*, *T. beigelii* and *Cr. neoformans*. Variation within *C. albicans* ranged from 0 to 10.7% of loci and between the *C. kefyr* type and reference strain at 5%. There were no fixed genetic differences between isolates 3630, RA1B, RA2B, RA3C, RA4C and ATCC 90029. The most genetically-similar species to *C. kefyr* was *S. cerevisiae*, which differed at 61% of loci. These two species

differed from *C. albicans* at 64.4% of loci. These three species differed from the basidiomycete species *T. beigelii* and *Cr. neoformans* at 76.3% of loci. *Cr. neoformans* var. *gattii* differed from *T. beigelii* at 38 to 53.5% of loci, with the *T. beigelii* type strain splitting the type and reference strains. These species, in turn, differed from *Cr. neoformans* var. *neoformans* at 65.2% of loci and the type and reference strain of this species differed from each other at 50% of loci. Genetic group 1 differs from *C. glabrata* at 80.3% of loci. The type and reference strains for this species differ at 13% of loci. These two clusters differ from the third at 81.5% of loci. The third cluster contains *C. parapsilosis*, *C. guilliermondii*, *C. norvegensis* and *C. dubliniensis*. The three type and reference isolates of *C. parapsilosis* differed at 0 to 5% of loci. The identical isolates of *C. parapsilosis* are derived from the same original clinical isolate, but obtained from different reference collections. This confirms the discriminatory power of the allozyme electrophoretic technique with strains of different origins being discriminated and the same strain exhibiting identical allelic profiles. The two isolates of *C. guilliermondii* differed at 8% of loci. The species *C. guilliermondii* and *C. norvegensis* differed from each other at 75.4% of loci and from *C. parapsilosis* at 75.4% of loci. The three species differed from *C. dubliniensis* at 78.2% of loci.

The three described clusters of isolates differed from the fourth and fifth at 87.5% of loci and the fourth differed from the fifth at 82.4% of loci. The fourth cluster contains five *Candida* species. *C. tropicalis* strains differed from each other at 11% of loci and from two identical *C. viswanathii* isolates at 66% of loci. Both *C. viswanathii* isolates originated from the same clinical infection but were sourced from different culture collections. *C. lusitaniae* isolates differed from each other at 18% of loci and from the *C. haemulonii* isolate and the *C. famata* isolate at 72% of loci. These species differed from the former two at 74.8% of loci. This cluster of species differed from the fifth, which contained the identical type and reference isolates of *C. krusei*, at 82.4% of loci. The two *C. krusei* isolates again originated from the same sample.

Table 3.10; Details of the reference and type strains used to assess the systematics of the genus *Candida* and related species using allozyme electrophoresis

Isolate	Species	Site of isolation [a]
CBS 562^{T} [b]	*C. albicans*	skin (interdigital mycosis), Uruguay
ATCC 90029	*C. albicans*	blood, Iowa
3630	*C. albicans*	lab strain
RA1B	*C. albicans*	mouse
RA2B	*C. albicans*	mouse
RA3C	*C. albicans*	mouse
RA4C	*C. albicans*	mouse
ATCC 90028	*C. albicans*	blood, Iowa
CBS 834^{T}	*Kluyveromyces marxianus (C. kefyr)* [c]	kefyr grains, Netherlands
ATCC 46764	*C. kefyr*	clinical isolate, Texas
ATCC 2601	*Saccharomyces cerevisiae*	
CBS 2466^{T}	*Trichosporon beigelii*	
ATCC 90113	*Cr. neoformans* var. *gattii*	cerebrospinal fluid, Pennsylvania
CBS 6289^{T}	*Cryptococcus neoformans* var. *gattii* [d]	spinal fluid of man, Zaire
CBS 132^{T}	*Cr. neoformans var. neoformans* [d]	fermenting fruit juice
ATCC 90112	*Cr. neoformans var. neoformans*	cerebrospinal fluid, Pennsylvania
CBS 138^{T}	*C. glabrata (Torulopsis glabrata)*	human faeces
ATCC 90030	*C. glabrata (Torulopsis glabrata)*	blood, Iowa
CBS 604^{T}	*C. parapsilosis*	case of sprue, Puerto Rico
ATCC 22019	*C. parapsilosis*	case of sprue, Puerto Rico
ATCC 90118	*C. parapsilosis*	blood, Virginia
CBS 566^{T}	*Pichia guilliermondii (C. guilliermondii)*	sputum
ATCC 6260	*C. guilliermondii*	bronchomycosis
CBS 6564^{T}	*Pichia norvegensis (C. norvegensis)*	pregnant woman's vagina, London
CBS 7987^{T}	*C. dubliniensis*	oral cavity, HIV-positive, Ireland
CBS 94^{T}	*C. tropicalis*	bronchomycosis
ATCC 13803	*C. tropicalis*	
CBS 4024^{T}	*C. viswanathii*	meningitis, cerebrospinal fluid, India
ATCC 22981	*C. viswanathii*	cerebrospinal fluid, India
CBS 6936^{T}	*Clavispora lusitaniae (C. lusitaniae)*	citrus essence, Israel
ATCC 42720	*C. lusitaniae*	blood, myelogenous leukaemia patient, California
CBS 5149^{T}	*C. haemulonii*	gut of *Haemulon sciurus* (fish), Florida
CBS 940^{T}	*C. famata*	air, Japan
CBS 573^{T}	*Issatchenkia orientalis (C. krusei)*	sputum, bronchitic convict, Sri Lanka
ATCC 6258	*C. krusei*	sputum, bronchitic convict, Sri Lanka

[a] Environmental/clinical origin of the strain
[b] ^{T} = type strain of the species
[c] Species names in brackets are the synonymous/teleomorph species names
[d] *Cr. neoformans* var. *gattii* and *Cr. neoformans* var., *neoformans* are the two serovars of the species.

Table 3.11; Allelic profiles at twenty enzyme loci for thirty-two isolates used to investigate the systematics of *Candida* using allozyme electrophoresis

	Enzyme Locus																			
Isolate Code	ADH	ALD	ENOL	EST	FDP	FUM	GDH	G6PD	GPI	HK1	HK2	IDH	NDPK	NP	PepA	PepB	PGAM	6PGD	PGM	PK
CBS 562	c	a	d	c	a	g	c	d	b	d	a	c	e	c	f	c	d	b	d	d
ATCC 90029	c	a	d	b	a	g	c	d	b	d	a	c	e	c	f	c	d	b	d	d
3630	c	a	d	b	a	g	c	d	b	d	a	c	e	c	f	c	d	b	d	d
RA1B	c	a	d	b	a	g	c	d	b	d	a	c	e	c	f	c	d	b	d	d
RA2B	c	a	d	b	a	g	c	d	b	d	a	c	e	c	f	c	d	b	d	d
RA3C	c	a	d	b	a	g	c	d	b	d	a	c	e	c	f	c	d	b	d	d
RA4C	c	a	d	b	a	g	c	d	b	d	a	c	e	c	f	c	d	b	d	d
ATCC 90028	c	a	d	b	a	g	c	c	b	d	a	c	e	c	f	c	d	b	d	b
CBS 834	d	ab	a	a	d	g	c	b	d	d	g	c	dh	b	f	f	b	a	a	d
ATCC 46764	d	a	a	a	d	g	c	b	d	d	g	c	d	c	f	f	b	a	a	d
ATCC 2601	a	a	ac	b	-	d	c	e	b	d	d	c	d	h	a	-	a	e	a	b
CBS 2466	-	c	-	b	-	c	c	f	a	-	i	a	c	b	b	b	-	b	d	f
ATCC 90113	d	a	c	b	-	c	c	a	a	d	a	-	b	b	-	f	c	b	d	f
CBS 6289	d	d	b	-	e	a	e	a	a	d	a	-	g	b	f	f	d	b	d	-
CBS 132	d	c	c	c	e	c	e	a	a	-	b	-	e	-	f	e	bc	c	e	e
ATCC 90112	e	c	d	b	e	-	e	a	a	c	c	-	b	-	c	e	bc	d	d	e
CBS 138	cd	c	b	a	-	g	f	e	d	e	d	-	a	c	d	a	c	-	b	e
ATCC 90030	d	c	b	b	-	-	f	e	d	e	d	-	d	c	d	-	c	b	b	e
CBS 604	a	d	b	b	a	ab	a	b	b	a	g	a	be	d	c	a	b	b	e	e
ATCC 22019	-	d	b	b	a	a	a	b	b	a	g	a	b	d	c	a	b	b	e	e
ATCC 90118	-	d	b	b	a	a	a	b	b	a	g	a	c	d	c	a	b	b	e	e
CBS 566	a	d	f	e	c	d	a	d	b	g	h	ac	ce	-	b	-	d	b	c	c
ATCC 6260	-	d	f	e	-	-	a	d	-	g	h	c	b	-	b	f	d	-	c	c
CBS 6564	b	d	d	e	ef	e	b	d	e	d	f	a	d	f	j	d	b	a	c	e
CBS 7987	a	a	c	a	b	f	b	b	b	b	f	c	b	e	e	d	c	b	c	a
CBS 94	d	b	e	f	d	hj	d	b	c	b	b	b	f	h	i	d	e	a	b	d
ATCC 13803	-	b	e	d	d	i	d	b	c	b	b	b	f	h	i	d	e	a	b	d
CBS 4024	c	c	e	b	e	h	c	bf	c	d	f	d	f	c	h	f	c	a	b	b
ATCC 22981	-	c	e	b	e	h	c	bf	c	d	f	d	f	c	h	f	c	a	b	b
CBS 6936	c	b	d	c	b	-	-	d	c	e	i	c	f	a	h	c	b	a	c	c
ATCC 42720	c	b	d	e	b	e	-	c	c	e	i	c	f	a	h	-	b	a	b	c
CBS 5149	c	e	g	a	d	e	c	e	e	f	g	c	f	g	k	e	e	a	c	b
CBS 940	c	f	h	c	-	f	e	f	f	f	i	-	f	cf	g	f	e	a	f	d
CBS 573	b	b	e	c	f	d	b	g	e	e	e	e	g	e	h	e	d	-	a	b
ATCC 6258	b	b	e	c	ef	d	b	g	e	e	e	e	g	e	h	e	d	a	a	b

Annotation as in Tables 3.3 and 3.5 (p., 71 and 79).

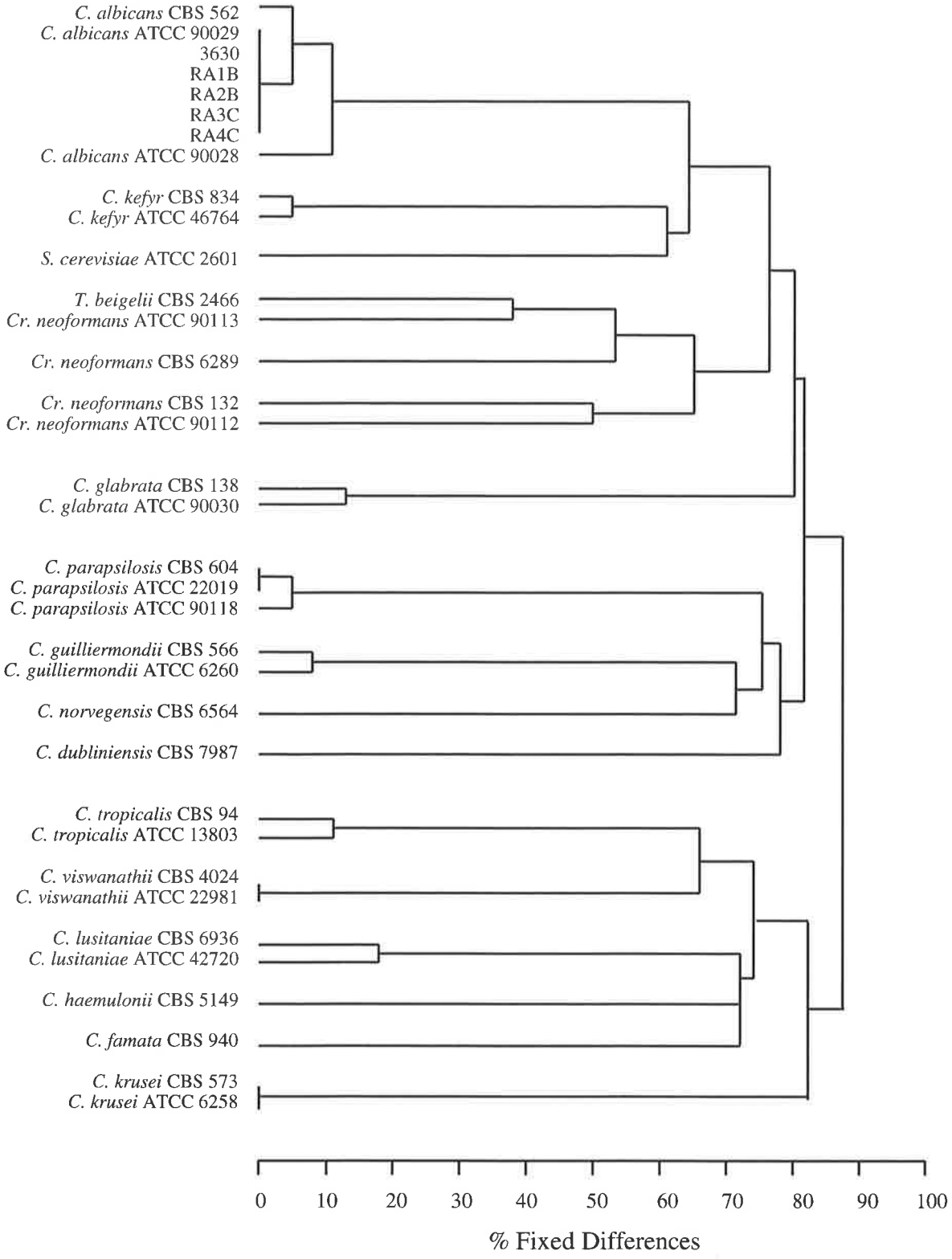


Figure 3.5; Phenogram depicting the genetic relationships between thirty-two type and reference strains of medically important *Candida* species and related genera used in the taxonomy study, as determined from an allozyme electrophoresis analysis using twenty loci.

3.2.1.d Interpretation

Rosa *et al.* (2000) recently investigated the usefulness of allozyme electrophoresis for determining the phylogenetic relationships of five *Candida* species. The authors found that based on fifteen enzyme loci the technique could be used to accurately discriminate among species of *Candida* but, in isolation it was not adequate for determining the systematics within the genus. Similarly herein, the thirteen *Candida* species investigated could be distinguished from one another but there was a high level of genetic variation observed above the species level. All species differed at over 60% of loci, which may be too high to accurately determine the systematics between *Candida* species. However, the levels of fixed differences within the currently recognized species included in the present study were relatively low, indicating that the species studied consisted of genetically related isolates. For many of the *Candida* species assessed a corresponding teleomorphic isolate was included, which clustered with the anamorphic isolates providing supportive evidence that they are morphological variants of a single species.

The levels of fixed differences between the species included in this thesis were equivalent to those used to discriminate bacterial species (eg., Selander *et al.* 1990). *Candida* species differed from each other at levels of variation above that used to discriminate bacterial genera; that is, 80% (eg., Selander *et al.* 1990). This extensive genetic heterogeneity is reflected in the variability in metabolic and morphological characters.

All of the *C. albicans* isolates examined electrophoretically were contained in a small group within genetic cluster 1 and had fixed genetic differences at 0 to 10.7% of loci. This supports the genetic cohesiveness of this species with the observed levels of heterogeneity between the *C. albicans* isolates representing strain variation within the species. Additionally, *S. cerevisiae* clustered with *C. albicans* and *C. kefyr* to the exclusion of other species of *Candida*. Thus the position of the type and reference strains for *S. cerevisiae*, *Cryptococcus neoformans* and *Trichosporon beigelii* within genetic cluster 1 questions the validity of the genus *Candida* or indicates that the resolving power of allozyme electrophoresis is limited to within species and does not extend to the genus level where genetic variability is too great to group species into genera.

Similarly, the two serovars of *Cryptococcus neoformans* were genetically distinct with *T. beigelii* separating them, raising the possibility that they actually constitute distinct species.

C. glabrata, the only *Candida* species within genetic group 2, has recently been transferred into the *Candida* genus from the genus *Torulopsis* (see Odds *et al.* 1997). However, in the present study it appears genetically distinct from all of the other *Candida* species tested and its inclusion in this genus may require further investigation.

Genetic groups 3, 4 and 5 were the most taxonomically-representative of the genus *Candida*; there are no other fungal genera within them. Group 4 contains *C. tropicalis*, the type species for the genus *Candida* and therefore, in phylogenetic investigations, would most accurately represent the *Candida* lineage.

Anamorphs and teleomorphs of *Candida* species are genetically clustered together according to the results obtained in this study. Furthermore, the current taxonomic framework of the genus *Candida* is also questionable. The genus does not appear succinct, being intermingled with other genera. Also, the relationships between *Candida* species are not in agreement with what has been published using traditional techniques (ie., the relative positions of different genera are intermingled with species of the genus *Candida* according to the allozyme electrophoresis results despite their phenotypic differences). The electrophoretic results also differ from the relationships defined using 18S rRNA gene (Hendriks *et al.* 1991). The applicability of the latter method has been questioned for a number of bacterial, algal, yeast, animal and plant taxa (refer to Section 1.1.2 p., 5), but it remains one of the most commonly used phylogenetic methods. This approach will also be used later in this thesis.

3.2.2 Genetic characterization of phenotypically diverse Candida clinical isolates

3.2.2.a Background

Most clinical isolates examined in the present study were identified as *C. albicans* using traditionally identification methods. This was as expected based on other predominance studies (eg., Schiodt *et al.* 1990). An investigation was made to assess the identities and relationships of the other clinically important species of *Candida*. This was possible due to

the relatively small number of isolates to be assessed. This study allowed comparative analysis of the identity and genetic relationships between these isolates on a single electrophoretic gel. This study also re-examined the upper limits of the discriminatory power of the allozyme electrophoresis technique since the previous study (Section 3.2.1 p., 93) indicated that relationships above the species level were not resolved.

The **aims** were;

- *to determine the level of genetic variation between clinical isolates that are morphologically and biochemically diverse and*
- *to assess the extent of discriminatory power of allozyme electrophoresis.*

3.2.2.b Material and methods

This study included a number of oral clinical isolates typed as *Candida* species (but not *C. albicans*), *C. albicans* from a range of clinical infections (eg., extraoral, radiotherapy patient, HIV-negative) and *C. albicans* isolates with different colony morphologies when grown on Sabouraud's dextrose agar (eg., rough colonies). A selection of type strains was also included so that the identity of the clinical isolates could be determined from their genetic relatedness to the type strains. A total of thirty-nine isolates (Tables 3.12a and b p., 104-5) was assessed at nineteen enzyme loci (Table 3.13 p., 106). The clinical isolates collected for this study were obtained from twelve HIV-positive individuals and five asymptomatic carriers.

3.2.2.c Results

Three clinical isolates exhibited multiple bands at loci suggesting that they were mixed cultures or heterozygous isolates. The inability to determine the cause of this banding pattern from this data, as has been discussed previously (p., 83), these three isolates were eliminated from the analysis.

The allelic profiles of the 36 isolates examined are shown in Table 3.13 (p., 106). The isolates had fixed genetic differences at 0 to 89.4% of the nineteen loci. Isolates clustered into species as occurred in Section 3.2.1 (p., 93). The isolates could be divided into six clusters distinguished at over 80% fixed genetic differences (Figure 3.6 p., 107). The first contains *C. albicans*, *C. lusitaniae*, *C. viswanathii*, and *C. dubliniensis*. The isolates within

the *C. albicans* cluster differed at 0 to 32.8% of loci. Two clinical isolates from different patients, 56A and 58A, were genetically identical. The *C. albicans* isolates differed from *C. lusitaniae*, *C. viswanathii* and clinical isolate 34A at 73% of loci. The clinical isolate differed from *C. lusitaniae* at 56% of loci and they, in turn, differed from *C. viswanathii* at 65.5% of loci. Clinical isolates S8 and 15526 differed from the *C. dubliniensis* type strain at 13 and 43% of loci, respectively. They then differed from the *C. parapsilosis* type strain at 66.3% of loci. This group of species differed from the first at 74.7% of loci and together constituted the first cluster.

The first cluster differed from the second at 80.2% of loci, the second containing a clinical isolate S1, the *C. guilliermondii* type strain and the *S. cerevisiae* type strain. The first two isolates differed from each other at 50% of loci and from the last at 69.5% of loci.

The third cluster differed from the first and second at 80.5% of loci. It contains *C. glabrata*, *C. tropicalis*, *T. beigelii* and both serovars of *Cr. neoformans*. The two strains of *Cr. neoformans* var. *neoformans* differed at 46% of loci, and differed from the clinical isolate 14130, subsequently identified as *T. beigelii* at 62% of loci. These isolates in turn differed from the *T. beigelii* type strain at 68.3% of loci and from *Cr. neoformans* var. *gattii* at 71% of loci. These species grouped with the *C. glabrata* and *C. tropicalis* type strains at 78.7% with these two strains differing at 56% of loci. The first three clusters differed from the second three clusters at 89.4% of loci. The fourth cluster consisted of *C. krusei* and *C. haemulonii*, which differed from each other at 65% of loci and from *C. tropicalis* at 75% of loci. This cluster differed at 82% of loci from the type strain of *C. norvegensis*. The clinical isolate S21 differed from the *C. famata* type strain at 60% of loci and this final cluster differed from the fourth and fifth cluster at 86.3% of loci.

Each recognised species could be distinguished from each other, with fixed genetic differences at above 50% of loci. This value appears to be sufficient for the designation of distinct species, whereas using a cutoff value of more than 80% fixed differences does not separate the *Candida* genus from other genera.

Of the seventeen clinical or asymptomatic carrier isolates, eleven were identified as belonging to the *C. albicans* species cluster; two were within the *C. dubliniensis* cluster with one having fixed differences exceeding 50% of loci; one was from the *C. guilliermondii* cluster; and three were from undetermined *Candida* species being most closely related to the

C. famata and *C. lusitaniae* type strains and the *Cr. neoformans* and *T. beigelii* cluster but with fixed differences at over 50% of loci. The clinical isolates that differed from type strains by over 50% of loci potentially represent cryptic species or are from species with higher levels of genetic variation within them than has been observed for *C. albicans*.

Table 3.12a; Details of the thirty-nine reference and clinical isolates used to estimate the genetic diversity of *Candida* clinical isolates using allozyme electrophoresis

Isolate Code	Sex	HIV status	Site of Isolation	Candidiasis Infection	Antifungal Resistance	Patient's Therapy	Other Notes
5B							
17130	F	+	Oesophageal Biopsy	+	Fluconazole, Itraconazole	Fluconazole	
56A.1 to 6	M	+	Oral Rinse	carrier			Post radiotherapy patient, not HIV-positive. 56A.2 and .4 growth on agar slopes very adhesive
58A.1 to 6	M	+	Oral Rinse	OHL			CD4<200, OHL tongue and coated with white plaques
ATCC 90028			Blood, Iowa	systemic			*C. albicans* reference strain
18502	M	+	Oral	+	-	Itraconazole trial	Low CD4 count
18735	F	?	Systemic; Blood	?	-		
18715	M	+	Oral Buccal Mucosa Swab	+	Fluconazole	Fluconazole	
CBS 562			skin (interdigital mycosis), Uruguay				*C. albicans* type strain
18527	M	?	Pancreas	?	-		Multiple abscess and on liver taken at laparotomy; from *C. albicans*, enterococci and multiple resistant *P. aeruginosa*
17484	M	+	Throat Swab	+	Fluconazole, Itraconazole, 5-flurocytosine	Ketoconazole	
12716	M	+	Oral Swab	+	Fluconazole, moderate 5-flurocytosine		Mixed infection with *C. krusei*; resistant to fluconazole, Itraconazole and 5-flurocytosine. Reidentified as *C. dubliniensis*
C1	M	-	Undiluted Saliva	asymptomatic carriage	-		
34A.1 to 6	M	+	Oral Rinse	?			Growth on agar slopes very adhesive and granular
CBS 6936			citrus essence, Israel				*Clavispora lusitaniae* (*C. lusitaniae*) type strain
CBS 4024			meningitis, cerebrospinal fluid, India				*C. viswanathii* type strain
CBS 7987		+	oral cavity, Ireland				*C. dubliniensis* type strain
S8							
15526	M	?	Oral	+	-	Ketoconazole	Germ-tube negative, recurrent infection
CBS 604			case of sprue, Puerto Rico				*C. parapsilosis* type strain
CBS 566			sputum				*Pichia guilliermondii* (*C. guilliermondii*) type strain
S1	M	-	Undiluted Saliva	asymptomatic carriage	-		species other than *C. albicans*

Annotation as in Tables 3.2 and 3.4 (p., 70 and 78).

Table 3.12b; Details of the thirty-nine reference and clinical isolates used to estimate the genetic diversity of *Candida* clinical isolates using allozyme electrophoresis

Isolate Code	Sex	HIV status	Site of Isolation	Candidiasis Infection	Antifungal Resistance	Patient's Therapy	Other Notes
ATCC 2601							*Saccharomyces cerevisiae*
CBS 6289			spinal fluid, Zaire				*Cryptococcus neoformans* var. *gattii* type strain
CBS 132			fermenting fruit juice				*Cr. neoformans* var. *neoformans* type strain
ATCC 90113			Cerebrospinal fluid, Pennsylvania	systemic			*Cr. neoformans* var. *gattii* reference strain
14130	M	+	Oral	+	-	Fluconazole	Outpatient. Reidentified WCH on 14/12/95 - *Trichosporon beigelii*
CBS 2466							*Trichosporon beigelii* type strain
CBS 138			human faeces				*C. glabrata* (*Torulopsis glabrata*) type strain
CBS 834			kefyr grains, Netherlands				*Kluyveromyces marxianus* (*C. kefyr*) b type strain
CBS 940			air, Japan				*C. famata* type strain
S21	F	-	Undiluted Saliva	asymptomatic carriage	-		species other than *C. albicans*
CBS 6564			pregnant woman's vagina, London				*Pichia norvegensis* (*C. norvegensis*) type strain
ATCC 6258			sputum, bronchomycosis, Sri Lanka				*C. krusei*
CBS 94			bronchomycosis				*C. tropicalis* type strain
CBS 5149			gut, *Haemulon sciurus* (fish), Florida				*C. haemulonii* type strain
40I.1 to 6	M	+	Oral Rinse	?			*Candida* species
62A.1 to 6	M	+	Mouth swab	?			HIV Ab+, immunosuppressed, clinical HSV of penile shaft
68A.1 to 6	M	+	Oral Rinse	?			species other than *C. albicans*

Annotation as in Tables 3.2 and 3.4 (p., 70 and 78).

Table 3.13; Allelic profiles of thirty-nine isolates analysed in the phenotypically-diverse *Candida* analysis at nineteen enzyme loci.

	Enzyme Locus																		
Isolate Code	ADH	ALD	ENOL	EST	FDP	FUM	GDH	G6PD	GPI	HK1	HK2	NDPK	NP	PepA	PepB	PGAM	6PGD	PGM	PK
5B	c	a	g	c	b	e	e	d	b	d	b	f	c	d	d	f	b	d	c
17130	c	a	g	c	b	e	e	d	b	e	c	f	c	d	d	f	b	d	c
56A.1 to 6	c	a	f	c	b	e	e	d	b	e	b	f	c	d	d	f	b	d	c
58A.1 to 6	c	a	f	c	b	e	e	d	b	e	b	f	c	d	d	f	b	d	c
ATCC 90028	c	a	f	c	b	e	e	d	b	e	b	f	c	d	d	-	b	d	b
18502	c	a	f	c	b	e	f	d	b	e	b	f	c	d	d	-	b	d	b
18735	c	a	f	c	b	e	-	d	b	e	b	f	c	d	d	f	b	e	b
18715	c	a	f	c	b	e	e	d	b	c	b	f	c	d	d	f	b	d	b
CBS 562	c	a	f	c	b	e	-	e	b	d	b	f	c	d	d	f	b	d	c
18527	c	a	f	c	b	e	e	e	b	e	b	f	c	d	d	f	b	f	d
17484	c	a	f	c	b	e	f	d	b	e	b	e	c	d	d	-	b	d	d
12716	f	a	f	c	b	e	d	d	b	e	b	f	c	d	d	f	b	e	c
C1	f	a	f	c	b	e	f	d	b	b	e	f	c	c	d	f	b	d	d
34A.1 to 6	-	a	f	-	f	g	-	c	b	a	b	f	i	f	d	f	a	b	f
CBS 6936	c	b	e	-	d	-	g	e	d	e	e	g	a	f	-	b	a	c	c
CBS 4024	c	d	f	c	g	f	c	ch	d	d	f	g	d	f	d	d	a	b	b
CBS 7987	a	a	d	a	d	d	-	c	b	b	f	b	e	c	f	d	b	c	a
S8	-	a	d	a	d	d	-	c	b	b	f	-	d	c	f	d	b	b	a
15526	b	a	d	-	-	d	-	c	b	e	c	e	d	c	d	d	b	d	b
CBS 604	a	e	c	c	b	a	e	c	b	a	g	b	d	b	a	d	b	d	d
CBS 566	a	d	f	f	-	b	e	e	b	g	i	e	-	a	c	-	b	c	c
S1	a	d	e	f	d	b	g	d	b	h	h	h	-	a	c	d	b	b	b
ATCC 2601	a	c	ad	c	e	b	-	e	b	d	a	e	-	c	b	a	-	a	b
CBS 6289	a	e	c	-	-	a	-	a	a	e	c	h	-	d	g	f	b	b	f
CBS 132	f	-	e	c	e	b	b	a	a	-	d	f	-	d	g	d	-	f	c
ATCC 90113	f	-	d	-	-	b	a	a	a	-	b	b	c	d	h	d	-	b	c
14130	a	a	g	c	a	b	a	g	a	d	h	i	d	b	b	d	b	b	c
CBS 2466	d	d	-	c	c	b	a	g	a	-	e	d	b	d	a	c	b	c	g
CBS 138	ef	d	c	a	-	e	-	h	e	e	h	a	c	b	e	c	-	b	c
CBS 834	f	ac	b	a	h	e	e	b	e	d	h	af	b	d	g	b	a	f	c
CBS 940	c	g	h	-	b	d	h	h	-	f	g	g	fi	e	h	g	c	g	d
S21	-	e	d	c	b	ac	d	c	b	e	f	c	c	b	a	c	b	d	d
CBS 6564	b	e	e	f	-	c	e	g	f	d	k	f	g	g	g	b	e	-	d
ATCC 6258	g	b	f	d	fi	b	h	i	f	e	j	h	e	e	g	g	e	a	b
CBS 5149	c	f	g	a	f	c	h	g	f	f	g	g	g	h	d	g	e	c	b
CBS 94	f	b	f	e	f	eg	f	c	c	b	d	g	h	g	d	g	a	b	e
40I.1 to 6	c	a	d	a	c	d	e	ce	b	b	eg	c	f	c	f	d	b	d	a
62A.1 to 6	c	a	e	c	h	e	e	df	b	e	b	f	c	d	d	d	b	d	d
68A.1 to 6	f	a	ce	f	f	e	f	ce	be	e	cg	c	b	d	g	f	b	-	b

Annotation as in Tables 3.3 and 3.5 (p., 71 and 79).

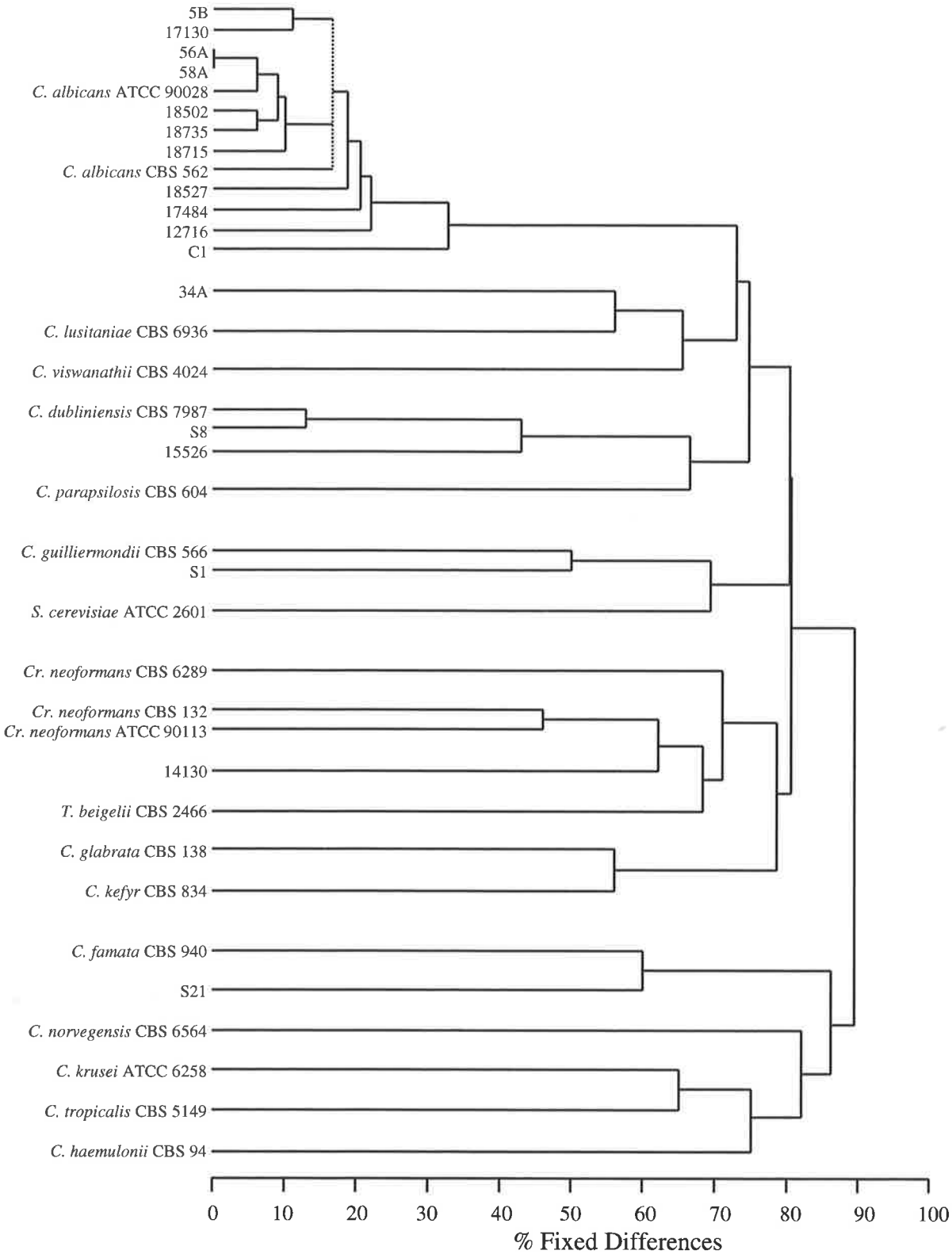


Figure 3.6; Phenogram depicting the genetic relationships between thirty-six isolates of *Candida* and related genera, as determined from an allozyme electrophoresis analysis using nineteen loci

Dashed lines represent unresolved genetic relationships between genetic groups at that taxonomic level.

3.2.2.d Interpretation

Allozyme electrophoresis can determine the species identity of clinical isolates that cluster with type strains. Isolates 56A and 58A from an HIV-negative radiotherapy patient and an HIV-positive patient with OHL, respectively, were not distinguishable in this study. These isolates could represent identical clones or closely related strains that were not distinguished using allozyme electrophoresis.

Clusters of clinical isolates that do not contain any of the assessed type strains may belong to previously described species for which the type or reference strains were not included in the study, or represent new species that have not been described previously.

Candida dubliniensis was described during the course of the present study (Sullivan *et al.* 1995) and so some isolates may initially have been incorrectly identified as *C. albicans* (eg., isolates S8 and 15526). The identity of a selection of those isolates identified as *C. albicans* that do not cluster with other *C. albicans* isolates were investigated further using 18S rRNA sequence comparisons as discussed elsewhere in this thesis (Section 3.5 p., 200).

Relationships between *Candida* species in this analysis varied slightly from those in the taxonomy analysis (Section 3.2.1 p., 93). This could be due to the inclusion of other isolates that alter the relationships between distantly related isolates and by the addition of the IDH enzyme locus in the latter study.

3.2.3 General Interpretation

A number of assumptions regarding the systematics of *Candida* and related genera can be made based on the results of studies 3.2.1 and 3.2.2;

- Different species of *Candida* can be accurately and reproducibly distinguished by allozyme electrophoresis and using, as a guide, a fixed genetic difference of greater than or equal to 50%.
- Anamorphs and teleomorphs of a *Candida* species cluster together, supporting their inclusion into a single taxonomic species.
- The taxonomy of the genus *Candida* is questionable on the basis of allozyme electrophoretic data, with isolates being interspersed with other yeast genera on phenograms.

- Identification of mixed or heterozygous clinical *Candida* samples was possible using allozyme electrophoresis.
- This approach also provided a means for the identification of samples of species other than *C. albicans*, cryptic species, or species not represented in the type and reference strains included in the analyses.

3.3 *Epidemiological analyses of isolates from HIV-positive patients using allozyme electrophoresis*

3.3.1 Background

Once the allozyme electrophoresis technique was optimised and the range of genetic diversity among isolates of *Candida* from clinical samples was estimated, the remaining approximately 80% of clinical isolates, that are germ-tube positive and were presumptively identified as *C. albicans*, were assessed.

The **aims** were:

- *to assess the suitability of the Candida type and reference strains used in Section 3.2 (p., 93) according to their genetic relatedness to clinical samples;*
- *to determine the extent of genetic heterogeneity of clinical isolates based on the framework established in Section 3.2.1 (p., 93); and*
- *to determine the genetic relatedness of Candida isolates obtained from the oral cavity of healthy carriers in relation to isolates obtained from infected individuals. This would provide information on the possibility that healthy carriers are potential sources of infection, and allow assessment of the heterogeneity of isolates from carriers.*

3.3.2 General materials and methods

The following analyses constitute comparisons of yeast species and strains isolated from ninety-nine different HIV-positive patients and from the same patient at different times. The isolates were taken during times of asymptomatic carriage of yeasts and when clinical infection was evident. Additional isolates were obtained from HIV-negative asymptomatic oral carriers of yeast species. Where possible, data for multiple isolates obtained from a single patient have been compared and conclusions drawn regarding the colonising strain. These 'serial' isolates are designated alphabetically in the order in which they were obtained ie., patient number then alphabetic order. So, if three samples were obtained from patient 1, they would be designated 1A, 1B and 1C. The number of individual colonies picked from

the original isolation plate whose lysate was combined for electrophoretic analysis is encoded in the suffix of the isolate code. For example, if isolate 1A consists of three colonies, it would be designated 1A.1-3.

From the ninety-nine HIV-positive patients, an average of two samples per patient were collected and up to six colonies were isolated from each sample and assessed in eleven independent analyses (following Sections). As has been discussed in the General Materials and Methods Chapter, one of the disadvantages of the allozyme electrophoretic technique is the inability to combine data across gels. As a consequence, each of the analyses conducted is discussed independently in the following eleven studies and there is a general discussion of all of the results at the end of this chapter. Information relating to the clinical isolates used in each analysis is provided within the relevant tables in each subsection. The genetic relationships between isolates were assessed using varying combinations of the twenty-three loci established in Section 3.1 (p., 65). The enzyme loci analysed remained consistent, but the staining intensity and resolution varied between experiments. This has resulted in a few differences in the enzymes scored and analysed in each experiment. Pairwise comparisons were made between all isolates in an analysis and the matrices of these comparisons are contained in Appendix 3. Excerpts from these matrices are included in some Sections. Isolates were identified according to their relatedness to type and reference strains included in the individual analyses, using a species cutoff value of fixed differences occurring at over 50% of the loci analysed. Each analysis is independently interpreted and a general interpretation of the analyses is included at the end of this Section.

3.3.3 Study 1

3.3.3.a Results

Isolates (Table 3.14 p., 113-4) differed at 0 to 92.1% of the fifteen loci assessed (Table 3.15 p., 115-6) forming twenty-two distinct clusters that differed at over 50% of the loci (Figure 3.7 p., 117).

The first cluster contained the *C. norvegensis* type strain CBS 6564 which differed to clinical isolate 34A at 57% of loci, the latter strain forming cluster 2. These two isolates differed from isolate S1 (cluster 3) at 62% if loci and from the *C. albicans* cluster (4) at 66.3% of loci. Isolates within the *C. albicans* cluster differed from each other at 0 to 29.3%

of loci. The fifth to eighth clusters differ from the first four at 83.5% of loci. The fifth cluster contained the type strains of *C. guilliermondii* and *T. beigelii*, which differed from each other at 50% of loci and from the sixth cluster containing isolate 70A (*C. glabrata*) at 68.5% of loci. The seventh cluster contained the type strain of *C. viswanathii* (CBS 4024) and the eighth contained the *C. lusitaniae* type strain (CBS 6936) which differed from each other at 54% of loci. The latter two clusters differed from the previously described fifth and sixth cluster at 80.5% of loci.

The first eight clusters differed from the next eleven clusters at 85.9% of loci. The ninth cluster contained the *C. glabrata* type strain, which had fixed differences at 74% of loci from both the tenth and eleventh cluster. The *Cr. neoformans* var *gattii* type strain had fixed differences at 62% of loci from the eleventh cluster, which contained the *C. parapsilosis* type strain and clinical isolate S21, differing from each other at 33% of loci.

This group of clusters differed from the twelfth to eighteenth clusters at 84.8% of loci. The twelfth cluster contained clinical isolates 15A and 15526, which differed at 23% of loci and from the *C. dubliniensis* type strain at 46% of loci. These isolates then differed from the *S. cerevisiae* type strain (cluster 13) at 66.3% of loci. These clusters differed from the *T. beigelii* reference strain (cluster 14) at 74.5% of loci. The type strain of *Cr. neoformans* var *neoformans* and a reference isolate of *Cr. neoformans* var *gattii* differed at 33% of loci, constituting cluster 15. The *C. kefyr* type strain and a clinical isolate (76A) differed at 60% of loci forming clusters 16 and 17, subsequently differed to cluster 15 at 79.9% of loci. These clusters differed from clusters 12 to 14 at 79.9% of loci. The *C. tropicalis* type strain differed from all of these clusters at 83.2% of loci, forming a distinct cluster. The precise relationship between clusters 19 to 21 could not be accurately determined on the basis of the available data. With the addition of more loci, this may be possible in the future. The results presented herein suggest that these three clusters differ from each other at approximately 70% of loci. Each of the clusters contain different *Candida* species, the first with a reference strain of *C. krusei*, the second containing the *C. haemulonii* type strain and the third with the *C. famata* type strain. These three clusters differed from the previously described eighteen clusters at 89.5% of loci.

The twenty-second cluster contains a single clinical isolate, 14130, subsequently identified as *T. beigelii*, which differed at 92.1% of loci from all other isolates assessed in this analysis.

Table 3.14a; Details of the forty-three reference and clinical samples analysed in study 1 using allozyme electrophoresis

Isolate Code	Sex	HIV status	Site of Isolation	Candidiasis	Antifungal Resistance	Patient's Therapy	Other Notes
CBS 6564			pregnant woman's vagina, London				Pichia norvegensis (*C. norvegensis*) type strain
34A.1 to 6	M	+	Oral Rinse	?			Growth on agar slopes very adhesive and granular
S1	M	-	Undiluted Saliva	-			Not *C. albicans*
58A.1 to 6	M	+	Oral Rinse	OHL			CD4<200, OHL tongue and coated with white plaques
CBS 562			skin (interdigital mycosis), Uruguay				*C. albicans* type strain
C1	M	-	Undiluted Saliva	-			
56A.1 to 6	M	+	Oral Rinse	carrier			Post radiotherapy patient, not HIV-positive. 56A.2 and .4 growth on agar slopes very adhesive
12716	M	+	Oral Swab	+	Fluconazole, moderate 5-flurocytosine		Mixed infection with *C. krusei*; resistant to fluconazole, Itraconazole and 5-flurocytosine. Reidentified as *C. dubliniensis*
18715	M	+	Oral Buccal Mucosa Swab	+	Fluconazole	Fluconazole	
18502	M	+	Oral	+	-	Itraconazole trial	Low CD4 count
ATCC 90028			Blood, Iowa	systemic			*C. albicans* reference strain
17484	M	+	Throat Swab	+	Fluconazole, Itraconazole, 5-flurocytosine	Ketoconazole	
18527	M	?	Pancreas	?	-		Multiple abscess and on liver taken at laparotomy; from *C. albicans*, enterococci and multiple resistant *P. aeruginosa*
17130	F	+	Oesophageal Biopsy	+	Fluconazole, Itraconazole	Fluconazole	
18735	F	?	Systemic; Blood	?	-		
5B	M	+	Oral Rinse	+			McCullough strain
CBS 566			sputum				*Pichia guilliermondii* (*C. guilliermondii*) type strain
CBS 2466							*Trichosporon beigelii* type strain
70A.1 to 6	M	+	Oral Rinse	?			*Candida glabrata*
CBS 4024			meningitis, cerebrospinal fluid, India				*C. viswanathii* type strain
CBS 6936			citrus essence, Israel				*Clavispora lusitaniae* (*C. lusitaniae*) type strain
CBS 138			human faeces				*C. glabrata* (*Torulopsis glabrata*) type strain
CBS 6289			spinal fluid, Zaire				*Cryptococcus neoformans* var. *gattii* type strain
CBS 604			case of sprue, Puerto Rico				*C. parapsilosis* type strain
S21	F	-	Undiluted Saliva	-			Not *C. albicans*

Annotation as in Tables 3.2 and 3.4 (p., 70 and 78).

Table 3.14b; Details of the forty-three reference and clinical samples analysed in study 1 using allozyme electrophoresis

Isolate Code	Sex	HIV status	Site of Isolation	Candidiasis	Antifungal Resistance	Patient's Therapy	Other Notes
15526	M	?	Oral	+	-	Ketoconazole	Germ-tube negative, recurrent infection
15A.1 to 6	M	+	?	?			*C. glabrata*
CBS 7987			Oral cavity, Ireland				*C. dubliniensis* type strain
ATCC 2601							*Saccharomyces cerevisiae*
ATCC 28592							*T. beigelii*
CBS 132			fermenting fruit juice				*Cr. neoformans* var. *neoformans* type strain
ATCC 90113			Cerebrospinal fluid, Pennsylvania	systemic			*Cr. neoformans* var. *gattii* reference strain
CBS 834			kefyr grains, Netherlands				*Kluyveromyces marxianus* (*C. kefyr*) b type strain
76A.1 to 6	M	+	Oral Rinse	?			Not *C. albicans*
CBS 94			bronchomycosis				*C. tropicalis* type strain
ATCC 6258			sputum, bronchomycosis, Sri Lanka				*C. krusei*
CBS 5149			gut, *Haemulon sciurus* (fish), Florida				*C. haemulonii* type strain
CBS 940			air, Japan				*C. famata* type strain
14130	M	+	Oral	+	-	Fluconazole	Outpatient. Reidentified WCH on 14/12/95 - *Trichosporon beigelii*
40I.1 to 6	M	+	Oral Rinse	?			*Candida* species
62A.1 to 6	M	+	Mouth swab	?			HIV Ab+, immunosuppressed, clinical HSV of penile shaft
68A.1 to 6	M	+	Oral Rinse	?			not *Candida albicans*, unidentified *Candida* species
S8	M	-	Undiluted Saliva	-			

Annotation as in Tables 3.2 and 3.4 (p., 70 and 78).

Table 3.15a; Allelic profiles of forty-three isolates analysed in study 1 at fifteen enzyme loci

	Enzyme Locus														
Isolate Code	ALD	ENOL	GDH	G6PD	GPI	HK1	HK2	IDH	MPI	NDPK	PepA	PepB	PGAM	PGM	PK
CBS 6564	c	d	c	c	-	b	e	e	g	g	g	d	b	f	g
34A.1 to 6	a	d	d	a	d	a	b	d	h	g	g	d	b	d	g
S1	c	d	b	a	d	i	l	b	a	g	a	d	e	c	g
58A.1 to 6	a	d	d	c	f	d	c	g	f	g	e	d	f	f	e
CBS 562	a	d	d	c	-	d	c	h	g	g	e	d	f	a	g
C1	a	d	d	c	d	d	c	h	g	g	e	d	f	f	g
56A.1 to 6	a	d	d	c	d	d	c	h	I	g	e	d	f	f	f
12716	a	d	d	c	d	d	c	h	dg	g	e	d	f	df	f
18715	a	d	d	c	d	d	c	h	g	g	d	d	f	f	e
18502	a	d	d	c	d	e	c	h	g	g	e	d	f	f	e
ATCC 90028	a	d	d	d	d	d	c	h	g	g	e	d	e	f	e
17484	a	d	-	d	d	d	c	h	g	g	e	d	f	f	e
18527	a	e	d	c	d	d	c	h	d	g	e	d	f	g	g
17130	a	e	d	c	d	d	c	h	d	g	e	d	f	f	e
18735	a	e	d	c	d	d	c	h	d	g	e	d	f	f	e
5B	-	e	d	c	-	d	c	h	d	g	e	d	f	f	f
CBS 566	c	e	b	c	g	b	j	cg	b	dg	a	e	-	eg	e
CBS 2466	c	-	g	a	-	-	-	c	b	d	a	b	c	d	i
70A.1 to 6	c	c	b	b	-	d	b	g	d	d	-	d	c	f	a
CBS 4024	c	e	d	bg	f	f	c	h	-	h	f	f	d	c	d
CBS 6936	c	d	-	d	f	f	e	g	b	h	f	f	b	e	e
CBS 138	c	b	f	f	-	g	d	a	b	j	b	e	d	i	f
CBS 6289	d	b	a	a	d	d	c	e	g	-	b	f	d	-	c
CBS 604	d	b	b	b	-	a	g	f	g	bd	b	-	b	-	g
S21	d	c	b	-	-	a	g	-	g	-	b	e	d	d	f

Annotation as in Tables 3.3 and 3.5 (p., 71 and 79).

Table 3.15b; Allelic profiles of forty-three isolates analysed in study 1 at fifteen enzyme loci

	Enzyme Locus														
Isolate Code	ALD	ENOL	GDH	G6PD	GPI	HK1	HK2	IDH	MPI	NDPK	PepA	PepB	PGAM	PGM	PK
15526	a	c	-	b	d	e	e	g	c	c	c	e	d	d	b
15A.1 to 6	a	c	c	b	d	e	e	b	b	d	c	-	d	d	b
CBS 7987	a	c	c	b	c	b	f	h	-	c	-	e	d	f	b
ATCC 2601	b	-	-	b	d	d	e	g	-	a	d	-	a	b	b
ATCC 28592	b	c	g	c	d	e	e	d	c	e	b	-	e	e	i
CBS 132	b	c	-	a	b	c	c	c	e	g	-	-	-	-	b
ATCC 90113	b	c	e	a	a	d	c	c	e	-	-	f	-	g	g
CBS 834	ab	a	-	b	-	f	h	c	-	cf	-	f	b	b	f
76A.1 to 6	b	c	f	f	h	d	e	f	h	-	b	f	b	f	f
CBS 94	b	e	e	b	e	d	a	f	j	h	g	d	e	d	h
ATCC 6258	b	f	c	h	h	c	e	i	e	i	f	e	e	b	c
CBS 5149	d	g	d	f	-	h	e	h	d	i	h	e	e	f	-
CBS 940	e	h	e	g	-	h	I	f	f	e	f	e	e	f	d
14130	a	g	h	e	c	f	I	b	-	k	c	c	c	d	c
40I.1 to 6	a	c	c	b	d	e	e	g	b	dg	c	d	de	d	a
62A.1 to 6	a	d	d	bc	g	d	c	h	g	g	be	d	e	f	g
68A.1 to 6	-	ad	d	ac	dg	d	ch	h	i	ac	e	e	f	b	d
S8	a	c	c	b	d	d	e	g	b	dg	c	d	d	d	b

Annotation as in Tables 3.3 and 3.5 (p., 71 and 79).

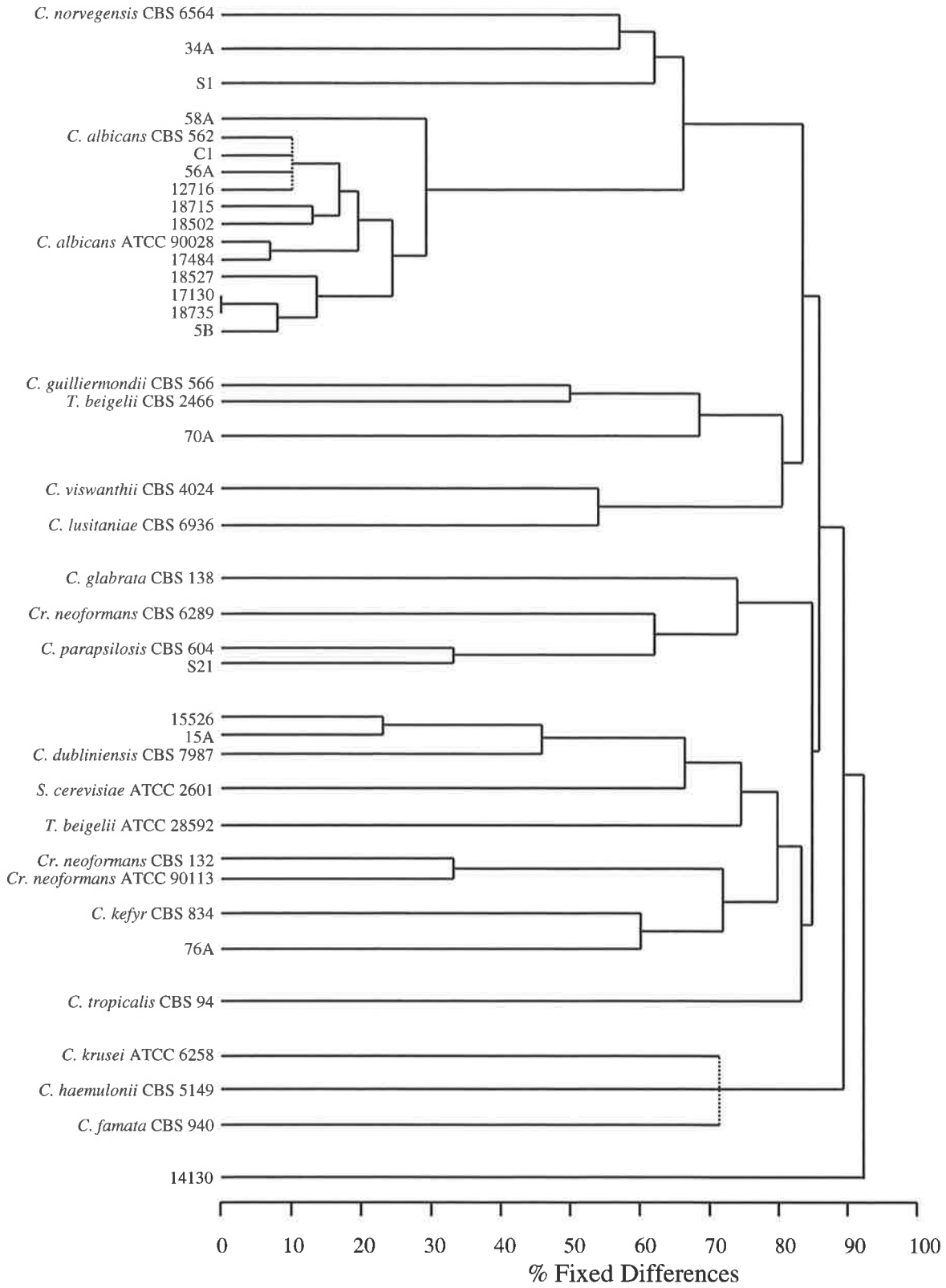


Figure 3.7; Phenogram depicting the genetic relationships between thirty-nine isolates of *Candida* and related genera used in Study 1, as determined by allozyme electrophoresis analysis using fifteen enzyme loci.

Dashed lines represent unresolved genetic relationships between genetic groups at that taxonomic level.

3.3.3.b Interpretation

Most isolates assessed in study 1 belonged to the *C. albicans* cluster (eleven of nineteen isolates), two belonged to *C. dubliniensis*, one to *C. parapsilosis* and five to undetermined *Candida* species. As discussed in Section 3.1.2 (p., 74) isolate 14130 was later identified as belonging to the species *Trichosporon beigelii*. However, the large genetic difference between this clinical isolate and the *T. beigelii* type strain CBS 2466 according to the current data question this identity.

Clinical isolates 15A and 70A were originally identified as *C. glabrata*, according to traditional characters, prior to their allozyme electrophoresis analysis. In this electrophoretic analysis, isolate 15A clustered with *C. dubliniensis*, which may be a consequence of the recent description of this species. Isolate 70A was genetically distinct from the other *Candida* species analysed, suggesting that it constitutes an alternative species to *C. glabrata*, and perhaps represents an, as yet, undescribed species.

The type and reference strains for the *Cryptococcus neoformans* serovars formed two distinct clusters (CBS 132 and ATCC 90113), suggesting that these subtypes may warrant a separate species designation. Additionally, the *Cr. neoformans* var *gattii* reference strain (CBS 6289) may have been improperly designated or was contaminated because it is not associated with the other *Cr. neoformans* isolates assessed in this analysis.

3.3.4 Study 2

3.3.4.a Results

The isolates assessed in study 2 (Table 3.16 p., 120-21) varied at 0 to 89.4% of the nineteen enzyme loci assessed (Table 3.17 p., 122). They formed nineteen distinct clusters with fixed differences between them at over 50% of the loci (Figure 3.8 p., 123).

The first cluster of isolates comprised nine clinical isolates, which exhibited fixed differences between them at 0 to 46.6% of loci. Since many of these isolates have been identified, using traditional methods, as *C. albicans* and have clustered in earlier experiments with the *C. albicans* type and reference strains, this cluster probably represents

this species. Isolate 68B differed from the first cluster at 53.6% of loci and constituted the second cluster. Isolates ATCC 22981 and 14130 differed from each other at 60% of loci, forming clusters 3 and 4, which differed from clusters 1 and 2 at 69.9% of loci.

These four clusters differed from clusters 5 and 6 at 79.3% of loci. Cluster 5 contained a single isolate ATCC 2601, the type strain for *S. cerevisiae* which differed from cluster 6 at 77.8% of loci. Cluster 6 contained four clinical isolates and the *C. dubliniensis* type strain that differed from each other at 8 to 27.3% of loci. The specific relationship between the three clinical isolates could not be accurately resolved on the basis of the nineteen loci assessed herein. However, the fixed differences between them ranged from 8 to 12.5% of loci and the relationship between them has been approximated and is represented with a dashed line. This cluster probably constitutes the isolates, in this analysis, that belong to the *C. dubliniensis* species.

Clusters 7 to 10 differ from the first six clusters at 84.2% of the nineteen enzyme loci assessed. Clinical isolate 76A and the *T. beigelii* type strain, which differ from each other at 50% of loci, constitute cluster 7. Cluster 8 contains the *Cr. neoformans* var. *neoformans* type strain CBS 132, which differs from cluster 7 at 59.5% of loci. Clusters 9 and 10 differ from each other at 62% and from clusters 7 and 8 at 74.8% of loci. They contain single isolates CBS 138, the *C. glabrata* type strain, and 86A. Clusters 11 to 16 contain single isolates, which differ from the previously described clusters at 86% of loci. These clusters differ from each other at 62% to 82% of loci, but the specific relationships between these clusters could not be reproducibly resolved using this data. For this reason, the relationship between these clusters has been approximated and is represented in the phenogram by a dashed line. Cluster 11 contains the *C. famata* type strain, CBS 940, cluster 12, contains the *C. guilliermondii* reference strain, ATCC 6260. Cluster 13 contains the *C. lusitaniae* type strain, cluster 14, the *C. norvegensis* type strain. Clusters 15 and 16 contain the *C. parapsilosis* type strain and clinical isolate S21, respectively.

Table 3.16a; Details of the thirty-four reference and clinical samples analysed in study 2 using allozyme electrophoresis

Isolate Code	Sex	HIV status	Site of Isolation	Candidiasis Infection	Antifungal Resistance	Patient's Therapy	Other Notes
M2A	M	+	Oral Rinse	+			
17484	M	+	Throat Swab	+	Fluconazole, Itraconazole, 5-flurocytosine	Ketoconazole	
P1			Patient with acute pseudomembranous candidiasis				Lesions induced in 60% of rats
16517	M	?	Systemic; Blood	?	-	penicillin	
56A	M	+	Oral Rinse	-			Post radiotherapy patient, not HIV-positive. 56A.2 and .4 growth on agar slopes very adhesive
M5A	M	+	Oral Rinse	+			
12716	M	+	Oral Swab	+	Fluconazole, moderate 5-flurocytosine		Mixed infection with *C. krusei*; resistant to fluconazole, Itraconazole and 5-flurocytosine
A2			Patient with chronic atrophic candidiasis				Lesions absent in rat.
91A	M	+	Oral Rinse	?			*Candida* species, germ tube + but possibly mixed with other yeast
68B	M	+	Oral Rinse	?			not *Candida albicans*, unidentified *Candida* species
ATCC 22981			cerebrospinal fluid, India				*C. viswanathii* reference strain
14130	M	+	Oral	+	-	Fluconazole	Outpatient. Reidentified WCH on 14/12/95 - *Trichosporon beigelii*
ATCC 2601							*Saccharomyces cerevisiae* type strain
40l	M	+	Oral Rinse	?			*Candida* species
15526	M	?	Oral	+	-	Ketoconazole	Germ-tube negative, recurrent infection
S8	M	-	Undiluted Saliva	-			
CBS 7987		+	oral cavity, Ireland				*C. dubliniensis* type strain
34B	M	+	Oral Rinse	?			Growth on agar slopes very adhesive and granular
CBS 2466							*T. beigelii* type strain
76A	M	+	Oral Rinse	?			Not *C. albicans*

Annotation as in Tables 3.2 and 3.4 (p., 70 and 78).

Table 3.16b; Details of the thirty-four reference and clinical samples analysed in study 2 using allozyme electrophoresis

Isolate Code	Sex	HIV status	Site of Isolation	Candidiasis Infection	Antifungal Resistance	Patient's Therapy	Other Notes
CBS 132			fermenting fruit juice				*Cr. neoformans* var. *neoformans* type strain
CBS 138			human faeces				*C. glabrata (Torulopsis glabrata)* type strain
86A	M	+	Oral Rinse	?			*Candida* species, not *C. albicans*
CBS 940			air, Japan				*C. famata* type strain
ATCC 6260			bronchomycosis				*C. guilliermondii* reference strain
CBS 6936			citrus essence, Israel				*Clavispora lusitaniae (C. lusitaniae)* type strain
CBS 6564			pregnant woman's vagina, London				*Pichia norvegensis* type strain
CBS 604			case of sprue, Puerto Rico				*C. parapsilosis* type strain
S21	F	-	Undiluted Saliva	-			Not *C. albicans*
CBS 834			kefyr grains, Netherlands				*Kluyveromyces marxianus (C. kefyr)* type strain
CBS 5149			gut, *Haemulon scirus* (fish), Florida				*C. haemulonii* type strain
ATCC 6258			sputum, bronchomycosis, Sri Lanka				*C. krusei* reference strain
CBS 573			sputum, bronchomycosis, Sri Lanka				*Issatchenkia orientalis (C. krusei)* type strain
54A	M	+	Oral Rinse	?			54A.1 growth on agar slopes very adhesive

Annotation as in Tables 3.2, 3.4 and 3.7 (p., 70, 78 and 85).

Table 3.17; Allelic profiles of thirty-four isolates analysed in study 2 at nineteen enzyme loci

	Enzyme Locus																		
Isolate Code	ALD	ENOL	EST	FDP	FUM	GDH	GOT	G6PD	GPI	HK1	HK2	NDPK	NP	PepA	PepB	PGAM	6PGD	PGM	PK
M2A	a	d	d	b	d	c	d	d	d	c	b	d	c	g	d	h	c	g	d
17484	a	d	d	b	d	c	d	d	d	c	b	d	c	g	d	h	c	g	d
P1	a	d	d	-	d	c	d	d	d	c	d	d	c	g	d	h	c	g	d
16517	a	d	d	b	d	c	d	e	d	a	d	d	c	g	d	h	c	g	d
56A	a	d	d	-	-	c	-	d	d	a	d	d	c	g	d	h	c	-	d
M5A	a	e	d	-	-	c	d	e	d	c	b	d	-	g	d	h	c	g	d
12716	a	d	d	b	d	c	d	d	d	d	c	d	-	g	g	h	c	eg	d
A2	a	d	d	-	d	-	d	e	d	c	b	-	c	g	e	h	c	g	f
91A	a	d	d	b	-	c	d	d	c	c	b	d	c	f	c	f	e	-	c
68B	a	d	d	-	d	f	-	d	e	e	d	f	c	f	d	f	c	-	e
ATCC 22981	d	e	d	-	g	f	f	f	d	a	a	-	d	e	-	g	c	d	c
14130	a	g	d	a	a	c	d	-	d	e	e	h	c	e	b	e	c	d	c
ATCC 2601	c	c	d	d	-	c	b	c	f	a	d	b	-	a	k	a	-	a	d
40I	a	c	b	c	c	b	b	c	d	b	d	c	e	f	g	e	c	-	b
15526	a	c	b	-	-	b	-	b	d	b	d	c	-	f	g	-	c	e	b
S8	a	c	-	-	-	b	b	c	d	b	e	-	-	f	g	e	c	e	b
CBS 7987	a	c	a	c	c	c	g	c	d	b	d	c	-	f	g	e	c	e	b
34B	a	c	b	c	-	b	-	c	h	b	d	c	-	f	g	-	d	f	b
CBS 2466	c	f	d	b	b	d	b	e	d	c	b	c	b	c	i	d	a	e	g
76A	d	c	d	-	-	-	c	g	g	c	b	f	-	e	i	d	-	e	g
CBS 132	c	c	d	-	-	e	b	b	b	c	b	d	-	g	h	-	-	h	g
CBS 138	c	b	a	-	d	e	c	g	g	d	c	-	-	d	-	d	-	e	c
86A	e	c	c	-	-	f	c	g	g	d	c	a	c	g	h	i	c	f	g
CBS 940	i	g	f	b	-	e	-	f	e	g	g	d	ce	h	-	j	f	e	c
ATCC 6260	f	d	f	b	-	a	b	d	e	g	h	c	-	b	i	f	-	d	e
CBS 6936	b	d	f	-	-	e	d	d	e	f	h	e	a	i	c	c	c	h	f
CBS 6564	g	d	f	-	c	b	d	e	a	f	e	d	-	j	-	c	-	-	g
CBS 604	g	b	-	b	ac	a	d	a	c	a	g	ce	e	d	f	c	b	g	g
S21	g	c	-	b	-	-	eh	b	c	e	d	a	-	h	-	d	c	g	g
CBS 834	ac	a	-	d	c	e	f	a	d	f	g	be	b	g	j	b	-	c	e
CBS 5149	h	f	a	d	d	c	h	f	-	e	g	f	f	k	f	j	g	c	d
CBS 573	b	e	e	e	-	b	g	-	h	f	e	g	-	h	-	h	g	c	d
ATCC 6258	b	e	e	-	c	b	g	-	c	f	e	g	-	h	h	h	g	b	c
54A	a	d	bd	c	d	bc	d	cd	d	b	d	cd	ce	fg	ae	-	b	e	ad

Annotation as in Tables 3.3, 3.5 and 3.8 (p., 71, 79 and 86)

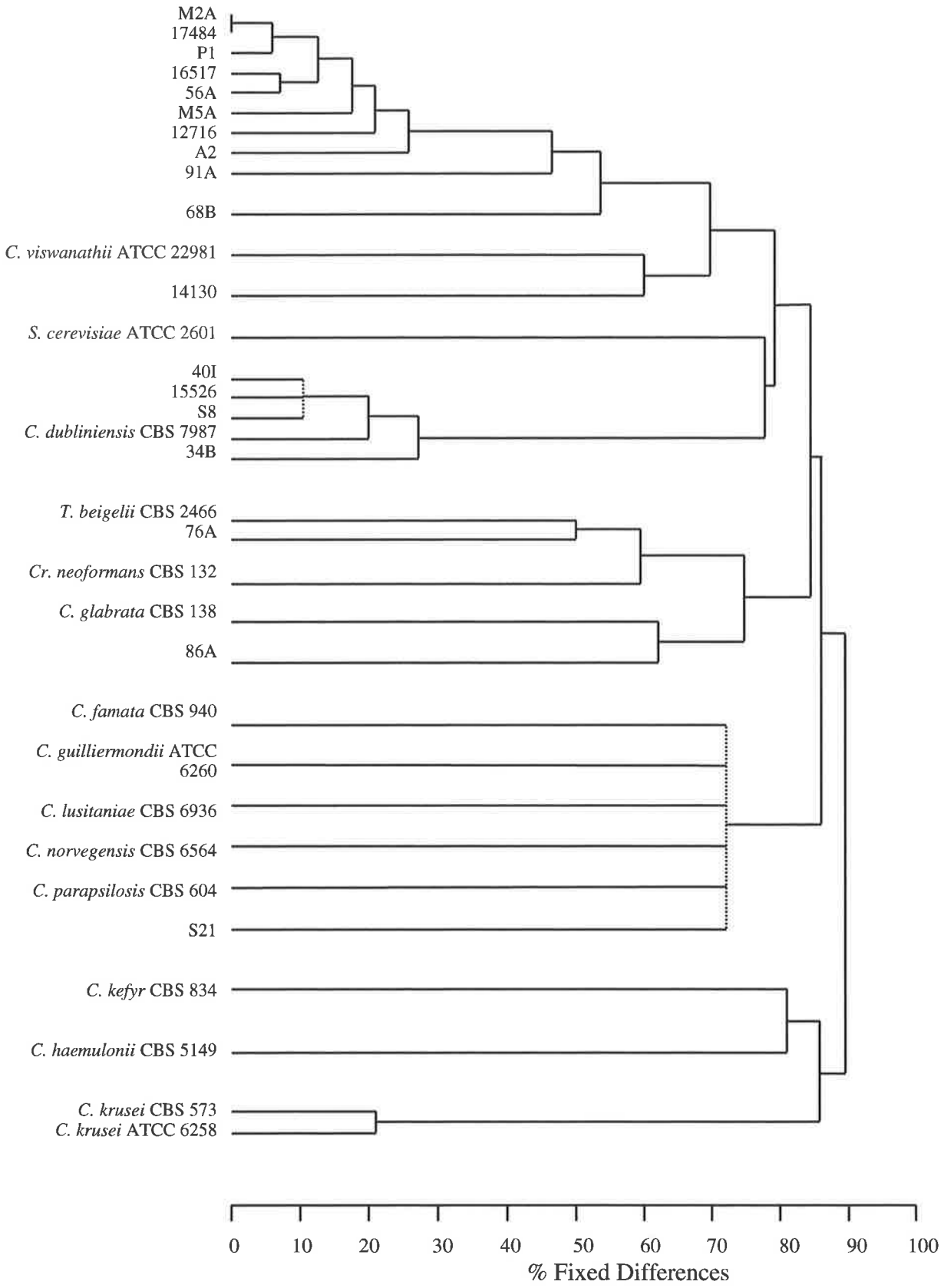


Figure 3.8; Phenogram depicting the genetic relationships between thirty-three isolates of *Candida* and related genera used in Study 2, as determined from an allozyme electrophoresis analysis using nineteen loci.

Dashed lines represent unresolved genetic relationships between genetic groups at that taxonomic level.

3.3.4.b Interpretation

Of the eighteen clinical or asymptomatic carrier isolates assessed, nine were contained within the *C. albicans* cluster. Although this cluster does not contain a type or reference strain for this species, it's identity can be invoked by the presence of other clinical isolates assessed in previous analyses that clustered with *C. albicans* type or reference strains (eg., 56A, 17484, 12716, 5A).

Isolates P1 and A2 (Allen and Beck 1987) were genetically distinct, but both belonged to the same cluster of isolates in this study. This result suggests that there are vast differences in the virulence of strains within a single genetic cluster.

Four clinical isolates were contained within the *C. dubliniensis* cluster. Another isolate was associated with the *Trichosporon beigelii* reference strain, but it was quite distinct and may belong to the species *C. guilliermondii*. The species identity of four clinical isolates could not be determined because they did not associate with any of the reference strains used in this analysis.

3.3.5 Study 3

3.3.5.a Results

Isolates (Table 3.18 p., 125) varied at 0 to 74.2% of the nineteen loci examined (Table 3.19 p., 126). Three clusters of isolates with fixed differences over 50% were evident (Figure 3.9 p., 127). The first contained the majority of clinical isolates assessed, which differed at 0 to 26.4% of loci.

This cluster also contained the *C. albicans* type and reference strains. Some of the relationships within the lower levels of this cluster were unable to be conclusively resolved using the available data (hence represented by a dotted line). This cluster differed from the second at 67.2% of loci. The second cluster contained two isolates, the *C. dubliniensis* type strain and clinical isolate 40H, which differed from each other at 44% of loci. The third cluster contained a single isolate, the *C. parapsilosis* type strain, which differed from all other isolates assessed herein at 74.2% of the loci.

Table 3.18; Details of the twenty-seven reference and clinical samples analysed in study 3 using allozyme electrophoresis.

Isolate Code	Sex	HIV status	Site of Isolation	Candidiasis	Antifungal Resistance	Patient's Therapy	Other Notes
71A.1 to 6			oral rinse				
71B.1 to 6			oral rinse				
CBS 562			skin(interdigital mycosis), Uruguay				*C. albicans* type strain
80A.1 to 6							
68D.1 to 6	M	+	Pharynx Swab	?			same time as 68C
ATCC 90029			Blood, Iowa	systemic			*C. albicans* reference strain
40B.1 to 6							
44A.1 to 6							
46B.1 to 6							
17130	F	+	Oesophageal Biopsy	+	Fluconazole, Itraconazole	Fluconazole	
ATCC 90028			Blood, Iowa	systemic			*C. albicans* reference strain
46A.1 to 6							
84B.1 to 6	M	+	Palate Swab	+			
40J.1 to 6							
12716	M	+	Oral Swab	+	Fluconazole, moderate 5-flurocytosine		Mixed infection with *C. krusei*; resistant to fluconazole, Itraconazole and 5-flurocytosine. Reidentified as *C. dubliniensis*
CBS 7987		+	oral cavity, Ireland				*C. dubliniensis* type strain
40H.1 to 6							
CBS 604			case of sprue, Puerto Rico				*C. parapsilosis* type strain
40A.1 to 6	M	+	?	?			Clinical and identification data not available
40C.1 to 6							
40D.1 to 6							
40E.1 to 6							
44B.1 to 6							
68C.1 to 6	M	+	Oral Rinse	?			
68E'	M	+					
68F.1 to 6	M	+	Oral Rinse	?			1 month later
84C.1 to 6	M	+	Tongue Swab	+		Fluconazole	*Candida* species, 7 months later

Annotation as in Tables 3.2, 3.4 and 3.7 (p., 70, 78 and 85).

Table 3.19; Allelic profiles of twenty-seven isolates analysed in study 3 at nineteen enzyme loci

	Enzyme Locus																		
Isolate Code	ADH	ALD	ENOL	EST	FDP	FUM	HK1	HK2	GDH	G6PD	GPI	NDPK	NP	PepA	PepB	PGAM.	6PGD	PGM	PK
71A.1 to 2	d	a	b	b	a	d	a	b	c	d	c	g	a	d	a	-	b	d	e
71B.1-2, 5. 6	d	a	b	b	a	d	a	b	c	d	c	g	a	d	a	c	b	d	e
CBS 562	d	a	b	b	a	d	a	c	c	c	c	g	a	d	a	c	b	d	e
80A.5	d	a	b	b	a	d	a	a	c	b	c	g	a	d	a	b	b	d	b
68D.1-4, 6	d	a	b	b	a	d	a	c	c	b	c	g	a	d	a	b	b	d	d
ATCC 90029	c	a	b	b	a	d	a	b	c	b	c	g	a	d	a	d	c	d	e
40B.1 to 6	d	a	b	b	a	d	a	b	c	b	c	g	a	d	c	-	c	e	c
44A.1 to 6	b	a	b	b	a	d	a	a	c	b	c	g	a	d	b	a	d	d	c
46B.1, 4	c	a	b	b	a	d	a	a	c	b	c	g	a	d	c	-	b	d	c
17130	c	a	c	b	a	d	a	b	c	b	c	g	a	d	b	a	c	d	c
ATCC 90028	d	a	b	b	a	d	a	b	c	b	c	g	a	d	b	a	b	d	c
46A.1 to 6	d	a	b	b	a	d	a	b	c	b	c	g	a	d	c	a	b	d	c
84B.2	de	a	b	b	a	d	a	b	c	b	c	g	a	d	b	a	b	d	d
40J.1	a	a	b	a	a	d	a	b	d	b	c	g	b	d	b	-	b	d	a
12716	c	a	a	b	a	d	a	b	b	b	c	h	a	e	b	c	c	ad	c
CBS 7987	a	a	a	b	b	c	a	a	a	a	b	d	b	c	c	a	c	c	a
40H.1 to 6	a	a	b	b	a	c	a	b	a	b	a	f	c	c	c	-	c	d	a
CBS 604	a	b	b	a	a	ab	b	a	b	a	a	cf	a	a	c	a	a	d	f
40A.1 to 6	a	a	b	a	b	c	a	b	a	a	a	ce	c	d	c	a	c	b	a
40C.2-3, 6	a	a	b	a	b	c	a	b	a	a	a	g	a	d	c	a	c	c	ac
40D.1-2, 4, 6	d	a	d	b	a	c	a	-	a	b	c	g	a	d	c	a	c	c	ac
40E.4-6	b	a	b	b	a	d	a	a	b	b	a	ch	b	c	bc	-	c	c	ac
44B.1 to 6	b	a	b	b	a	d	a	a	c	a	c	g	a	d	c	a	c	d	ac
68C.2 to 5	bd	a	b	b	a	d	a	c	c	b	c	g	a	d	a	b	b	d	c
68E'.1 to 6	df	a	b	b	a	d	a	b	c	b	c	g	a	d	a	a	b	d	d
68F.1 to 3	df	a	b	b	a	d	a	b	c	b	c	g	a	d	a	a	d	d	d
84C.1 to 6	d	a	a	b	a	d	a	b	c	b	c	eg	a	d	b	a	b	d	d

Annotation as in Tables 3.3, 3.5 and 3.8 (p., 71, 79 and 86)

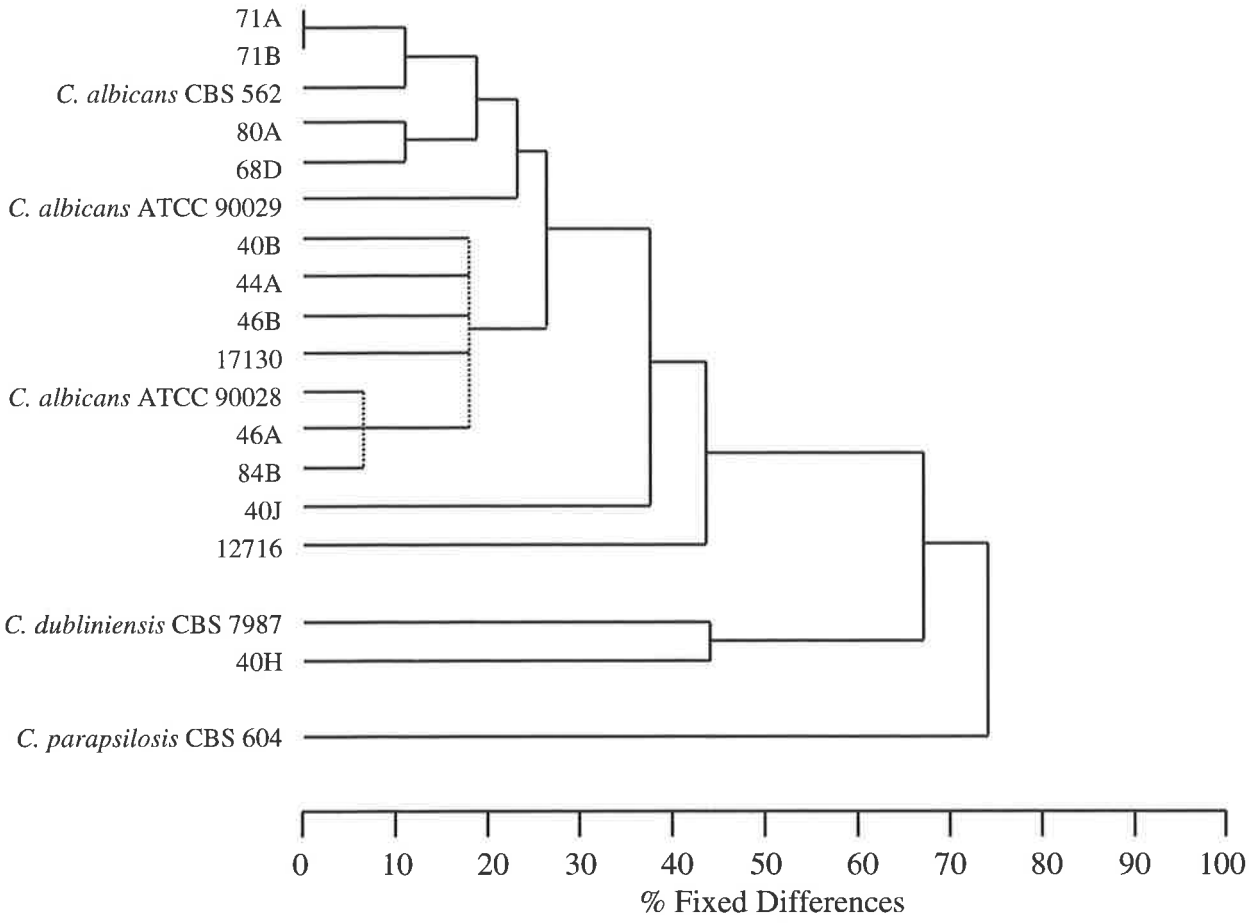


Figure 3.9; Phenogram depicting the genetic relationships between eighteen isolates of *Candida* used in Study 3, as determined from an allozyme electrophoresis analysis using nineteen loci.

Dashed lines represent unresolved genetic relationships between genetic groups at that taxonomic level.

Table 3.20; Matrix of the percentages of fixed genetic difference between pairs of isolates from 5 individual patient in study 3.

Isolate	40B	40H	40J	46A	46B	71A
40H	**50**[a]					
40J	**44**	**50**				
46A	11	50	33			
46B	22	56	39	**11**		
71A	28	61	39	17	28	
71B	28	61	39	21	28	**0**[b]

a; Percentages of loci at which fixed differences occurred between isolates from the same patient are shown in bold, other values are the percentages of fixed differences between unrelated isolates.
b; When fixed differences do not occur at any of the loci examined (ie., the isolates differ at 0%), the isolates are closely related but not necessarily identical. Allozyme electrophoresis provides a conservative estimation of the genetic diversity between isolates.

3.3.5.b Interpretation

Nine clinical isolates exhibited multiple banding patterns due to being mixed or heterozygous and were therefore eliminated from the analysis. Two clinical strains, derived from a single colony, were multiple banded (12716 and 84B.2) suggesting that they are heterozygous or contaminated. The same was also noted for the *C. parapsilosis* type strain at the PGM enzyme locus. Their purity was confirmed using a Gram stain, where only yeast cells were evident. The implications of this result are that either the *C. parapsilosis* Type strain has become contaminated (unlikely since there was only one enzyme with multiple bands) or that this strain is heterozygous for the PGM locus. Twelve of the thirteen remaining clinical isolates assessed belonged to the *C. albicans* cluster and one belonged to the *C. dubliniensis* cluster.

Using an excerpt from the matrix of pairwise comparisons in Appendix 3 (Table 8), multiple isolates from a single patient were compared independent of all other isolates assessed. Isolates from patient 40 were all very distinct, differing at 44 to 50% of loci (Table 3.20 p., 128). Isolate 40H was identified as *C. dubliniensis* and the other isolates assessed were *C. albicans*. Isolate 40B was taken on the 10th of May 1995, 40H on the 17th of January 1996 and 40J on the 13th of March 1996. All three isolates were from oral rinses. Isolates 40A, C, D and E were mixed/heterozygous and were therefore eliminated from the analysis. All of the isolates were obtained from oral rinse samples in the absence of obvious lesions and were all taken at different times. Patient 40 participated in an amphotericin B lozenge trial, commenced prior to the collection of isolate J. Following this treatment, patient 40 was no longer colonized with *C. dubliniensis*, as they were when the previous sample was obtained (isolate 40H), but were instead colonized by another *C. albicans* strain. This patient illustrates the complexity of oropharyngeal candidiasis and asymptomatic carriage since on no occasion was the same strain obtained from two oral rinse samples. The clinical implications of this and other similar observations in this thesis are further explored in the Discussion Chapter (p., 254).

Isolates A and B from patient 46 differed at 11% of loci, constituting distinct *C. albicans* strains. They were both isolated from oral rinse samples taken on the 22nd of May 1995 and the 16th of August 1995, respectively.

Patient 68 participated in an amphotericin B lozenge trial during the time that isolates D to G were collected. The isolate obtained just prior to the trial was mixed/heterozygous and

at the commencement of the trial it was a single/homozygous isolate. Subsequent isolates were mixed/heterozygous, suggesting that the amphotericin B lozenges may have contributed to the increased incidence of a mixed colonization or the appearance of heterozygous strains in this patient. The lozenges contain 100mg of Amphotericin B and patients in the trial took 4 per day. This is equivalent to the 400mg per day recommended for the treatment of oral fungal infections including oropharyngeal candidiasis (see http://www.thebody.com/pwa/ampho.html) but the slower delivery of the drug may lead to a fungiostatic rather than a fungicidal effect on *Candida.*

Clinical isolates 71A and B were genetically identical according to the allozyme electrophoresis results, which suggests maintenance of the same strain of *C. albicans*. The two samples were taken one week apart on the 9th and the 16th of August 1995, respectively, both arising from oral rinses.

3.3.6 Study 4

3.3.6.a Results

Four clusters of isolates (Table 3.21 p., 131-2) that differed at over 50% of the nineteen loci assessed (Table 3.22 p., 133-4) are evident in study 4 (Figure 3.10 p., 135). The first contains the majority of clinical isolates, along with the *C. albicans* type and reference strains, with fixed differences occurring at 5 to 40.9% of loci. It can be assumed that this cluster constitutes the *C. albicans* species in this analysis. The second and third clusters contain single isolates CBS 604, the *C. parapsilosis* type strain, and clinical isolate 34A. These two clusters differ from each other at 65% of loci and from cluster 1 at 72.4% of loci. The fourth cluster differs from the other three at 76.9% of loci. It contains two clinical isolates and the *C. dubliniensis* type strain CBS 7987 with fixed differences between them at 24 to 41% of loci. This cluster probably constitutes the *C. dubliniensis* isolates in this analysis. The described clustering pattern was accurate and reproducible. However, with repeat analyses, the genetic relationships between isolates within the first cluster that differed at less than 30.7% of loci (subcluster A) were not. This is due to there being tied values in the matrix of fixed genetic differences (see Richardson *et al.* 1986 for a more detailed discussion). The percentage of loci at which multiple isolates from a single patient have fixed differences, taken from the matrix (Appendix 3, Table 9) is discussed.

Table 3.21a; Details of the forty-six reference and clinical samples analysed in study 4 using allozyme electrophoresis

Isolate Code	Sex	HIV status	Site of Isolation	Candidiasis	Antifungal Resistance	Patient's Therapy	Other Notes
CBS 562			skin (interdigital mycosis), Uruguay				*C. albicans* type strain
22A.1 to 6	M	+	Oral Rinse	?			
22B.1 to 6	M	+	Oral Rinse	+	Fluconazole	100mg/day fluconazole	Fluconazole resistant candidiasis, Germ tube positive, FCZ resistance required
17D.1 to 6	M	+	Palate swab	?			
17A.1 to 6	M	+	?	?			
17E.1 to 6	M	+	Oral Rinse	+			
46B.1 to 6	M	+	Oral Rinse	?			
84A.1 to 6	M	+	Oral Rinse	+			
ATCC 90028			Blood, Iowa	systemic			*C. albicans* reference strain
80A.1 to 6	M	+	Oral Rinse	?			
17130	F	+	Oesophageal Biopsy	+	Fluconazole, Itraconazole	Fluconazole	
19A.3	M	+	Oral Rinse	?			
12716	M	+	Oral Swab	+	Fluconazole, moderate 5-flurocytosine		Mixed infection with *C. krusei* - resistant to fluconazole, Itraconazole and 5-flurocytosine. Reidentified as *C. dubliniensis*
71A.1 to 6	M	+	Oral Rinse	?			
71B.1 to 6	M	+	Oral Rinse	?			
ATCC 90029			blood, Iowa				*C. albicans* reference strain
23B.1 to 6	M	+	OHL Tongue swab	OHL		fungilin	
23A.1 to 6	M	+	Oral Rinse	+			
23C.1 to 6	M	+	Oral Rinse	?			
17C.1 to 6	M	+	Oral Rinse	?			
88D.1 to 6	M	+	Oral Rinse	OHL			candidiasis, oral hairy leukoplakia
31A.1 to 6	M	+	Oral Rinse	?			
40D.1 to 6	M	+	Oral Rinse	?			
68C.1 to 6	M	+	Oral Rinse	?			
19D.1 to 6	M	+	Oral Rinse	?			
31E.1 to 6	M	+	Oral Rinse	?			
CBS 604			case of sprue, Puerto Rico				*C. parapsilosis* type strain
34A.1 to 6	M	+	Oral Rinse	?			Growth on agar slopes very adhesive and granular
CBS 7987		+	oral cavity, Ireland				*C. dubliniensis* type strain
30A.1 to 6	M	+	Oral Rinse	+	Fluconazole	On 50mg fluconazole a day	Previous isolate fluconazole sensitive
40F.1 to 2	M	+	Oral Rinse	?			

Annotation as in Tables 3.2 and 3.4 (p., 70 and 78).

Table 3.21b; Details of the forty-six reference and clinical samples analysed in study 4 using allozyme electrophoresis

Isolate Code	Sex	HIV status	Site of Isolation	Candidiasis	Antifungal Resistance	Patient's Therapy	Other Notes
32A.1 to 6	M	+	Swab OHL Tongue	OHL			
32B.1 to 6	M	+	Oral Rinse	?			
33A.1 to 6	M	+	Oral Rinse	?			
40C.1 to 6	M	+	Oral Rinse	?			
40E.1 to 6	M	+	Oral Rinse	?			
68A.1 to 6	M	+	Oral Rinse	?	not *C. albicans*		
68D.1 to 6	M	+	Pharynx Swab	?	obtained at the same time as 68C		
84B.1 to 6	M	+	Palate Swab	+	obtained at the same time as 84A		
88B.1 to 6	M	+	Oral Rinse	+			Oral pseudomembranous candidiasis, sensitivity to amphotericin B requested
88C.1 to 6	M	+	Oral Rinse	+			Oral pseudomembranous candidiasis, sensitivity to amphotericin B requested
88E.1 to 6	M	+	Left Buccal Mucosal swab	+			candidiasis, oral hairy leukoplakia, sensitivity to amphotericin B requested
88F.1 to 6	M	+	Right Buccal mucosal swab	+			candidiasis, oral hairy leukoplakia, sensitivity to amphotericin B requested
88G.1 to 6	M	+	Left Buccal Mucosal Swab	Pseudomembranous candidiasis over OHL			pseudomembranous candidiasis, oral hairy leukoplakia, sensitivity to amphotericin B requested
88H.1 to 6	M	+	Oral Rinse	Pseudomembranous candidiasis over OHL			pseudomembranous candidiasis, oral hairy leukoplakia, sensitivity to amphotericin B requested
88I.1 to 6	M	+	Oral Rinse	?			germ tube positive

Annotation as in Tables 3.2, 3.4 and 3.7 (p., 70, 78 and 85).

Table 3.22a; Allelic profiles for the forty-six isolates analysed in study 4 at nineteen loci

	Enzyme Locus																		
Isolate Code	ADH	ALD	ENOL	EST	FDP	FUM	GDH	G6PD	GPI	HK1	HK2	NDPK	NP	PepA	PepB	PGAM	6PGD	PGM	PK
CBS 562	e	a	c	d	a	d	-	c	c	c	b	f	a	f	b	-	b	d	e
22A.1 to 6	e	a	c	d	a	d	c	c	c	c	f	f	a	f	b	-	b	d	e
22B.1 to 6	e	a	c	d	a	d	c	c	c	a	d	f	a	f	b	-	b	d	e
17D.1 to 6	e	a	c	d	a	d	c	c	c	c	d	f	a	f	b	-	b	d	c
17A.1 to 6	e	a	c	d	a	d	c	b	c	e	b	f	a	f	b	-	c	d	c
17E.1 to 6	-	a	c	d	a	d	-	c	c	e	b	f	a	f	b	-	-	-	d
46B.1 to 6	b	a	c	d	a	d	c	c	c	c	b	f	a	f	d	b	c	d	d
84A.1 to 6	e	a	c	d	a	d	c	c	c	c	b	f	a	f	c	-	c	d	d
ATCC 90028	e	a	c	d	a	d	c	b	c	c	b	f	a	f	c	b	b	d	c
80A.1 to 6	e	a	c	d	a	d	c	b	c	c	b	f	a	f	c	b	c	d	c
17130	e	a	d	d	a	d	c	b	c	c	b	f	a	g	c	b	c	d	c
19A.3	e	a	c	d	a	d	c	b	c	b	c	f	a	f	c	c	c	d	c
12716	c	a	c	d	a	d	b	b	c	c	b	f	a	g	c	b	c	bc	c
71A.1 to 6	-	a	c	-	a	d	-	d	-	c	a	f	a	f	c	-	c	b	d
71B.1 to 6	e	a	c	d	a	d	c	d	c	c	a	f	a	f	c	-	c	d	d
ATCC 90029	e	a	c	d	a	d	b	b	c	c	a	e	a	f	b	b	c	d	d
23B.1 to 6	e	a	c	d	a	d	-	c	c	c	e	e	a	f	b	b	c	d	d
23A.1 to 6	e	a	d	d	a	d	c	b	c	c	b	h	a	f	b	b	c	d	d
23C.1 to 6	e	a	d	d	a	d	-	b	c	b	f	g	a	f	b	b	c	d	d
17C.1 to 6	e	a	c	a	a	d	e	b	c	b	f	f	a	f	b	b	b	d	c
88D.1 to 6	e	a	c	d	a	d	-	b	c	b	f	f	a	g	b	-	c	d	c
31A.1 to 6	-	a	c	d	a	d	d	d	d	c	f	f	a	f	d	-	c	d	c
40D.1 to 6	e	a	c	-	a	-	-	c	c	d	e	f	a	f	d	b	c	-	c
68C.1 to 6	e	a	c	d	a	f	-	c	-	c	d	f	-	f	d	b	-	d	c
19D.1 to 6	b	a	c	d	a	e	c	b	c	b	c	f	a	h	c	b	b	d	c
31E.1 to 6	-	a	c	d	-	d	-	c	c	e	a	f	a	g	c	-	b	d	c
CBS 604	c	a	b	c	a	ab	b	a	a	a	g	ag	a	b	d	c	a	d	f
34A.1 to 6	d	a	d	-	d	f	c	a	c	b	a	g	a	f	c	-	a	b	f
CBS 7987	-	a	b	b	b	c	a	a	b	a	b	f	b	e	d	b	c	b	a
30A.1 to 6	e	a	b	b	a	c	-	a	b	a	g	c	b	d	d	b	c	b	a
40F.1 to 2	a	a	b	b	b	c	a	a	c	b	f	c	b	d	d	-	c	d	b

Annotation as in Tables 3.3 and 3.5 (p., 70 and 78)

Table 3.22b; Allelic profiles for the forty-six isolates analysed in study 4 at nineteen loci

	Enzyme Locus																		
Isolate Code	ADH	ALD	ENOL	EST	FDP	FUM	GDH	G6PD	GPI	HK1	HK2	NDPK	NP	PepA	PepB	PGAM	6PGD	PGM	PK
32A.1 to 6	e	a	d	d	a	d	-	c	c	b	bf	ch	a	d	b	b	b	d	e
32B.1 to 6	e	a	d	d	a	c	f	c	c	b	bf	ch	a	d	b	b	b	d	c
33A.1 to 6	e	-	c	d	a	d	c	b	c	c	bf	f	a	f	b	c	a	d	d
40C.1 to 6	e	a	c	d	a	ce	c	ac	d	b	af	cf	ab	d	d	b	c	d	bc
40E.1 to 6	e	a	bc	-	a	d	c	c	c	b	f	f	a	f	d	-	c	c	be
68A.1 to 6	d	a	ac	d	c	e	c	ac	ac	c	bg	cf	a	f	e	b	c	d	d
68D.1 to 6	e	b	c	bd	a	d	c	c	c	c	be	f	a	f	d	-	c	d	d
84B.1 to 6	ce	a	c	d	a	d	-	c	c	c	d	f	a	f	c	-	c	d	c
88B.1 to 6	e	a	c	d	a	d	c	b	c	b	bf	f	a	eg	b	b	b	d	c
88C.1 to 6	e	a	c	d	a	d	d	b	c	b	bf	f	a	eg	b	b	b	d	c
88E.1 to 6	e	a	c	d	a	d	-	b	c	b	f	f	a	eg	b	-	a	-	c
88F.1 to 6	e	a	c	d	a	d	-	b	c	b	f	f	a	eg	b	b	b	d	c
88G.1 to 6	e	a	c	d	a	d	c	b	c	b	f	f	a	cg	b	b	b	d	c
88H.1 to 6	-	a	c	d	a	d	-	c	c	e	c	f	a	cg	b	-	-	-	c
88I.1 to 6	e	a	c	d	a	d	-	b	c	b	bf	f	a	cg	b	bd	b	d	c

Annotation as in Tables 3.3, 3.5 and 3.8 (p., 71, 79 and 86)

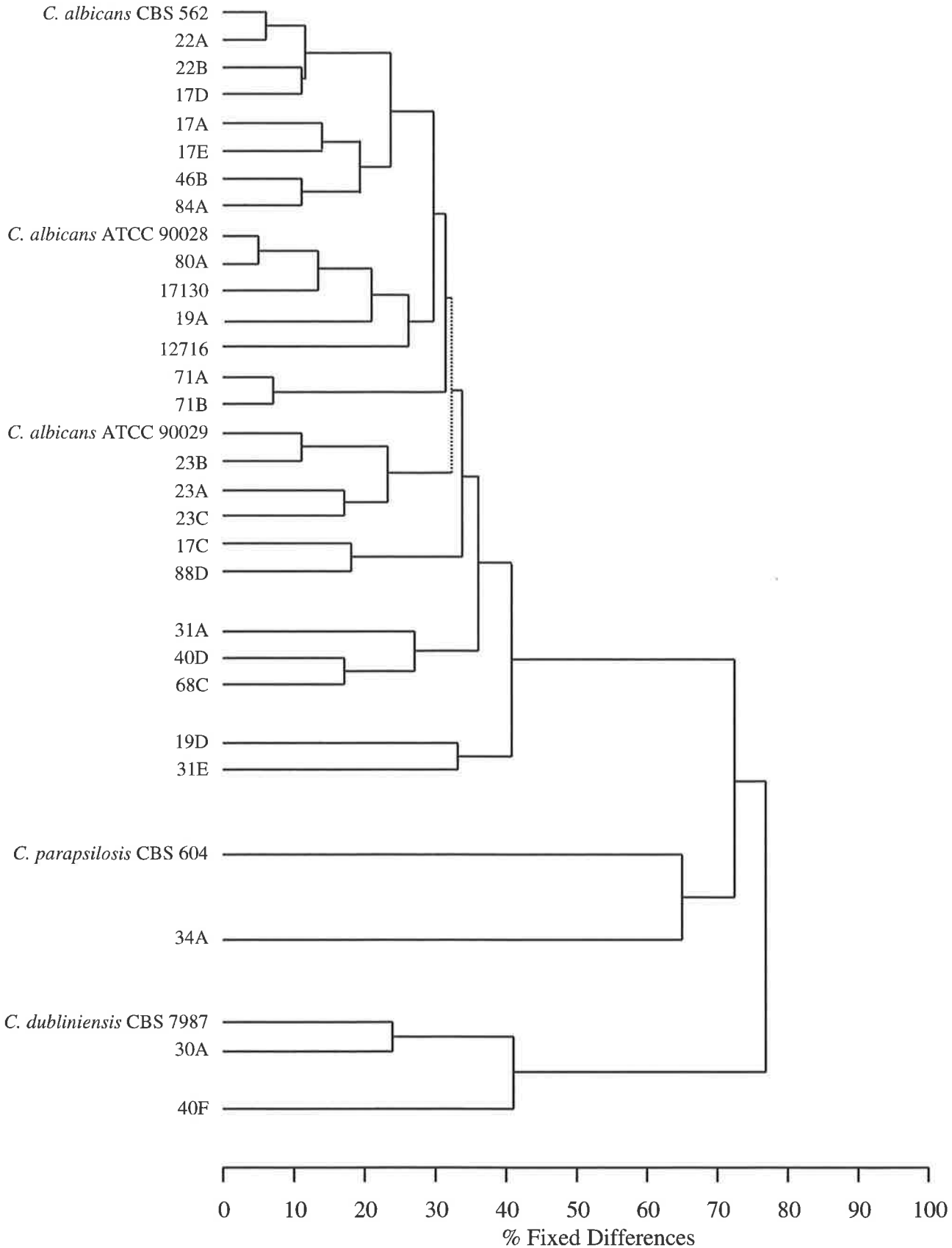


Figure 3.10; Phenogram depicting the genetic relationships between thirty-one isolates of *Candida* used in Study 4, as determined from an allozyme electrophoresis analysis using nineteen loci.

Dashed lines represent unresolved genetic relationships between genetic groups at that taxonomic level.

Table 3.23; Matrix of the percentages of fixed genetic difference between multiple isolates from 6 individual patients in study 4

Isolate	17A	17C	17D	17E	19A	19D	23A	23B	23C	31A	31E	40D	40F	71A
17C	**28**													
17D	**22**	**28**												
17E	**14**	**36**	**21**											
19A	17	32	28	36										
19D	39	37	39	50	**26**									
23A	22	42	33	29	37	53								
23B	29	39	24	21	39	56	**22**							
23C	29	28	41	36	33	50	**17**	**28**						
31A	35	41	35	43	35	53	47	38	44					
31E	33	40	27	31	33	33	60	47	60	**47**				
40D	29	33	39	33	33	47	47	27	47	31	42			
40F	78	72	83	86	72	78	78	76	65	71	80	**71**		
71A	43	50	43	33	36	57	43	36	50	29	46	42	86	
71B	28	44	28	29	22	44	28	24	35	29	33	36	78	**7**

Annotation as in Table 3.20 (p., 128).

3.3.6.b Interpretation

Of the twenty-six clinical or asymptomatic isolates assessed, twenty-three were placed within the *C. albicans* cluster. Of the remaining clinical isolates, two were within the *C. dubliniensis* cluster and one was from an undetermined species of *Candida*. Eighteen of the clinical *C. albicans* isolates were contained in the subcluster A population, which had unresolvable genetic relationships among them. This result indicates a lower limit in the resolving power of allozyme electrophoresis in this particular analysis. The resolving power of allozyme electrophoresis in this case differs to that of other present analyses because it is severely affected by the larger number of alleles that could not be unequivocally interpreted and the close genetic relationship between the 19 isolates in subcluster A. However, the other relationships within this analysis were resolvable due to the additional loci that could be scored.

Table 3.23 (p., 136) contains a portion of the matrix in Appendix 3 (Table 9) designating the percentage of loci at which fixed differences occur between isolates obtained from a single patient. Different isolates from patient 17 differed at 14 to 36% of the loci assessed, representing distinct *C. albicans* strains. Isolate 17A was collected for the preliminary investigation, prior to June 1995, C and D were collected on the 29th, and E on the 30th of August 1995. The origin of isolate 17A was not provided. Isolates 17C and E were oral rinse samples, D was a palatal swab of an obvious lesion. As with patient 23, the strain causing the infection in this patient is distinct from the predominant strain obtained from an oral rinse sample. The predominant isolate from the oral rinses on consecutive days also differed. This patient was colonized by at least three distinct *C. albicans* strains at the end of August, 1995.

The multiple isolates from patient 23 differed at 17 to 28% of loci, constituting distinct *C. albicans* strains. Isolate 23B was taken from a tongue swab and C from an oral rinse in this patient when oral hairy leukoplakia was noted on the 19th of May 1995. Isolate 23A was from a previous oral rinse sample taken on the 11th of April 1995. This result suggests that the strain causing the oral hairy leukoplakia infection is genetically distinct from the previous strain and from the predominant strain in the oral cavity, assuming that the strain obtained from an oral rinse sample represents the most predominant one.

Isolates 31A and E differed at 47% of loci. They were both oral rinse samples, obtained on the 3rd of May 1995 and the 29th of November 1995, respectively. Isolates 19A and D

differed at 26% of loci. They were also both oral rinse samples obtained on the 5th of April 1995 and the 24th of January 1996, respectively. In both of these cases, the patient became colonised by a different strain over the course of a few months.

Isolates 40D and F differed at 71% of loci, with 40D belonging to the *C. albicans* cluster and 40F belonging to the *C. dubliniensis* cluster. These isolates were both from oral rinse samples obtained on the 22nd of November and the 13th of December 1995, respectively. The predominant species in this patient's oral cavity changed from *C. albicans* to *C. dubliniensis* within three weeks. Isolates 40C and E were mixed or heterozygous isolates. They were obtained from oral rinses at different times to all other isolates. They may have included both *C. albicans* and *C. dubliniensis*. Resolution of this requires reanalysis of the individual colonies from each of these isolates.

Isolates 71A and B, from oral rinses on the 9th and the 16th of August 1995, which were identical in study 3 (Section 3.3.5 p., 124), now differ at the PGM enzyme locus. This could be due to an increased resolution obtained in this analysis or the increased number of colonies assessed in this analysis compared to the previous one where one of the additional colonies was a different strain. This observation suggests that within one week the patient was colonized by two closely related but distinct strains of *C. albicans*.

Seven of eight isolates obtained from patient 88 and analysed herein were mixed or heterozygous. Some of them were obtained from oral rinses and some were from concurrent oral swabs of obvious lesions. This patient appears to have been colonized by multiple strains at times of both asymptomatic carriage and infection, indicating the maintenance of a complex community of *Candida* strains.

3.3.7 Study 5

3.3.7.a Results

Isolates in study 5 (Table 3.24 p., 140) differed at 14 to 78% of the nineteen loci assessed (Table 3.25 p., 141). Six distinct clusters of isolates differing at over 50% of loci can be identified (Figure 3.11 p., 142). The first cluster contains a number of clinical isolates and the type and reference strains for *C. albicans*, with fixed differences occurring at 15 to 46.2% of loci. Isolate 12716 differed at 50.7% of loci from this cluster, forming a unique cluster. The third cluster contained three clinical isolates which differed at 59% of loci from the first two. Fixed differences in this cluster occurred at 27 to 36.5% of loci. Clinical isolate 34A again formed a distinct cluster differing at 70.7% of loci from the three described. These clusters differed from the remaining two at 78% of loci. The fifth cluster contained the *C. dubliniensis* type strain and three clinical isolates that differed at 14 to 50% of loci. This cluster differed at 63% of loci from the *C. parapsilosis* type strain, which constituted the final cluster.

Table 3.24; Details of the nineteen reference and clinical samples analysed in study 5 using allozyme electrophoresis

Isolate Code	Sex	HIV status	Site of Isolation	Candidiasis	Antifungal Resistance	Patient's Therapy	Other Notes
CBS 562			skin (interdigital mycosis), Uruguay				*C. albicans* type strain
87A.1 to 6	M	+	Tongue Swab	?		none	
ATCC 90028			blood, Iowa	systemic			*C. albicans* reference strain
29A.1 to 6	M	+	Oral Rinse	?			
28B.1 to 6	M	+	?	?			
28A.1 to 6	M	+	Throat Swab	red throat			red throat
ATCC 90029			blood, Iowa	systemic			*C. albicans* reference strain
12716	M	+	Oral Swab	+	Fluconazole, moderate 5-flurocytosine		*C. dubliniensis* from a mixed infection with *C. krusei*; Resistant to fluconazole, Itraconazole and 5-flurocytosine.
21B.1, 2, 4-6	F	+	Oral Rinse	+			
24A.1 to 6	M	+	Swab	+		Nilstat drops	Resection for Floor of Mouth scc & rnd & rt to bilateral neck.
25A.1 to 6	M	+	Swab	+			
34A.4	M	+	Oral Rinse	?			Growth on agar slopes very adhesive and granular Reidentified as *C. parapsilosis* or *C. tropicalis*
27A.1 to 6	M	+	Oral Rinse	?			Identity confirmed as *C. albicans*
27B.1 to 6	M	+	Tongue Swab	?			
CBS 7987		+	oral cavity, Ireland				*C. dubliniensis* type strain
34B.1 to 6	M	+	Oral Rinse	?			Growth on agar slopes very adhesive and granular Identity confirmed as *C. albicans*
CBS 604			case of sprue, Puerto Rico				*C. parapsilosis* type strain
20A.1, 3-6	M	+	?	?			
26A.1 to 6	M	+	Oral Rinse	?			Identity confirmed as *C. albicans*

Annotation as in Tables 3.2, 3.4 and 3.7 (p., 70, 78 and 85).

Table 3.25; Allelic profiles of nineteen isolates analysed in study 5 at nineteen enzyme loci

	Enzyme Locus																		
Isolate Code	ADH	ALD	ENOL	EST	FDP	FUM	GDH	G6PD	GPI	HK1	HK2	NDPK	NP	PepA	PepB	PGAM	6PGD	PGM	PK
CBS 562	d	e	c	c	a	d	c	c	c	b	b	b	c	b	a	-	b	c	c
87A.1 to 6	d	-	c	c	-	d	a	-	c	c	c	b	-	b	b	-	b	c	b
ATCC 90028	b	d	d	c	a	d	c	c	c	c	a	c	c	c	b	-	b	-	-
29A.1 to 6	d	-	d	c	a	d	-	c	c	c	a	c	-	c	c	c	b	c	-
28B.1 to 6	d	c	-	-	a	d	c	c	c	c	a	d	c	d	b	-	b	-	a
28A.1 to 6	d	d	d	c	c	d	-	c	c	e	a	c	c	d	b	-	b	-	a
ATCC 90029	d	d	c	c	a	d	-	c	c	c	a	d	c	c	b	b	c	c	c
12716	d	d	b	c	a	d	a	b	c	d	a	b	c	c	c	b	c	ad	-
21B.1, 2, 4-6	c	e	e	c	b	e	c	c	c	c	a	e	c	d	d	-	c	-	-
24A.1 to 6	-	e	d	c	b	e	c	c	c	e	a	c	c	d	d	-	d	d	b
25A.1 to 6	b	-	d	c	-	e	c	c	c	c	d	-	b	d	b	-	-	d	b
34A.5	d	b	e	-	d	f	a	c	c	a	a	a	a	e	c	-	b	e	c
27A.1 to 6	-	e	b	a	b	c	-	a	b	e	a	b	c	b	d	c	d	d	b
27B.1 to 6	-	e	-	a	b	c	c	-	b	e	a	c	d	b	d	c	-	d	b
CBS 7987	a	d	b	a	b	c	c	a	a	e	a	-	d	b	c	b	c	a	a
34B.1 to 6	b	-	b	a	b	c	-	c	b	e	c	b	a	a	c	a	c	d	-
CBS 604	b	a	a	c	b	ab	c	a	a	e	a	ab	c	a	a	-	a	b	b
20A.1, 3-6	d	f	d	ac	a	d	c	d	c	e	e	d	c	d	b	-	c	-	-
26A.1 to 6	-	-	d	c	-	c	a	a	b	c	ad	c	c	d	b	-	c	a	b

Annotation as in Tables 3.3, 3.5 and 3.8 (p., 71, 79 and 86)

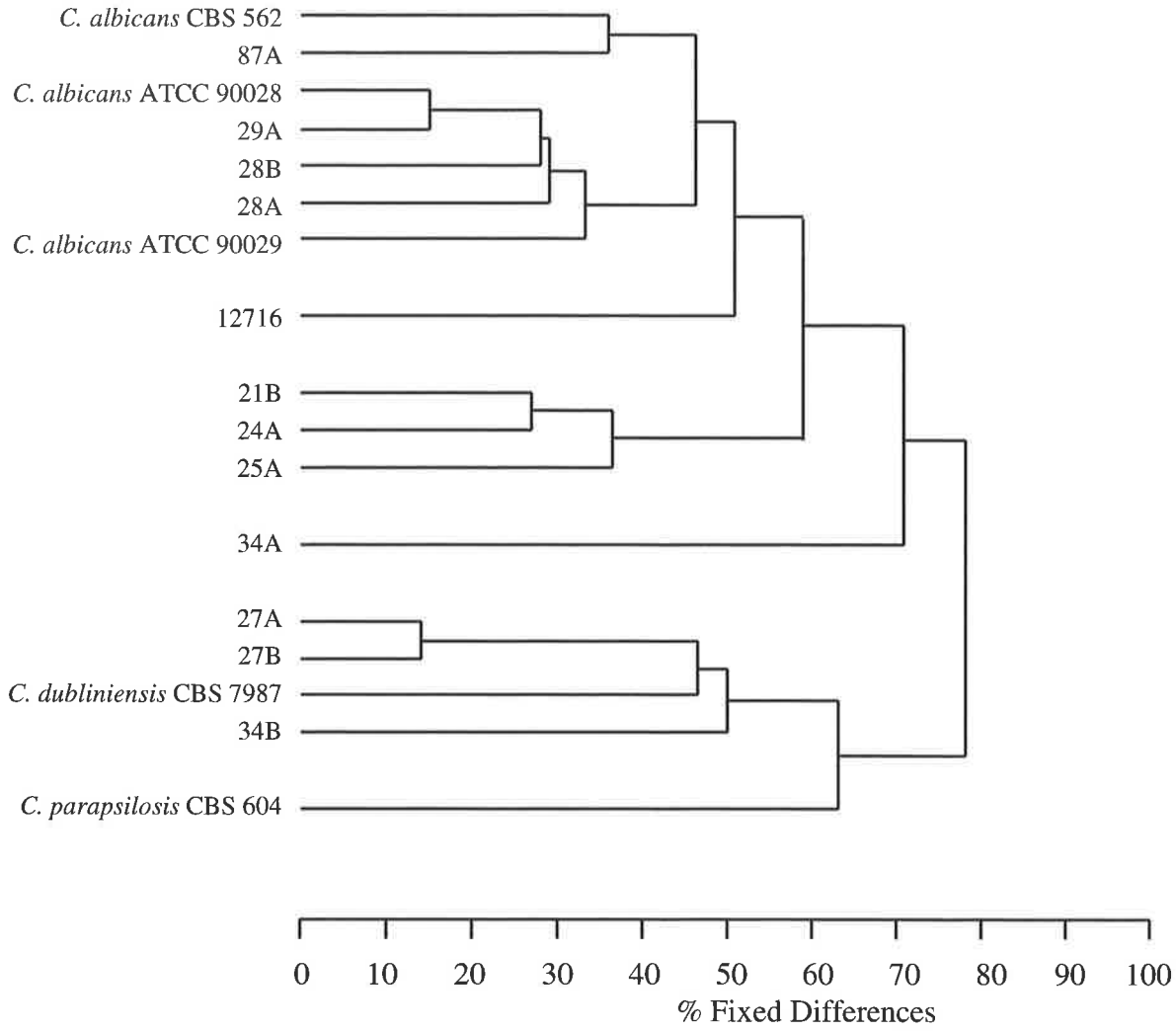


Figure 3.11; Phenogram depicting the genetic relationships between seventeen isolates of *Candida* used in Study 5, as determined from an allozyme electrophoresis analysis using nineteen loci.

Table 3.26; Matrix of the percentages of fixed genetic difference occurred between isolates from 3 individual patients in study 5

Isolate	27A	27B	28A	28B	34A
27B	**14**				
28A	80	75			
28B	85	83	**29**		
34A	93	92	67	67	
34B	47	50	86	92	**79**

Annotation as in Table 3.20 (p., 128).

3.3.7.b Interpretation

Of the twelve clinical isolates assessed, four belonged to the *C. albicans* cluster. Isolate 12716, which had previously been contained within this cluster, is now external to it. This result suggests that it may have become contaminated during reculturing between electrophoretic runs or that the resolution in this study had increased providing more ensymes for analysis. Three isolates were within the *C. dubliniensis* cluster and five isolates belonged to three undetermined *Candida* species, two in unique clusters and three forming a cluster of isolates. The latter cluster contained isolates all taken from patients that had oral candidiasis at the time the sample was obtained, perhaps constituting a species of *Candida* or a *C. albicans* subpopulation with increased pathogenicity.

An excerpt of the matrix of pairwise comparisons containing multiple isolates from the same patient is contained within Table 3.26 (p., 143). Isolates 27A and B differed at 14% of loci suggesting that they are distinct strains. They were from an oral rinse and tongue swab, respectively; both belonging to the cluster containing *C. dubliniensis*. Isolate 27A was collected on the 21st and B on the 24th of April 1995. This patient was probably colonized by both *C. dubliniensis* strains during infection, with the 27B strain causing their lesion and 27A being the most predominant in their oral cavity.

Isolates 28A and B differed at 29% of loci, suggesting that they constitute different *C. albicans* strains. Isolate 28A was taken from a throat swab but the clinical origin of isolate 28B was not provided. Isolate 28A was collected on the 24th of April 1995 but the time at which isolate B was collected was also not provided, although it can be assumed that it was after this date.

Isolates 34A and B differed at 79% of loci, the former placed within the *C. albicans* cluster and the latter within the *C. dubliniensis* cluster. Both isolates were obtained from oral rinse samples taken on the 5th of April and the 29th of September 1995, respectively. This patient was colonized by two different *Candida* species over the course of sample collection.

3.3.8 Study 6

3.3.8.a Results

Nine clusters of isolates (Table 3.27 p., 146-7) that differ at more than 50% of the sixteen loci assessed (Table 3.28 p., 148-9) could be discriminated in this analysis (Figure 3.12 p., 150). Cluster 1 contained the majority of isolates, including the *C. albicans* type strain, with fixed differences between isolates in this cluster ranging from 13 to 47.9% of loci. This cluster probably constitutes the *C. albicans* species. The genetic relationships between isolates within a subcluster of cluster 1, when the isolates varied at less than 30.6% of the loci, could not be accurately and reproducibly resolved in this analysis based on the available data. As in study 4, this is due to tied fixed genetic difference values.

The second cluster differs from the first at 50.2% of loci and contains four clinical isolates with fixed differences ranging from 31 to 44.5% of loci. The third cluster differs from the first two at 54.6% of loci. It contains the two *C. albicans* reference strains, which differ from each other at 33% of loci.

These clusters differ from clinical isolate 34A, forming cluster 4, at 57.4% of loci. Clinical isolates 20A and 45D differ at 45% of loci and constitute cluster 5. They differ from the other four clusters described at 59.6% of loci. Clinical isolate 22A forms cluster 6, which differs from the other clusters at 61.6% of loci.

Clusters 7 and 8 differ from clusters 1 to 6 at 74.3% of loci and cluster 9 differs at 81% of loci. Cluster 7 contains six clinical isolates with fixed differences between them at 14 to 42.9% of loci. Cluster 8 differs from cluster 7 at 54.3% of loci and contains a single isolate, the *C. dubliniensis* type strain CBS 7987. Cluster 9 contains only the *C. parapsilosis* type strain CBS 604.

The genetic identities of multiple isolates from several patients are discussed below on the basis of the number of fixed differences between them, extracted from the matrix (Appendix 3, Table 10).

Table 3.27a; Details of the forty-six reference and clinical samples analysed in study 6 using allozyme electrophoresis

Isolate Code	Sex	HIV status	Site of Isolation	Candidiasis	Antifungal Resistance	Patient's Therapy	Other Notes
CBS 562			skin (interdigital mycosis), Uruguay				*C. albicans* type strain
88A.2-4, 6	M	+	Oral Rinse	?			
88C.2	M	+	Oral Swab	+			Oral pseudomembranous candidiasis, sensitivity to amphotericin B requested
17G.1, 3, 4, 6	M	+	Oral Rinse	+			
17H.1, 2, 5	M	+	Palate Swab	+			Previous cultures taken - A *Candida* strain resistant to fluconazole
31C.1, 4, 5	M	+	Tongue Swab	OHL			
12716	M	+	Oral Swab	+	Fluconazole, moderate 5-flurocytosine		Mixed infection with *C. krusei* - resistant to fluconazole, Itraconazole and 5-flurocytosine. Reidentified as *C. dubliniensis*
17C.2	M	+	Oral Rinse	?			
17D.3	M	+	Palate Swab	?			
17C.3, 4	M	+	Oral Rinse	?			
17D.5, 6	M	+	Palate Swab	?			
19A.3	M	+	Oral Rinse	?			
31B.1 to 6	M	+	Oral Rinse	OHL			
17F.2-4	M	+	Oral Rinse	+			
88D.2	M	+	Oral Rinse	+			candidiasis, oral hairy leukoplakia
19B.1-4, 6	M	+	Buccal Mucosal Swab	?			
19C.1 to 5	M	+	Oral Rinse	?			
18A.1	M	+	Mouth Swab	?			
31A.2	M	+	Oral Rinse	?			
32A.4	M	+	OHL Tongue Swab	OHL			
34B.1	M	+	Oral Rinse	?			Growth on agar slopes very adhesive and granular
87A.1, 4-6	M	+	Tongue Swab	?		none	
28B.1 to 5	M	+	?	?			
32B.5	M	+	Oral Rinse	?			
24A.1	M	+	Swab Floor of Mouth	+		Nilstat Oral Drops	Resection for Floor of Mouth scc & rnd & rt to bilateral neck.
23C.1, 4, 5	M	+	Oral Rinse	?			
28A.2	M	+	Throat Swab	red throat			red throat
21B.1, 2, 5	M	+	Oral Rinse	+			

Annotation as in Tables 3.2 and 3.4 (p., 70 and 78).

Table 3.27b; Details of the forty-six reference and clinical samples analysed in study 6 using allozyme electrophoresis

Isolate Code	Sex	HIV status	Site of Isolation	Candidiasis	Antifungal Resistance	Patient's Therapy	Other Notes
23B.1 to 5	M	+	OHL Tongue Swab	OHL		Fungilin	
31E.1, 3, 5, 6	M	+	Oral Rinse	?			
25A.2 to 6	M	+	Oral Swab	+			
33B.2, 4, 6	M	+	Oral Rinse	?			
ATCC 90028			Blood, Iowa	systemic			*C. albicans* reference strain
ATCC 90029			blood, Iowa	systemic			*C. albicans* reference strain
34A.1, 3-6	M	+	Oral Rinse	?			Growth on agar slopes very adhesive and granular
19D.1	M	+	Oral Rinse	?			
20A.5	M	+	?	?			
22A.2, 3, 5, 6	M	+	Oral Rinse	?			
21A.1, 2, 4-6	M	+	Oral Rinse	+			
23D.2, 3, 5	M	+	Oral Rinse	?			
23A.1, 5	M	+	Oral Rinse	+			
27A.1, 2, 5, 6	M	+	Oral Rinse	?			
27B.1 to 5	M	+	Tongue Swab	?			
27C.2 to 6	M	+	Oral Rinse	?			
CBS 7987		+	oral cavity, Ireland				*C. dubliniensis* type strain
CBS 604			case of sprue, Puerto Rico				*C. parapsilosis* type strain

Annotation as in Tables 3.2 and 3.4 (p., 70 and 78).

Table 3.28a; Allelic profiles of forty-six isolates analysed in study 6 at sixteen enzyme loci

	Enzyme Locus															
Isolate Code	ALD	ENOL	EST	FDP	FUM	G6PD	GPI	HK1	HK2	NDPK	NP	PepA	PepB	PGAM	6PGD	PK
CBS 562	b	c	c	c	d	b	b	c	e	-	a	d	b	e	b	c
88A.2-4, 6	b	c	c	-	d	c	b	c	e	-	a	d	d	e	b	c
88C.2	b	c	c	c	d	c	b	c	e	-	a	d	d	e	b	c
17G.1, 3, 4, 6	b	c	c	c	d	b	b	c	e	-	a	d	d	e	c	c
17H.1, 2, 5	b	c	c	c	d	b	b	c	e	b	a	d	d	c	c	d
31C.1, 4, 5	b	d	c	-	d	b	b	b	j	b	a	d	d	e	b	c
12716	b	c	c	b	d	b	b	d	b	-	a	d	d	e	b	c
17C.2	b	c	b	c	d	b	b	f	c	b	a	d	d	f	d	d
17D.3	b	c	c	c	d	b	b	d	e	-	a	d	d	f	d	c
17C.3, 4	b	c	c	c	d	b	b	f	c	b	a	d	d	f	c	c
17D.5, 6	b	c	c	c	d	b	b	f	j	-	a	d	d	f	c	c
19A.3	-	c	c	c	d	d	b	f	c	b	-	d	e	a	-	c
31B.1 to 6	b	d	c	-	d	c	b	f	c	-	a	d	d	e	b	c
17F.2-4	b	c	c	c	d	b	b	f	c	-	a	d	d	g	b	c
88D.2	-	c	c	c	d	c	b	f	c	b	a	d	d	b	b	c
19B.1-4, 6	b	c	c	c	d	c	b	e	c	b	a	d	d	c	b	c
19C.1 to 5	b	c	c	c	d	c	b	e	c	-	a	d	d	g	c	c
18A.1	b	c	c	c	d	c	b	f	c	b	a	d	d	c	c	e
31A.2	b	d	c	a	d	c	b	e	f	b	a	d	d	h	b	c
32A.4	b	e	c	-	d	c	b	e	f	b	a	d	d	h	b	e
34B.1	b	c	c	a	d	c	a	e	f	b	a	-	d	h	b	c
87A.1, 4-6	b	c	c	a	d	c	b	e	f	b	a	d	d	f	c	c
28B.1 to 5	b	d	c	-	d	b	b	e	f	b	a	d	e	c	b	c
32B.5	b	d	c	d	d	b	b	e	f	b	a	d	d	h	b	d
24A.1	b	d	c	-	d	b	b	e	f	a	b	d	d	e	c	c
23C.1, 4, 5	b	e	c	c	d	b	b	c	h	b	b	e	d	e	b	d
28A.2	b	d	-	-	d	b	b	f	g	b	-	d	d	b	b	d
21B.1, 2, 5	b	e	c	c	d	b	c	c	e	b	a	f	d	b	b	e

Annotation as in Tables 3.3 and 3.5 (p., 71 and 79)

Table 3.28b; Allelic profiles of forty-six isolates analysed in study 6 at sixteen enzyme loci

	Enzyme Locus															
Isolate Code	ALD	ENOL	EST	FDP	FUM	G6PD	GPI	HK1	HK2	NDPK	NP	PepA	PepB	PGAM	6PGD	PK
23B.1 to 5	b	b	c	-	d	d	b	f	c	b	b	e	d	g	b	e
31E.1, 3, 5, 6	b	d	c	-	d	d	b	f	h	-	a	d	d	g	-	e
25A.2 to 6	b	d	-	-	c	d	b	g	c	-	a	b	d	c	-	e
33B.2, 4, 6	b	c	c	a	d	b	b	g	c	b	a	g	d	h	b	e
ATCC 90028	b	c	c	b	d	b	b	b	d	-	b	d	c	e	c	b
ATCC 90029	b	c	c	b	d	b	c	f	c	-	b	d	c	e	b	e
34A.1, 3-6	b	d	c	c	e	d	c	c	a	b	e	d	d	i	b	c
19D.1	a	c	c	-	d	d	a	f	f	-	-	d	d	i	d	e
20A.5	b	c	-	-	d	d	a	c	f	b	a	f	e	e	-	e
22A.2, 3, 5, 6	b	c	-	c	d	b	b	g	a	b	-	b	a	b	a	f
21A.1, 2, 4-6	b	b	a	c	c	a	b	c	i	-	c	c	d	-	c	a
23D.2, 3, 5	b	b	a	b	c	a	b	c	h	-	d	c	e	h	c	a
23A.1, 5	b	b	-	-	d	a	b	c	e	-	d	d	e	e	b	a
27A.1, 2, 5, 6	b	c	a	c	c	a	b	c	k	b	d	b	e	f	b	a
27C.2 to 6	b	c	a	b	c	a	b	c	k	b	d	d	e	d	b	d
27B.1 to 5	b	c	a	b	c	a	b	c	k	-	d	b	e	g	-	a
CBS 7987	c	a	a	c	c	a	a	c	e	c	e	b	e	e	c	a
CBS 604	d	a	c	b	ab	a	a	a	f	-	a	a	d	a	a	f

Annotation as in Tables 3.3 and 3.5 (p., 71 and 79).

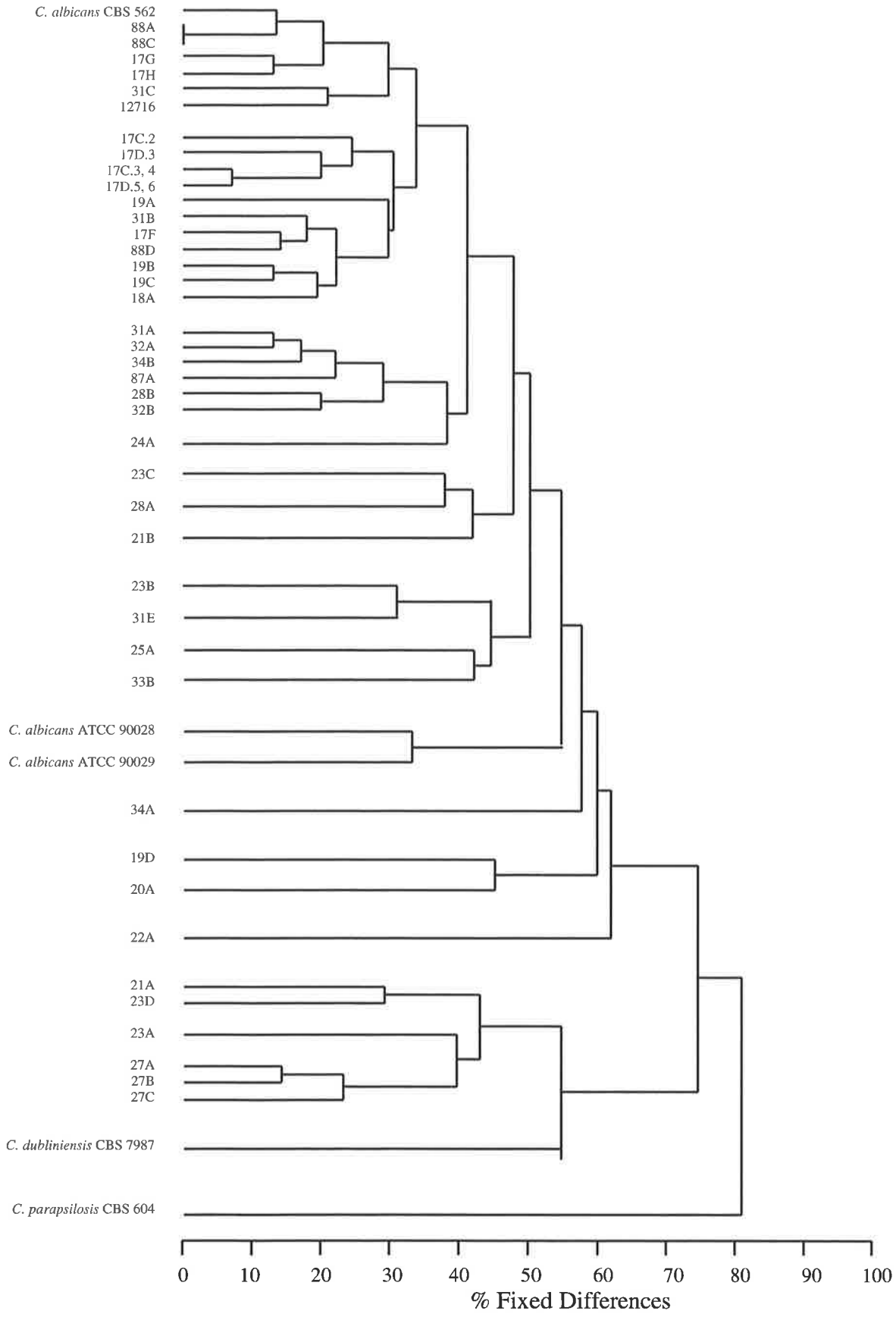


Figure 3.12; Phenogram depicting the genetic relationships between forty-six *Candida* isolates used in Study 6, as determined from an allozyme electrophoresis analysis using sixteen loci.

Table 3.29; Matrices of the percentages of fixed genetic difference occurred between isolates from individual patients in study 6

Isolate	17C.2	17C.3, 4	17D	17D.3	17F	17 G
17C.3, 4	19					
17D	27	7				
17D.3	27	20	20			
17F	27	13	20	27		
17G	40	20	20	20	27	
17H	31	25	27	27	33	13

Isolate	19A	19B	19C
19B	31		
19C	33	13	
19D	45	62	62

Isolate	21A
21B	71

Isolate	23A	23B	23C
23B	62		
23C	54	40	
23D	38	79	73

Isolate	27A	27B
27B	14	
27C	25	21

Isolate	28A
28B	38

Isolate	31A	31B	31C
31B	21		
31C	27	21	
31E	38	31	38

Isolate	32A
32B	20

Isolate	34A
34B	60

Isolate	88A	88C
88C	0	
88D	23	21

Annotation as in Table 3.20 (p., 128).

3.3.8.b Interpretation

There were no mixed or heterozygous clinical isolates eliminated from this analysis but it was noted that the *C. parapsilosis* type strain CBS604 is heterozygous at the fumarase locus, exhibiting three distinct bands of enzyme activity. Heterozygosity at this locus was also noted in study 4 (Section 3.3.6 p., 130).

Table 3.29 (p., 151) contains a number of excerpts from the matrix of pairwise comparisons for this study for individual patients (Appendix 3 Table 11). Isolates from patient 17 differed at 7 to 40% of loci, representing different *C. albicans* strains. Samples C.2 and C.3, 4 are different strains from the same oral rinse sample, isolates D.3 and D.5, 6 are different strains from the same palate swab. Individual colonies of each of these isolates were run separately. Both of them were mixed with the two sets of colonies differing at 19 and 20% of the loci, respectively. Isolates F and G are from oral rinse samples and H is from a palate swab of a lesion. The patient had obvious clinical candidiasis when all samples were taken. Isolates C and D were obtained on the 29th of August, F on the 14th of September and G and H on the 10th of November 1995. Each isolate was genetically distinct. The *Candida* cultures taken were noted as clinically resistant to fluconazole. The first infection was caused by at least four different *C. albicans* strains, with two evident in the lesion and two different strains in the oral rinse sample. The isolates from the patient's palatal lesion also differed from that obtained in the concurrent oral rinse.

The multiple isolates from patient 19 differed at 13 to 62% of loci, isolates A, B and C representing different strains within the *C. albicans* cluster and D from an unidentified *Candida* species. Isolates A, C and D were from oral rinse samples and B was a buccal mucosal swab of an obvious lesion. Isolate A was obtained on the 5th of April, B on the 10th of June, C on the 15th of November 1995 and D on the 24th of January 1996. Isolate D yielded a single colony. Patient 19 took part in an amphotericin B lozenge trial during the time isolates B to D were collected. This treatment resulted in the low level colonization of this patient with a *Candida* species other than *C. albicans* that was clinically resistant to amphotericin B, perhaps *C. lusitaniae*.

Isolates A and B from patient 21 differed at 71% of loci. They were both from oral rinse samples taken when the patient had obvious candidiasis infection, on the 12th of April and

the 1st of December 1995. The first was placed with the *C. dubliniensis* cluster, the second with the *C. albicans* cluster.

The multiple isolates from patient 23 differed at 40 to 79% of loci, representing distinct strains belonging to two distinct species, A and D from *C. albicans* and B and C from *C. dubliniensis*. Isolate A was from an oral rinse sample when the patient had candidiasis on the 11th of April 1995, B a tongue swab in the presence of oral hairy leukoplakia on the 19th of May 1995 and C and D oral rinse samples on the 22nd of May and the 9th of July 1995, respectively. This patient was infected and asymptomatically colonized by both *C. albicans* and *C. dubliniensis* strains, which were all genetically distinct. A *C. albicans* strain caused candidiasis, whilst a *C. dubliniensis* strain caused oral hairy leukoplakia. The *C. dubliniensis* species persisted after the oral hairy leukoplakia infection, although it was a different strain, but was eventually replaced by a second *C. albicans* strain.

Isolates from patient 27 differed at 14 to 25% of loci, representing different *C. dubliniensis* strains. Isolates A and C were oral rinse samples, B was a tongue swab of oral hairy leukoplakia. They were taken on the 21st and the 24th of April and the 17th of October 1995, respectively. The *C. dubliniensis* species persisted in this patient in the absence of a *C. albicans* strain, however a different *C. dubliniensis* strain caused the oral hairy leukoplakia lesion than was isolated in the oral rinse sample.

Isolates 28A and B differed at 38% of loci, being different strains of *C. albicans*. Isolate 28A was from a throat swab when the patient had an inflamed throat and the origin of sample B is unknown other than it being isolated from the oral cavity. These isolates were also analysed in study 5 (Section 3.3.7 p., 139) where they differed at 29% of loci, however, the enzyme loci ADH, GDH and PGM were not used in this analysis. The two isolates were identical at the ADH locus but could not be compared due to insufficient staining intensity or resolution at the other two loci. As a consequence, these two isolates appear more closely related in the latter study. This patient's inflamed throat was caused by a different *C. albicans* strain than was obtained by oral rinse at a later date.

The isolates from patient 31 differed at 21 to 38% of loci, constituting distinct *C. albicans* strains. Isolate A was from an oral rinse sample on the 5th of March 1995, B from oral rinse in the presence of oral hairy leukoplakia, C a tongue swab of the oral hairy leukoplakia lesion, both taken on the 29th of June 1995 and E from an oral rinse sample on

the 29th of November 1995. The patient's oral hairy leukoplakia infection was caused by a strain other than that obtained from an oral rinse at the same time. Four different *C. albicans* strains were identified in this patient, with the strain isolated from the oral hairy leukoplakia lesion differing from the oral rinse samples, including sample B taken on the same day. This result indicates patient 31 had a mixed infection.

Isolates A and B from patient 32 differed at 20% of loci, representing different strains of *C. albicans*. Isolate A was from a tongue swab when the patient had oral hairy leukoplakia on the 4th of May 1995 and isolate B was an oral rinse sample taken on the 29th of September 1995. The colonies were adhesive and granular, but this morphological characteristic, again, does not appear to be restricted to a genetic subset of strains.

Isolates 34A and B differed at 60% of loci, isolate B being identified as a *C. albicans* strain and A from an undetermined species of *Candida*. Both samples were from oral rinses and, in each, the colonies were noted as adhesive to agar and granular. This result implies that this characteristic is not genetically restricted to a subset of strains or a single species. They were obtained on the 4th of May and the 29th of September 1995, respectively. During this time, patient 34 was colonized by two different species.

The multiple isolates from patient 88 had fixed genetic differences at 0 (A and C) to 23% of loci. All isolates formed a cluster with *C. albicans*. Isolates A and D were from oral rinse samples and C was from a lesion swab. Isolate A was taken when the patient had candidiasis on the 30th of November 1995, C on the 4th of March and D on the 4th of October 1996. The patient had clinical candidiasis infection when isolates C and D were taken and the patient also had oral hairy leukoplakia when sample D was obtained. This patient was reinfected by the same *C. albicans* strain within 3½ months and a second strain caused a candidiasis and oral hairy leukoplakia infection later. Patient 88 participated in an amphotericin B lozenge trial during the time that isolates A to L were collected. Isolates E to I were assessed in study 4 and J to L were analysed in study 10. Isolates 88E to I were mixed/heterozygous and isolates J to L were different *C. albicans* strains. The persistence of one of the strains of *C. albicans* during this time suggests that it may have a decreased susceptibility to amphotericin B.

Overall, twenty-seven of the forty-one clinical or asymptomatic carrier isolates were within the *C. albicans* cluster. Eleven of them were contained within the subcluster A

population with unresolvable genetic relationships within it. Four clinical isolates clustered in an undetermined *Candida* species cluster.

The *C. albicans* reference strains clustered outside of these two clusters of isolates. These isolates may represent the extent of genetic variation that exists within the *C. albicans* species or the reference strains may have become contaminated again (as in Section 3.1.4 p., 89), although all of them continued to exhibit single bands on the electrophoretic gels. These reference isolates were recultured from their original glycerol stocks for future analyses.

Six clinical isolates clustered, but differed from the *C. dubliniensis* type strain at more than 50% of the loci assessed. These isolates may still belong to the *C. dubliniensis* species, representing a genetically distinct population.

Three other *Candida* species clusters exist, two containing single isolates and one with two isolates. Their identity was not determined in the present study.

3.3.9 Study 7

3.3.9.a Results

Isolates in study 7 (Table 3.30 p., 156-7) differed at 0 to 74.8% of the sixteen loci assessed (Table 3.31 p., 158-9). The isolates formed six distinct clusters with fixed differences between them at over 50% of loci (Figure 3.13 p., 160). All but the first cluster contained a single isolate. The first cluster contained the majority of clinical isolates with the type and reference strains for *C. albicans*, with fixed differences ranging from 0 to 49.7% of the loci. Clinical isolate 15A differed from this cluster at 59.8% of loci. These two clusters differed from the four others at 74.8% of loci. The *C. dubliniensis* type strain differed from the *C. parapsilosis* type strain at 55% of loci and from clinical isolates 42C and 42E at 65% of loci. The latter two clinical isolates differed from each other at 62% of loci.

Table 3.30a; Details of the fifty-seven reference and clinical samples analysed in study 7 using allozyme electrophoresis

Isolate Code	Sex	HIV status	Site of Isolation	Candidiasis	Antifungal Resistance	Patient's Therapy	Other Notes
38A.1	M	?	Oral Rinse	?			
42D.1, 4	M	+	Oral Rinse	?			
ATCC 90028			Blood, Iowa	systemic			*C. albicans* reference strain
41A.2 to 6	M	+	Oral Rinse	?			
CBS 562			skin (interdigital mycosis), Uruguay				*C. albicans* type strain
16A.1 to 6	M	+	?	?			
22B.1 to 6	M	+	Oral Rinse	+	Fluconazole	100mg/day Fluconazole trial	Fluconazole resistant candidiasis, Germ tube positive, FCZ resistance required
22A.1, 4	M	+	Oral Rinse	?			
22D.1	M	+	Oral Swab Floor of Mouth	+			Fluconazole resistant candidiasis, Germ tube positive, FCZ resistance required
42A.1 to 6	M	+	Oral Rinse	?		not previously treated	
42B.1 to 6	M	+	Tongue Swab	?		not previously treated	
9C.1 to 6	M	+	Swab Palate/Throat	?	Fluconazole	Fluconazole	Low CD4+ count
9E.1 to 6	M	+	Pharynx Swab	Oesophageal?		Fluconazole	sensitivity to fluconazole requested
9F.1 to 6	M	+	Oral Rinse	Oesophageal?		Fluconazole	sensitivity to fluconazole requested
9G.1 to 6	M	+	Oral Swab	?			sensitivity to fluconazole requested
11A.1 to 6	M	+	Oral Rinse	+			
9D.1 to 6	M	+	Oral Rinse	?			
9I.1 to 6	M	+	?	?			
12A.1 to 6	M	+	Mouth Swab	?			
29A.5	M	+	Oral Rinse	?			
29B.1, 2	M	+	Oral Rinse	?			
ATCC 90029			blood, Iowa	systemic			*C. albicans* reference strain
18A.1, 3-6	M	+	Mouth Swab	?			
12716	M	+	Oral Swab	+	Fluconazole, moderate 5-flurocytosine		Mixed infection with *C. krusei*; resistant to fluconazole, Itraconazole and 5-flurocytosine. Reidentified as *C. dubliniensis*
39C.2 to 6	M	+	Oral Rinse	?			
42D.2, 3, 5, 6	M	+	Oral Rinse	?			
15A.1 to 6	M	+	?	?			
42C.1 to 6	M	+	Oral Rinse	?			
42E.1 to 6	M	+	Oral Rinse	?			
CBS 7987		+	oral cavity, Ireland				*C. dubliniensis* type strain
CBS 604			case of sprue, Puerto Rico				*C. parapsilosis* type strain

Annotation as in Tables 3.2 and 3.4 (p., 70 and 78).

Table 3.30b; Details of the fifty-seven reference and clinical samples analysed in study 7 using allozyme electrophoresis

Isolate Code	Sex	HIV status	Site of Isolation	Candidiasis	Antifungal Resistance	Patient's Therapy	Other Notes
4A.1 to 6	M	?	Oral Rinse	?			
8A.1 to 6	M	+	Oral Rinse	+			
8B.1, 4-6	M	+	Oral Rinse	+			
8C.1 to 6	M	+	Oral Rinse	?			
9A.1 to 6	M	+	?	+			
9B.2 to 6	M	+	Oral Rinse	?			
9H.1 to 6	M	+	Oral Rinse	?			sensitivity to fluconazole requested
9J.1, 3-6	M	+	?	?			
10A.1 to 6	M	+	?	+			
13A.1 to 6	M	+	Mouth Swab	?			
14A.1 to 6	M	+	Oral Swab	+			
22C.1 to 6	M	+	Oral Swab Hard Palate	+			Fluconazole resistant candidiasis, Germ tube positive, FCZ resistance required
22E.1 to 6	M	+	Oral Rinse	+			Resistance to fluconazole, ketoconazole and amphotericin B requested
22F.1 to 6	M	+	Retromolar swab	Psedomembranous candidiasis			Resistance to amphotericin B requested
22G.1 to 6	M	+	Hard Palate Swab	Pseudomembranous candidiasis on hard palate			Resistance to amphotericin B requested
22H.1 to 6	M	+	Oral Rinse	Pseudomembranous candidiasis on hard palate			Resistance to amphotericin B requested
22I.1 to 6	M	+	Oral Rinse	?			
22J.1-4, 6	M	+		?			
35A.1 to 6	M	+	Buccal Mucosal Swab	?			
35B.1 to 6	M	+	Oral Swab	?			
35C.1 to 6	M	+	Oral Rinse	?			
36A.1 to 6	M	+	Oral Rinse	?			
39A.1 to 6	M	+	Oral Rinse	?			
39B.1-4, 6	M	+	Oral Rinse	?			
39D.1 to 6	M	+	Oral Rinse	+		Fluconazole	Chemotherapy for lymphoma, fluconazole and miconazole sensitivities requested
39E.1 to 6	M	+	Oral Swab	+		Fluconazole	Chemotherapy for lymphoma, fluconazole and miconazole sensitivities requested

Annotation as in Tables 3.2, 3.4 and 3.7 (p., 70, 78 and 85).

Table 3.31a; Allelic profiles for fifty-seven isolates of *Candida* analysed in study 7 as determined from an allozyme electrophoresis analysis using sixteen loci

	Enzyme Locus															
Isolate Code	ALD	ENOL	EST	FUM	GDH	G6PD	GPI	HK1	HK2	NDPK	NP	PepA	PepB	PGAM	PGM	PK
38A.1	e	c	d	e	b	c	c	b	a	c	b	d	b	e	c	d
42D.1, 4	d	c	d	e	b	c	b	b	a	c	a	d	b	f	c	d
ATCC 90028	e	c	d	e	b	c	a	c	a	-	-	d	b	f	-	d
41A.2 to 6	e	c	d	e	b	c	a	c	b	c	b	d	b	i	c	d
CBS 562	e	c	d	e	b	c	a	c	a	c	b	d	a	i	c	d
16A.1 to 6	d	c	d	e	b	c	a	c	a	c	-	-	b	k	-	d
22B.1 to 6	e	c	d	e	-	c	b	c	a	c	-	d	b	j	c	e
22A.1, 4	e	c	d	e	-	c	b	c	a	c	b	d	b	k	c	e
22D.1	e	c	d	e	b	bc	b	c	a	c	b	d	b	fk	c	e
42A.1 to 6	e	c	d	e	b	c	b	b	c	c	a	d	b	i	c	d
42B.1 to 6	e	c	d	e	b	c	b	c	b	c	a	d	d	i	c	d
9C.1 to 6	c	c	d	e	-	c	a	c	d	c	b	d	b	i	c	-
9E.1 to 6	c	c	d	e	-	c	a	c	d	c	-	d	b	-	c	e
9F.1 to 6	c	c	d	e	-	c	a	c	d	c	-	d	b	-	-	e
9G.1 to 6	c	-	d	e	-	c	a	c	d	c	-	d	b	-	-	g
11A.1 to 6	c	c	d	e	-	c	a	c	d	c	b	d	b	d	-	b
9D.1 to 6	c	c	b	e	-	c	a	c	d	c	-	d	b	-	c	-
9I.1 to 6	c	c	d	e	-	c	d	c	d	c	-	d	b	i	c	e
12A.1 to 6	c	c	d	e	-	c	c	c	d	c	-	d	c	k	b	c
29A.5	c	c	d	e	-	c	b	b	a	c	b	d	a	c	c	d
29B.1, 2	c	b	d	e	-	c	a	b	a	c	-	d	-	c	c	d
ATCC 90029	e	b	d	e	-	c	a	c	a	c	-	e	-	f	c	e
18A.1, 3-6	e	c	-	e	b	c	a	d	a	c	c	d	b	f	e	e
12716	e	c	d	e	b	b	b	c	a	d	c	e	b	f	bc	d
39C.2 to 6	e	b	d	d	b	c	c	c	b	c	b	d	b	g	c	d
42D.2, 3, 5, 6	e	d	d	f	-	b	c	c	d	c	a	d	-	k	c	d
15A.1 to 6	c	b	c	e	b	b	e	c	e	c	b	b	b	-	-	e
42C.1 to 6	e	c	a	d	a	b	a	b	a	a	a	b	b	i	a	b
42E.1 to 6	d	c	b	d	-	a	b	c	d	a	b	-	-	c	a	b
CBS 7987	e	a	b	c	-	b	b	c	a	a	-	a	d	i	-	-
CBS 604	e	a	d	ab	c	b	b	a	d	ab	b	-	b	h	c	f

Annotation as in Tables 3.3 and 3.5 (p., 71 and 79).

Table 3.31b; Allelic profiles for fifty-seven isolates of *Candida* analysed in study 7 as determined froman allozyme electrophoresis analysis using sixteen loci

	Enzyme Locus															
Isolate Code	ALD	ENOL	EST	FUM	GDH	G6PD	GPI	HK1	HK2	NDPK	NP	PepA	PepB	PGAM	PGM	PK
4A.1 to 6	e	b	d	e	a	cd	a	d	c	c	b	d	b	i	-	c
8A.1 to 6	b	b	d	e	a	bc	a	c	a	c	f	c	be	k	c	f
8B.1, 4-6	e	b	a	e	a	bc	a	c	a	ac	-	c	be	k	-	c
8C.1 to 6	a	c	ad	c	a	bc	a	c	a	ac	-	c	be	k	c	f
9A.1 to 6	e	c	d	e	-	c	a	c	a	ac	-	d	b	c	-	d
9B.2 to 6	ce	c	d	e	-	c	a	c	d	c	-	d	b	-	c	d
9H.1 to 6	c	-	d	e	-	c	a	c	a	c	-	d	be	-	c	g
9J.1, 3-6	e	c	d	e	b	bc	c	c	a	c	b	d	b	f	c	d
10A.1 to 6	e	c	d	e	b	bc	c	c	a	c	b	d	b	-	e	d
13A.1 to 6	c	d	a	g	b	b	d	a	e	-	d	f	b	k	b	ac
14A.1 to 6	ce	c	d	e	c	b	e	c	d	c	b	d	b	cf	b	b
22C.1 to 6	e	c	d	e	b	bc	b	c	a	c	b	d	b	fk	c	e
22E.1 to 6	ce	c	d	e	b	c	c	c	a	c	b	d	b	fk	c	eg
22F.1 to 6	e	c	d	e	b	c	c	c	a	c	b	d	b	fl	c	-
22G.1 to 6	e	c	d	e	b	c	c	c	a	c	b	d	b	fi	d	-
22H.1 to 6	e	c	d	e	b	bc	b	c	a	c	b	d	b	h	d	e
22I.1 to 6	e	c	d	e	b	bc	b	c	a	c	b	d	b	h	d	e
22J.1-4, 6	c	c	d	e	-	bc	b	b	a	c	-	d	b	c	c	d
35A.1 to 6	e	c	d	e	b	ab	a	b	a	c	b	d	b	a	c	d
35B.1 to 6	e	c	d	e	b	ab	a	b	a	c	b	d	b	a	c	d
35C.1 to 6	e	c	d	e	b	ab	a	b	d	c	b	d	b	f	c	d
36A.1 to 6	e	c	d	e	b	ab	a	b	a	c	b	d	b	e	c	d
39A.1 to 6	e	c	d	e	bc	bc	a	c	b	c	b	d	b	h	c	d
39B.1-4, 6	e	c	d	e	b	bc	a	c	b	c	b	d	b	e	c	d
39D.1 to 6	e	c	d	e	b	bc	a	c	b	c	b	d	b	e	c	d
39E.1 to 6	e	c	d	e	b	bc	a	c	b	c	b	d	b	e	c	d

Annotation as in Tables 3.3, 3.5 and 3.8 (p., 71, 79 and 86).

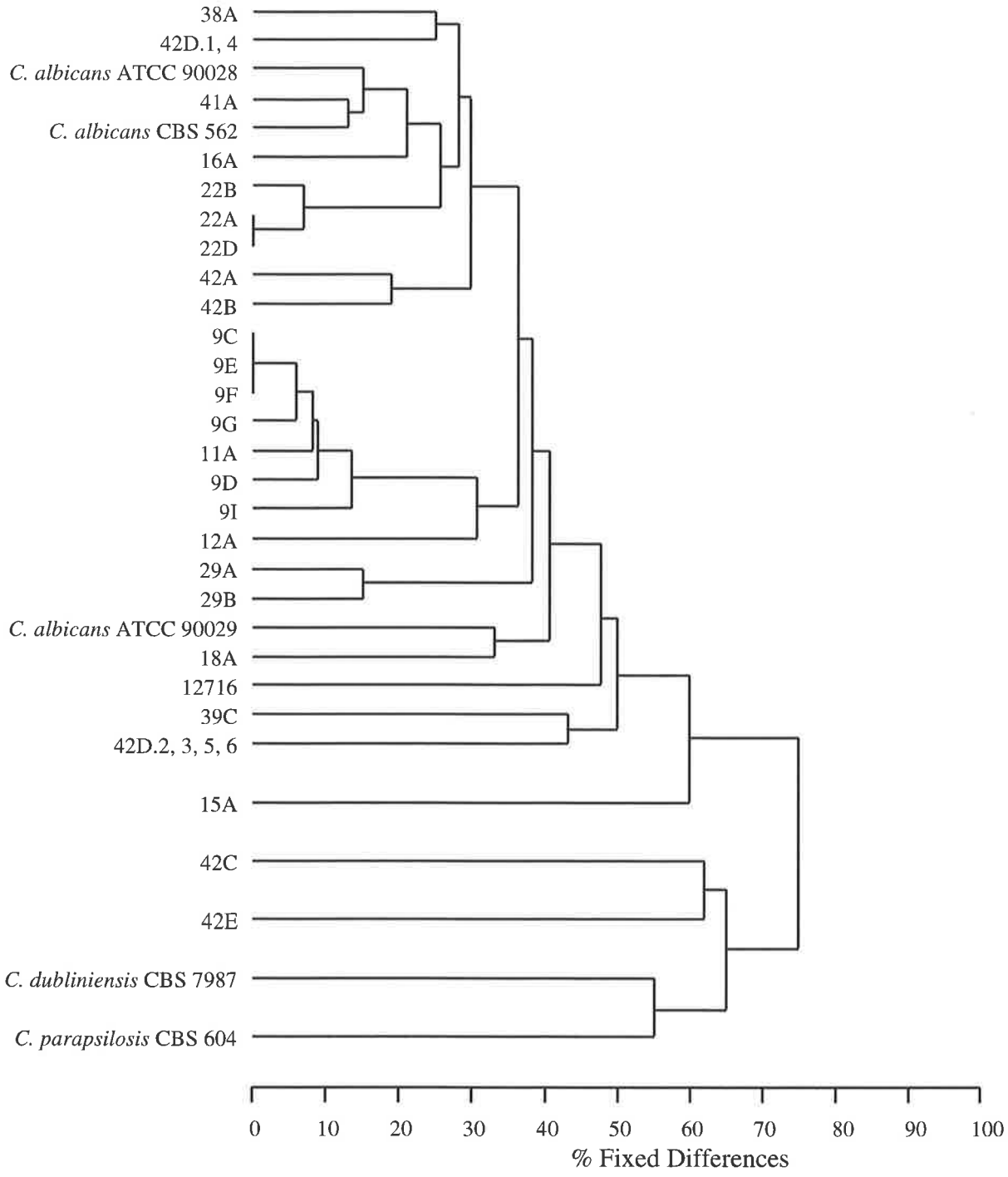


Figure 3.13; Phenogram depicting the genetic relationships between thirty-one isolates of *Candida* used in Study 7, as determined from an allozyme electrophoresis analysis using sixteen loci.

Table 3.32; Matrix of the percentages of fixed genetic difference between isolates from 4 individual patients in study 7

Isolate	9C	9D	9E	9F	9G	9I	22A	22B	22D	29A	29B	42A	42B	42C	42D	42D'
9D	**8**															
9E	**0**	**8**														
9F	**0**	**9**	**0**													
9G	**0**	**10**	**9**	**9**												
9I	**8**	**17**	**8**	**8**	**18**											
22A	29	33	23	25	36	29										
22B	31	33	23	25	36	29	**7**									
22D	29	33	23	25	36	29	**0**	**7**								
29A	36	42	38	42	45	43	33	36	33							
29B	33	36	33	36	30	46	46	46	46	**15**						
42A	36	42	38	42	45	36	33	29	31	33	38					
42B	36	42	38	42	45	36	33	29	31	40	46	**19**				
42C	71	75	77	75	82	79	73	71	69	80	77	**63**	**75**			
42D	54	55	50	55	50	54	50	54	43	64	62	**50**	**43**	**79**		
42D'	43	42	38	42	45	43	33	29	25	27	31	**19**	**31**	**69**	**57**	
42E	67	60	73	70	78	75	69	75	69	69	92	**85**	**77**	**62**	**85**	**77**

Annotation as in Table 3.20 (p., 128).

3.3.9.b Interpretation

Twenty-seven of the fifty-three clinical isolates assessed had multiple banding patterns indicating that they are mixed or heterozygous and were eliminated from this interpretation. The *C. parapsilosis* type strain CBS604 was again heterozygous at FUM and NDPK in this analysis.

Twenty-three of the twenty-six clinical isolates assessed were contained within the *C. albicans* cluster, the remaining three isolates, 15A, 42C and 42E, potentially belonged to unique *Candida* species. The matrix in Table 3.32 (p., 161) is an excerpt of the matrix of pairwise comparisons for this analysis in Appendix 3 (Table 12)for several patients. Isolates C, E and F from patient 9 were identical and differed from isolates D, G and I with fixed genetic differences at 8 to 18% of loci. All isolates represented different *C. albicans* strains. Isolate C was from a palate swab; D, F and H were from oral rinse samples, E was from a pharynx swab and G was from a swab of an oral lesion. Isolates B and C were obtained on the 14th of September 1995, D on the 26th of March, E and F on the 1st of July, G on the 10th of July 1996 and A and I on unknown dates. Isolates 9A, B and H were mixed/heterozygous. Isolates B (oral rinse) and C (swab) were taken during a fluconazole resistant infection caused by mixed *C. albicans* isolates. Patient 9 was being treated with fluconazole at the time isolates B, C, E and F were taken. The latter three strains are all genetically identical, possibly indicating persistence of a fluconazole resistant strain. Additionally, the patient participated in an amphotericin B lozenge trial during the time that isolates E to J were collected. The same or a very closely related strain of *C. albicans* persisted during this time, until eventually a mixed infection was detected. While the patient was being treated with fluconazole, the infection was fluconazole resistant and mixed. When treatment ceased, a different *C. albicans* strain predominated. When fluconazole treatment was recommenced, one of the original infecting strains from the previous fluconazole resistant infection returned. This strain not only predominated in the oral cavity, but was also responsible for the infection, being isolated from both the oral rinse and the lesion swab. Within ten days this strain was replaced by another *C. albicans* strain.

Isolates 22A and D were identical but had fixed genetic differences when compared to isolate 22B at 7% of loci. All 3 isolates could be assigned to *C. albicans*. Isolates A and D were obtained form oral rinse samples while B was obtained from a tongue swab. Isolate 22A was taken on the 12th of April 1995, B on the 28th of May and D on the 19th of June

1996. Isolate B was clinically resistant to fluconazole. This patient had candidiasis at the time when isolates B and D were obtained. Isolate D was a single isolated colony and was heterozygous. It shared alleles in common with isolate A, hence there was no fixed differences between the two isolates. Isolates 22C, D and E were obtained on the 19th of June 1996, C and D being obtained from lesion swabs and E from an oral rinse. Isolates F, G and H were taken on the 3rd of July 1996; F and G from lesions, H from an oral rinse. Isolate I was taken one week later from an oral rinse and J was taken after this time on an unknown date. Isolates C, E, F, G, H, I and J were all mixed/heterozygous. Patient 22 participated in an amphotericin B lozenge trial during the time that isolates C to J were collected. The amphotericin B lozenge contained a lower concentration of the antifungal than may be taken via other methods. This may have caused the mixed/heterozygous colonization of this patient. Since isolates A and D are indistinguishable, this implies that the patient was recolonized with the same strain during the time these samples were collected, the later colonization resulting in candidiasis. This observation supports the evidence provided in Sections 3.1.2 and 3.1.3 (p., 74 and 83), that clinical *C. albicans* infection is host-mediated and opportunistic rather than the result of colonization by a pathogenic organism. Between these two colonizations, the patient was infected with a closely related strain that was clinically resistant to fluconazole, but that strain was not maintained.

The two isolates (A and B) from patient 29 had fixed genetic differences at 15% of loci. The allelic profiles of these isolates suggeseted that they represent different strains of *C. albicans*. These isolates were both obtained from oral rinse samples collected on the 26th of April and the 28th of July 1995, respectively, during which time the patient appeared to be recolonised by a distinct, but closely related *C. albicans* strain.

Isolates from patient 42 differed at 19 to 85% of loci with isolates A, B and D belonging to the *C. albicans* cluster whereas isolates C and E belonged to separate but as yet undetermined species of *Candida*. Isolates 42D.1, 4 and 42D.2, 3, 5, 6 were obtained from a single oral rinse sample with each group of single colonies comprising a distinct subset of strains which differed at 57% of loci. This sample represents a mixed colonization in this patient. Isolates A, C, D and E were oral rinse samples from this patient and B was from a tongue swab. Isolates A and B were taken on the 12th of May 1995 prior to any antifungal treatment, C on the 9th of August and D and E on the 14th of November 1995. The patient's original lesion was caused by a different strain than was obtained by oral rinse. After

antifungal treatment, these strains were replaced with another *Candida* species. The later oral rinses (D and E taken on the 14th of November 1995) indicated that the patient was colonized by a number of different strains from both *C. albicans* and another *Candida* species.

3.3.10 Study 8

3.3.10.a Results

Isolates within study 8 (Table 3.33 p., 165-6) differed at 0 to 75% of the seventeen loci scored (Table 3.34 p., 167-8). The isolates formed six distinct clusters having fixed genetic differences at over 50% of loci (Figure 3.14 p., 169). The first cluster contains the majority of isolates, including the *C. albicans* type and reference strains and the *C. dubliniensis* type strain. Variation within this cluster ranged from 0 to 45.4% of loci. The presence of the *C. dubliniensis* type strain in this cluster for the first time in this thesis suggests its possible contamination with a *C. albicans* isolate. For this reason, this and the other reference strains were recultured from the original glycerol stocks for the ensuing allozyme electrophoresis analyses. The presence of numerous *C. albicans* reference isolates in this cluster continues to suggest that the first cluster represents the *C. albicans* species. Isolates within the second cluster differed from the first cluster at 52.4% of loci and these two clusters differed from the third and fourth at 71.4% of loci. These latter two clusters differed from each other at 56.5% of loci. The fifth and sixth cluster differed from each other at 59.8% of loci and from the other clusters at 75% of loci.

Table 3.33a; Details of the forty-nine reference and clinical samples analysed in study 8 using allozyme electrophoresis

Isolate Code	Sex	HIV status	Site of Isolation	Candidiasis	Antifungal Resistance	Patient's Therapy	Other Notes
ATCC 90028			Blood, Iowa	systemic			*C. albicans* reference strain
ATCC 90029			blood, Iowa	systemic			*C. albicans* reference strain
12716	M	+	Oral Swab	+	Fluconazole, moderate 5-flurocytosine		Mixed infection with *C. krusei*; resistant to fluconazole, Itraconazole and 5-flurocytosine. Reidentified as *C. dubliniensis*
17130	F	+	Oesophageal Biopsy	+	Fluconazole, Itraconazole	Fluconazole	
CBS 562			skin (interdigital mycosis), Uruguay				*C. albicans* type strain
CBS 7987		+	oral cavity, Ireland				*C. dubliniensis* type strain
50A.1 to 6	M	+	Oral Rinse	?			
50B.1-3, 5, 6	M	+	Oral Rinse	?			
50C.1 to 6	M	+	Oral Rinse	?			
52A.1 to 6	M	+	Oral Rinse	?			Colony 52A.6 Labelled 26A.6 on McCartney, 2 colony types - one very adhesive
52B.1 to 6	M	+	Tongue Swab	?			White lesions on tongue
45A.1 to 6	M	+	Swab from OHL on Tongue	OHL			
46B.2, 3, 5, 6	M	+	Oral Rinse	?			
52C.1 to 6	M	+	Oral Rinse	+			candidiasis - dorsum of tongue.
44D.1 to 6	M	+	Oral Rinse	?			
46D.1 to 6	M	+	Oral Rinse	+			
46F.1 to 4	M	+	Oral Rinse	?			Amp-B sensitivity requested Trial completed
46G.1 to 6	M	+	Oral Swab	?			
47A.1 to 6	M	+	Oral Rinse	?			
32B.5	M	+	Oral Rinse	?			
46C.1 to 6	M	+	Oral Rinse	+			
53C.1 to 6	M	+	Oral Rinse	?			
55B.1 to 6	M	+	Oral Rinse	?			
57A.1 to 6	M	+	Tongue Scrape	?			

Annotation as in Tables 3.2 and 3.4 (p., 70 and 78).

Table 3.33b; Details of the forty-nine reference and clinical samples analysed in study 8 using allozyme electrophoresis

Isolate Code	Sex	HIV status	Site of Isolation	Candidiasis	Antifungal Resistance	Patient's Therapy	Other Notes
23A.1, 5	M	+	Oral Rinse	+			
23D.1-3, 5, 6	M	+	Oral Rinse	?			
44G.1 to 6	M	+	Oral Rinse	?			
44H.1	M	+	Oral Rinse	?			
31D.1 to 6	M	+	Oral Rinse	?			
43A.1-3, 5, 6	M	+	Tongue Swab	?		Fluconazole, dosage recently increased	43A.3-6 Growth on agar slopes very adhesive and granular
43B.1, 6	M	+	Oral Rinse	?			Growth on agar slopes very adhesive
44E.1 to 6	M	+	Oral Rinse	?			
44F.1 to 6	M	+	Oral Rinse	?			
48A.1 to 6	M	+	Tongue Swab, OHL	OHL			OHL lateral side tongue
48B.1 to 6	M	+	Oral Rinse	OHL			OHL lateral side tongue
49A.1 to 6	M	+	Oral Rinse	?			
51A.1 to 6	M	+	Oral Rinse	?			
51B.1 to 6	M	+	Oral Rinse	?			
53A.1 to 6	M	+	Oral Rinse	?			53A.1-6 growth on agar slopes very adhesive
53B.1 to 6	M	+	Oral Rinse	?			
54B.1 to 6	M	+	Oral Rinse	?			
54C.1 to 6	M	+	Oral Rinse	?			
54D.1 to 6	M	+	Oral Rinse	?			
54E.2 to 6	M	+	Oral Rinse	?			
54F.1 to 6	M	+	Oral Rinse	?			Amphotericin B trial
54G.1 to 5	M	+	Oral Rinse	?			
55A.1 to 6	M	+	Oral Rinse	?			
57B.1 to 6	M	+	Oral Rinse	?			
57C.1 to 6	M	+	Oral Rinse	?			

Annotation as in Tables 3.2, 3.4 and 3.7 (p., 70, 78 and 85).

Table 3.34a; Allelic profiles of forty-nine isolates analysed in study 8 at seventeen enzyme loci

	Enzyme Locus																
Isolate Code	ALD	ENOL	EST	FUM	GDH	G6PD	GPI	HK1	HK2	NDPK	NP	PepA	PepB	PGAM	6PGD	PGM	PK
ATCC 90028	c	g	d	e	b	d	b	d	c	d	-	-	b	d	b	-	d
ATCC 90029	c	d	d	e	-	d	b	d	c	d	a	-	-	-	b	c	-
12716	c	d	d	e	-	d	b	d	c	d	-	d	d	b	b	-	d
17130	c	-	d	e	b	e	b	d	c	d	-	d	b	-	-	b	c
CBS 562	c	b	d	e	b	e	b	d	c	d	a	c	c	b	b	c	-
CBS 7987	c	-	b	b	b	d	b	d	c	d	e	-	d	a	-	-	d
50A.1 to 6	c	c	d	d	b	e	b	e	c	d	a	c	-	j	-	a	a
50B.1-3, 5, 6	c	d	d	d	d	e	c	-	-	d	a	c	c	-	b	a	c
50C.1 to 6	c	d	d	f	b	d	b	e	c	d	a	c	c	b	-	c	d
52A.1 to 6	c	d	d	f	b	d	b	e	e	d	a	c	c	b	-	a	d
52B.1 to 6	c	d	d	f	b	e	b	e	c	d	a	c	c	b	-	a	-
45A.1 to 6	c	d	e	d	a	d	b	e	c	d	a	c	b	-	b	c	d
46B.2, 3, 5, 6	c	d	e	d	b	e	b	e	c	d	a	c	b	-	b	-	-
52C.1 to 6	c	d	-	d	d	e	b	-	-	d	b	c	-	b	-	c	e
44D.1 to 6	c	d	d	f	d	d	d	d	c	d	a	c	c	e	b	c	a
46D.1 to 6	c	d	d	d	a	d	b	d	c	d	a	c	c	e	b	c	e
46F.1 to 4	c	d	d	d	a	d	-	d	c	d	a	c	c	e	-	c	e
46G.1 to 6	-	d	d	c	a	e	b	d	d	d	-	c	c	h	b	-	b
47A.1 to 6	-	d	d	d	c	e	b	d	g	d	a	c	c	h	b	c	d
32B.5	c	d	e	e	a	c	b	b	d	d	a	-	b	e	-	-	f
46C.1 to 6	c	d	e	e	a	d	b	d	b	d	a	c	b	e	-	c	e
53C.1 to 6	-	f	d	c	d	e	c	c	b	c	b	d	c	i	b	a	d
55B.1 to 6	c	b	d	e	b	e	a	c	a	b	b	d	c	i	a	c	d
57A.1 to 6	c	b	d	d	b	e	a	c	a	b	a	d	a	c	b	c	d

Annotation as in Tables 3.3 and 3.5 (p., 71 and 79).

Table 3.34b; Allelic profiles of forty-nine isolates analysed in study 8 at seventeen enzyme loci

	Enzyme Locus																
Isolate Code	ALD	ENOL	EST	FUM	GDH	G6PD	GPI	HK1	HK2	NDPK	NP	PepA	PepB	PGAM	6PGD	PGM	PK
23A.1, 5	c	c	-	d	b	c	b	d	d	b	-	-	c	g	-	-	a
23D.1-3, 5, 6	a	c	a	d	b	c	c	c	c	b	-	a	c	e	-	a	a
44G.1 to 6	a	b	a	b	b	a	b	c	d	b	-	b	b	e	-	a	a
44H.1	a	b	b	b	b	d	b	a	a	b	b	c	b	b	b	a	a
31D.1 to 6	c	ad	d	d	a	e	b	d	c	d	a	-	b	e	b	-	d
43A.1-3, 5, 6	ac	-	a	d	b	c	c	c	c	b	-	a	c	e	-	a	a
43B.1, 6	c	c	d	d	b	e	b	d	c	bd	b	c	b	-	-	c	a
44E.1 to 6	a	d	ad	f	b	d	c	d	c	b	-	-	c	-	-	c	d
44F.1 to 6	a	c	ad	b	b	b	b	d	c	b	-	a	c	e	-	a	-
48A.1 to 6	c	d	d	d	b	be	d	d	b	d	a	d	c	e	b	c	e
48B.1 to 6	c	d	d	d	b	be	b	d	b	d	a	d	c	f	b	c	d
49A.1 to 6	c	b	cd	b	b	be	b	c	d	a	d	a	c	f	-	a	b
51A.1 to 6	b	e	b	b	b	d	b	c	a	a	-	a	e	f	c	a	ce
51B.1 to 6	c	be	c	b	b	d	b	c	a	a	c	b	f	e	-	-	b
53A.1 to 6	c	e	bd	b	c	e	b	d	c	a	-	-	c	e	b	a	c
53B.1 to 6	c	bf	d	e	d	e	c	c	b	c	b	d	c	i	b	c	d
54B.1 to 6	c	b	b	f	-	b	b	c	c	bd	-	b	d	e	-	a	b
54C.1 to 6	-	d	d	a	-	e	b	e	f	d	a	-	c	bd	-	b	-
54D.1 to 6	c	d	d	f	d	e	b	e	c	d	a	c	c	be	-	c	b
54E.2 to 6	-	d	d	b	b	d	e	f	c	d	a	-	c	bf	b	c	-
54F.1 to 6	c	d	d	e	b	e	e	e	c	d	a	d	b	be	-	c	bf
54G.1 to 5	c	d	d	e	b	de	e	d	c	d	a	d	b	be	-	bc	d
55A.1 to 6	c	bf	d	e	b	e	c	c	b	c	b	d	c	i	b	c	d
57B.1 to 6	c	b	d	d	b	be	a	c	a	b	a	d	a	c	-	a	d
57C.1 to 6	c	b	d	d	b	ce	a	c	a	b	a	d	a	c	b	a	d

Annotation as in Tables 3.3, 3.5 and 3.8 (p., 71, 79 and 86).

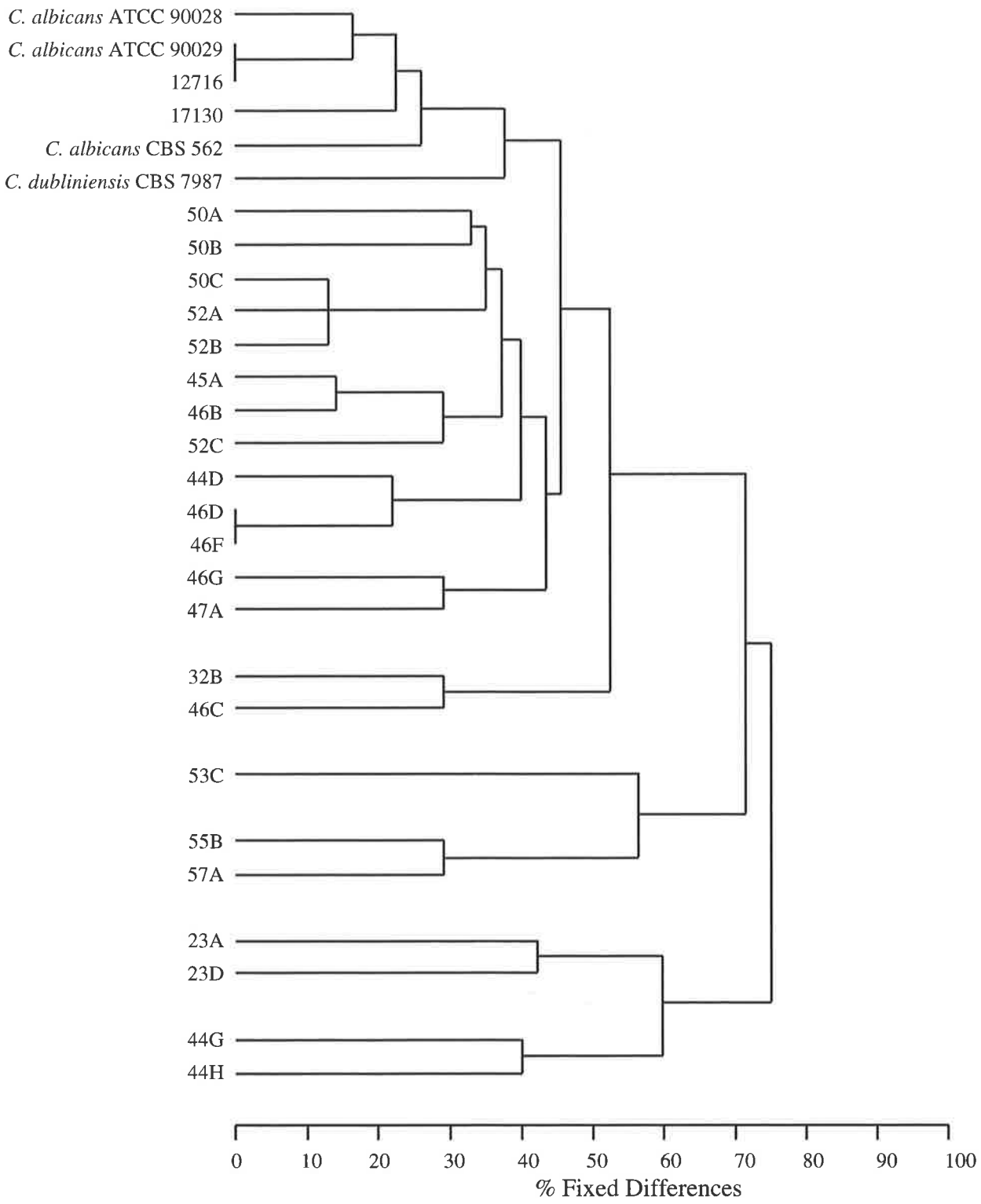


Figure 3.14; Phenogram depicting the genetic relationships between twenty-eight isolates of *Candida* used in Study 8, as determined from an allozyme electrophoresis analysis using seventeen loci.

Table 3.35; Matrix of the percentages of fixed genetic difference between isolates from 5 individual patients in study 8

Isolate	23A	23D	44D	44G	44H	46B	46C	46D	46F	46G	50A	50B	50C	52A	52B
23D	**42**														
44D	67	73													
44G	58	47	**87**												
44H	67	67	**76**	**40**											
46B	60	75	50	75	64										
46C	75	93	44	80	75	**38**									
46D	58	73	24	87	76	**36**	**25**								
46F	64	71	20	93	87	**42**	**27**	**0**							
46G	64	92	50	85	79	**50**	**54**	**36**	**42**						
50A	45	57	53	71	67	17	67	47	50	58					
50B	67	67	36	92	79	33	62	36	33	36	**33**				
50C	67	80	31	87	69	31	50	31	33	54	**40**	**46**			
52A	67	80	44	80	63	38	56	44	47	54	40	38	13		
52B	64	71	40	79	67	23	60	40	43	42	21	25	13	**13**	
52C	67	91	50	91	67	22	42	33	36	44	50	36	42	**50**	**36**

Annotation as in Table 3.20 (p., 128).

3.3.10.b Interpretation

Twenty-one of the forty-five clinical isolates examined in this analysis were mixed or heterozygous. This includes isolates B to G from patient 54. This patient was participating in an amphotericin B lozenge trial when isolates D to G were collected, perhaps contributing to the persistence of the mixed/heterozygous colonization.

Fifteen of the remaining twenty-four isolates were positioned within the *C. albicans* cluster while isolates 32B and 46C of cluster 2 probably representing a subpopulation of *C. albicans* or a closely related species of *Candida*. Nine clinical isolates were placed external to this cluster and formed five distinct clusters. Four of these clusters contained two isolates and the fifth contained a single isolate. The species designation of the isolates comprising these 5 clusters could not be determined in this analysis because there were no corresponding type or reference isolates included on the gel.

Isolates ATCC 90029 and 12716 were identical in this analysis but differed at a number of these same loci in previous analyses. This indicates contamination of one or both of these isolates, or a decrease in resolution of the loci in question in this analysis. Since the lysates used herein for both isolates were prepared prior to study 7, in which they were very different to each other, it appears that the resolution in study 8 is decreased. All but one enzyme locus (6PGD in study 8) was scored in both analyses. Differences in the electrophoretic mobility of enzymes may vary due to for example, the age of the lysates, the buffers used and/or the running conditions used on the day. The levels of genetic variation detected between other isolates were consistent in both studies suggesting that the decrease in polymorphism was associated only with isolates ATCC 90029 and 12716.

The interpretations below are derived from excerpts of the matrix of pairwise comparisons for this analysis within Appendix 3 (Table 13) and relate to comparisons of multiple samples derived from the same patients. Isolates 23A and D had fixed genetic differences at 42% of loci representing different strains of a *Candida* species but their species designation could not be esablished. Both isolates were collected using an oral rinse. The patient had candidiasis when isolate A was collected on the 11th of April 1995. Isolate D was collected on the 7th of September 1995. This patient showed persistence of different strains of a species other than *C. albicans* during both asymptomatic carriage and infection.

Isolates 44D, G and H differed at 40 to 87% of loci, with 44D being in the *C. albicans* cluster and G and H belonging to a unique but as yet undetermined species of *Candida*. All three isolates were from oral rinse samples. Isolate D was taken on the 14th of June, G on the 11th of September, and H was taken on the 9th of October 1996. Isolates E and F, from oral rinses on the 26th of June and the 7th of August 1996 respectively, were mixed/heterozygous. This patient was initially colonized with *C. albicans*, then by a mixed/heterozygous culture and finally by two strains from another *Candida* species. Patient 44 took part in an amphotericin B lozenge trial when isolates D to H were collected. This trial could have caused the mixed infection when isolates E and F were taken and could have selected for the alternate *Candida* species present when isolates G and H were obtained.

The multiple isolates from patient 46 had fixed genetic differences at 0 to 54% of the loci. In this analysis, isolates 46D and F were genetically identical and all of the strains collected belonged to the cluster comprising *C. albicans*. However, isolate 46C may represent either an extremity of genetic variation within *C. albicans* or may constitute a distinct species. Isolates B, C, D and F were from oral rinse samples, G was from an oral swab. Patient 46 was candidiasis positive when isolates C and D were taken. The patient was participating in an amphotericin B lozenge trial during the time isolates D to G were taken. These results suggest the possible selection of isolate D/F with amphotericin B treatment. Isolate B was obtained on the 16th of August, C on the 22nd of November, D on the 6th of December 1995, F on the 21st of February and G on the 27th of March 1996. The same strain D/F persisted after resolution of the infection and was replaced by another strain causing the lesion isolate G was taken from. Although strain D/F persisted, it did not cause infection.

For patient 50, isolates A, B and C differed at 33 to 46% of loci. These isolates represented different *C. albicans* strains. They were obtained from oral rinse samples taken on the 25th of May, 29th of August and the 21st of November 1995, respectively. The patient's predominant colonizing strain varied between each sample collection time.

Similarly, isolates A, B and C from patient 52 had fixed genetic differences at 13 to 50% of loci, suggesting that they represent different *C. albicans* strains. Isolates A and C were from oral rinse samples and B was a tongue swab sample. Isolates A and B were obtained on the 25th of May and C on the 20th of November 1995. This patient's oral hairy

leukoplakia infection was caused by a strain different from that obtained by oral rinse and after resolution of the oral hairy leukoplakia lesion, a third strain predominated.

3.3.11 Study 9

3.3.11.a Results

Isolates within study 9 (Table 3.36, p., 174-5) differed from each other at 0 to 73.7% of the fifteen loci analysed (Table 3.37 p., 176-7). They formed seven distinct clusters, which differed from each other at more than 50% of the loci assessed (Figure 3.15 p., 178). The first cluster contains most of the clinical isolates and the *C. albicans* type strain with fixed differences within it at 0 to 45.8% of loci. This cluster probably represents the members of this species. They differed from the second cluster, containing isolate 63B, at 50.1% of loci. Four clinical isolates fall into cluster 3, which differs from clusters 1 and 2 at 51.3% of loci and has fixed differences within it at 22 to 38.5% of loci. Another four clinical isolates form cluster 4, which differs at 54.2% of loci from the first three and has fixed differences within it at 17 to 41% of loci. Cluster 5 contains two clinical isolates, which differ at 45% of loci and from the other clusters at 61.1% of loci. The *C. parapsilosis* type strain forms cluster 6 and the *C. dubliniensis* type strain forms cluster 7 which differ from clusters 1 to 5 at 70.1 and 73.7% of loci, respectively.

The genetic relationships between a sub-population of isolates within cluster 1 that differed at less than 31.5% of loci were not reproducible upon repeat UPGMA analyses, as has been observed in previous studies 4 and 6 (p., 130 and 145). Again, this is due to tied fixed difference values.

Table 3.36a; Details of the fifty-one reference and clinical samples analysed in study 9 using allozyme electrophoresis

Isolate Code	Sex	HIV status	Site of Isolation	Candidiasis	Antifungal Resistance	Patient's Therapy	Other Notes
54I	M	+	Oral Rinse	?			AmpB trial
59A	M	+	Oral Rinse	?			59A.4 Growth on agar slopes very adhesive and granular
59B	M	+	Tongue Swab	Median Rhomboid lesion			
58C	M	+	Oral Rinse	?			
56B	M	+	Oral Rinse	carrier			Post radiotherapy patient
60B	M	+	Oral Rinse	?			
54J	M	+	Oral Rinse	Oral pseudomembranous candidiasis			AmpB trial
60C	M	+	Tongue Swab	?			
59C	M	+	Palate Swab	Erythematous candidiasis hard palate			
2A	M	+	Buccal mucosal swab	+		Was on Itraconazole trial (placebo?), 14 days Ketoconazole, now fungilin lozenges	Oropharyngeal and oesophageal *Candida*
59I	M	+	Oral Rinse	?			
59D	M	+	Oral Rinse	+			
61A	M	+	Tongue Swab	?			
60A	M	+	Mouth Swab	?			
59H	M	+	Oral Rinse	?			
58B	M	+	Tongue Swab	OHL			CD4<200, OHL tongue and coated with white plaques. 58B.5-6 Growth on agar slopes very adhesive and granular
1E	M	+	Palate Swab	Oropharyngeal candidiasis		nil at present	Sensitivities requested especially ketoconazole
60D	M	+	Palate Swab	?			
54H	M	+	Oral Rinse	?			AmpB trial
59G	M	+	Oral Rinse	?			
63G	M	+	Oral Rinse	?			
1A	M	+	Tongue Swab OHL	OHL			Previous microscopy +ve for fungal elements but not cultured - recultured
1C	M	+	Oral Rinse	+			
37B	M	+	Tongue Swab	?			

Annotation as in Table 3.2 (p., 70).

Table 3.36b; Details of the fifty-one reference and clinical samples analysed in study 9 using allozyme electrophoresis

Isolate Code	Sex	HIV status	Site of Isolation	Candidiasis	Antifungal Resistance	Patient's Therapy	Other Notes
2B	M	+	Oral Rinse	+		Was on Itraconazole trial (placebo?), 14 days Ketoconazole, now fungal lozenges	Oropharyngeal and oesophageal *Candida*
5A	M	-	Tongue swab	?			Radiotherapy, Xerostomia Glossitis and coated tongue
CBS 562			skin (interdigital mycosis), Uruguay				*C. albicans* type strain
63I	M	+	Oral Rinse	?			
63J	M	+	Oral Swab	?			
63E	M	+	Tongue Swab	?			
63M	M	+	Oral Rinse	Pseudomembranous Candidiasis			
63N	M	+	Oral Swab, lateral border of tongue	pseudomembranous candidiasis, OHL likelihood			
63R	M	+	Oral Swab	Angular Chelitis			
63C	M	+	Oral Swab	?			
63B	M	+	Oral Rinse	OHL and Candidiasis			
7A	M	+	Oral Rinse	+			
37C	M	+	Oral Rinse	?			
1B	M	+	Oral Rinse	+			
27C	M	+	Oral Rinse	?			
6A	M	?	Oral Rinse	+			
63S	M	+	Oral Rinse	?			
63Q	M	+	Oral Rinse	?			
63P	M	+	Oral Rinse	?			
63F	M	+	Palate Swab	?			
63H	M	+	Oral Rinse	?			
CBS 604			case of sprue, Puerto Rico				*C. parapsilosis* type strain
CBS 7987		+	oral cavity, Ireland				*C. dubliniensis* type strain
1D	M	+	Oral Rinse	?			
3A	M	+	Floor of Mouth Swab	+			Floor of Mouth. candidiasis
59E	M	+	Oral Rinse	?			
59F	M	+	Palate Swab	?			

Annotation as in Tables 3.2, 3.4 and 3.7 (p., 70, 78 and 85).

Table 3.37a; Allelic profile of fifty-one isolates analysed in study 9 at fifteen enzyme loci

	Enzyme Locus														
Isolate Code	ADH	ALD	ENOL	EST	FDP	FUM	GDH	G6PD	GPI	HK1	HK2	PepA	PepB	PGAM	PK
54I	-	b	e	c	-	c	e	b	c	d	c	g	-	c	c
59A	-	b	e	c	a	c	e	c	d	d	c	g	d	c	c
59B	-	b	-	c	a	c	a	-	c	d	b	g	d	c	c
58C	-	-	-	c	a	c	a	c	-	d	b	g	-	e	c
56B	-	b	e	c	a	c	e	c	c	d	b	g	d	d	c
60B	-	b	e	c	-	c	e	c	c	d	b	g	c	d	c
54J	-	-	e	c	-	c	e	c	c	d	b	g	d	c	c
60C	-	b	e	c	-	c	e	c	c	d	b	g	c	c	c
59C	-	b	e	c	-	c	e	-	-	d	b	g	d	a	c
2A	-	b	-	c	a	c	b	-	c	d	b	-	c	c	-
59I	-	-	e	c	a	c	b	-	c	d	b	c	c	c	c
59D	-	b	e	c	-	c	e	-	-	d	b	b	c	c	c
61A	-	b	e	c	-	c	e	c	-	d	b	c	c	c	c
60A	-	b	-	b	-	c	e	c	c	d	b	g	c	c	c
59H	-	b	c	c	-	c	e	c	c	c	b	g	c	c	c
58B	-	b	d	-	-	c	e	c	d	d	b	g	d	c	-
1E	a	-	e	c	-	-	c	c	b	d	b	-	c	d	c
60D	-	b	e	-	-	c	e	c	b	d	b	g	c	d	c
54H	-	b	e	c	a	-	c	c	b	d	b	-	d	c	c
59G	b	b	e	c	-	c	e	c	c	c	d	b	c	d	c
63G	a	-	b	c	-	-	a	c	c	b	b	-	b	d	c
1A	a	-	e	c	-	-	d	c	c	b	b	-	c	d	e
1C	a	b	e	c	-	-	c	c	c	b	b	-	c	d	c
37B	a	b	-	c	a	b	b	c	c	-	-	-	d	c	e

Annotation as in Tables 3.3 and 3.5 (p., 71 and 79).

Table 3.37b; Allelic profile of fifty-one isolates analysed in study 9 at fifteen enzyme loci

	Enzyme Locus														
Isolate Code	ADH	ALD	ENOL	EST	FDP	FUM	GDH	G6PD	GPI	HK1	HK2	PepA	PepB	PGAM	PK
2B	a	-	f	c	-	-	a	c	c	d	b	e	c	c	-
5A	a	-	-	c	a	-	e	c	c	d	b	-	-	d	b
CBS 562	-	b	d	c	-	-	d	-	c	d	b	c	-	d	d
63I	a	-	b	c	-	-	d	c	c	d	b	-	b	d	c
63J	a	b	-	c	a	-	-	b	c	d	b	-	b	d	c
63E	a	-	d	b	-	-	d	c	c	d	b	d	b	d	c
63M	a	b	d	c	-	-	d	-	b	d	b	-	-	d	c
63N	a	-	-	c	a	-	b	c	b	d	b	-	a	d	c
63R	a	b	d	a	-	-	b	-	b	d	b	d	b	d	c
63C	a	-	d	-	-	c	b	c	c	d	a	g	-	d	c
63B	-	b	e	c	-	b	a	c	-	c	b	d	-	b	c
7A	-	b	-	a	-	-	e	b	c	d	d	-	e	c	c
37C	a	b	d	a	-	-	e	b	-	d	c	-	d	c	c
1B	a	b	-	a	-	-	d	b	b	d	b	-	c	c	c
27C	a	-	d	c	-	-	a	b	c	d	b	f	c	c	c
6A	a	b	-	c	a	-	a	c	c	e	c	-	c	d	c
63S	a	b	d	c	a	-	a	c	b	e	d	c	c	d	c
63Q	a	b	d	c	a	-	e	-	b	e	d	-	e	d	c
63P	a	b	-	a	-	-	e	c	-	e	d	-	a	e	c
63F	a	-	a	c	a	-	b	-	b	d	d	-	b	c	c
63H	a	a	d	c	a	a	d	a	b	a	a	e	b	d	c
CBS 604	a	-	d	a	b	-	b	a	-	f	e	-	c	d	c
CBS 7987	a	c	c	c	a	b	a	-	a	b	d	a	a	d	-
1D	a	-	-	ac	a	-	a	b	-	d	b	g	c	c	a
3A	a	b	d	c	a	-	a	c	c	e	b	-	c	d	bd
59E	-	b	d	c	-	c	e	c	-	c	b	g	d	d	bd
59F	-	bd	e	c	-	c	e	c	b	c	b	g	d	d	e

Annotation as in Tables 3.3, 3.5 and 3.8 (p., 71, 79 and 86).

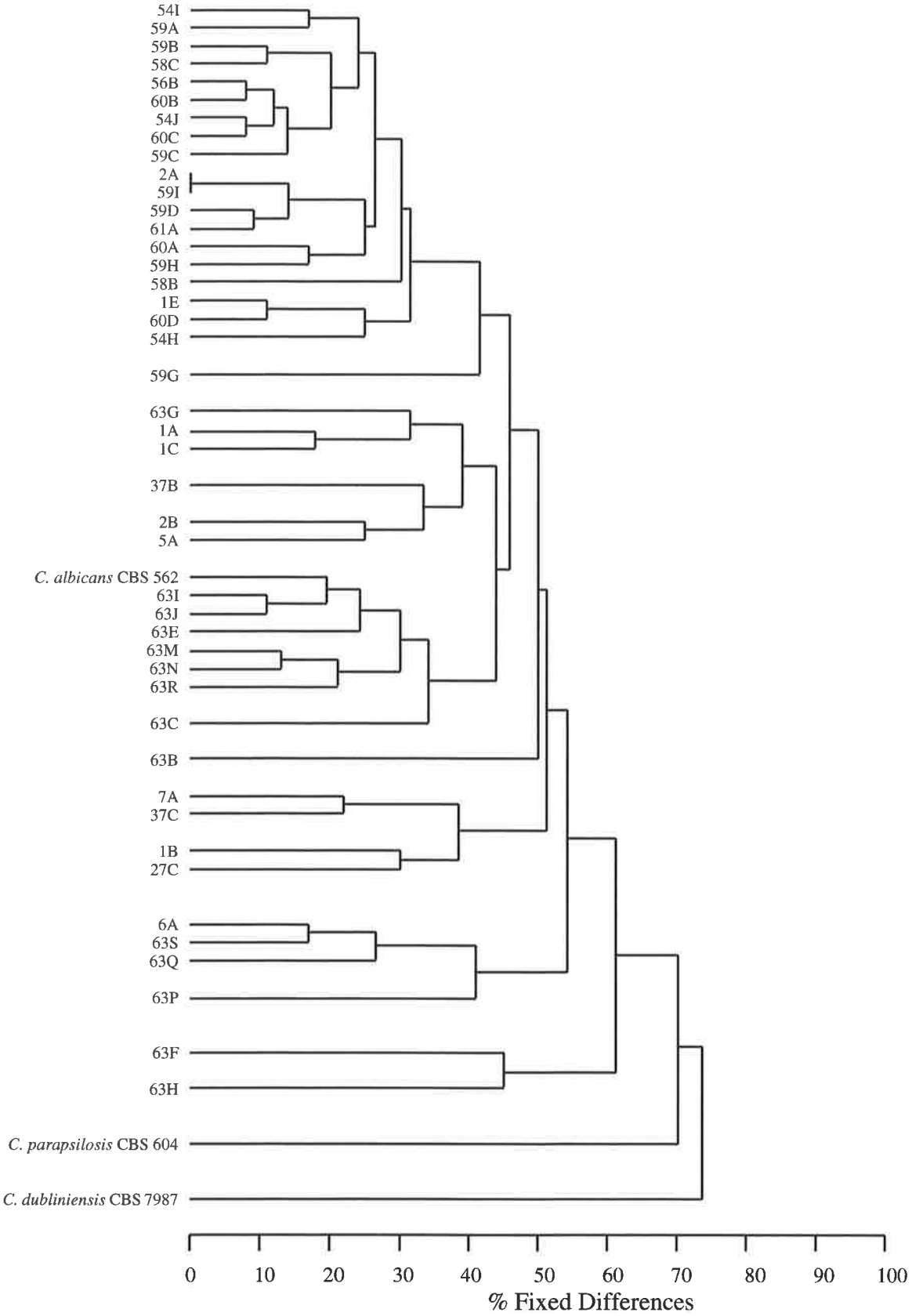


Figure 3.15a; Phenogram depicting the genetic relationships between forty-seven isolates of *Candida* used in Study 9, as determined from an allozyme electrophoresis analysis using fifteen loci.

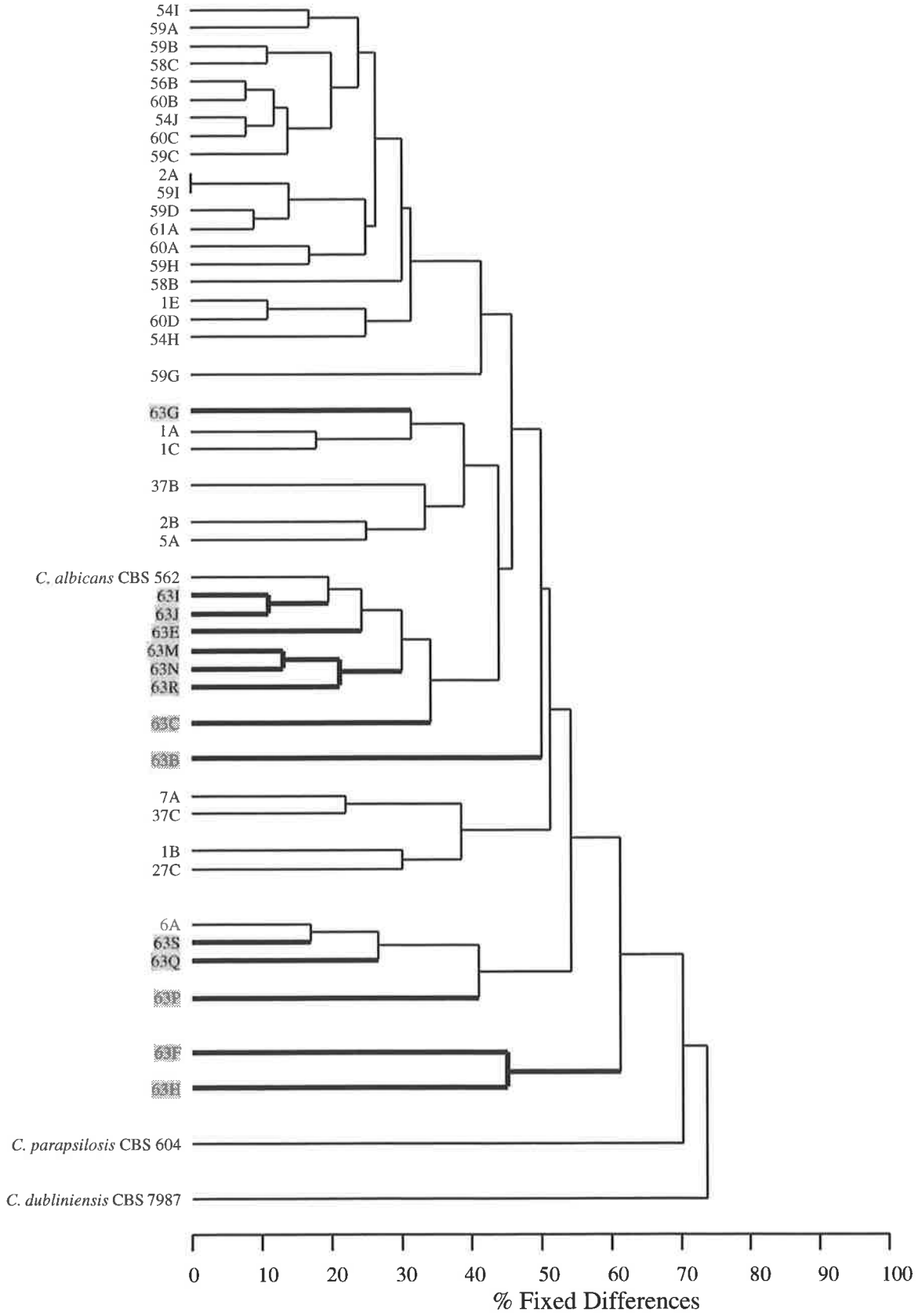


Figure 3.15b; Phenogram highlighting the genetic relationships between serial isolates obtained from patient 63 assessed in Study 9, as determined from an allozyme electrophoresis analysis using fifteen loci.

Table 3.38; Matrices of the percentage of fixed genetic difference between isolates from individual patients in study 9

Isolate	1A	1B	1C
1B	60		
1C	18	55	
1E	36	40	18

Isolate	2A
2B	14

Isolate	37B
37C	50

Isolate	54H	54I
54I	40	
54J	20	18

Isolate	58B
58C	29

Isolate	59A	59B	59D	59G	59H
59B	25				
59D	27	30			
59G	46	55	27		
59H	38	27	27	31	
59I	42	27	20	45	36

Isolate	60A	60B	60C
60B	17		
60C	8	8	
60D	18	8	17

Annotation as in Table 3.20 (p., 128).

Isolate	63B	63C	63E	63F	63G	63H	63I	63J	63M	63N	63P	63Q	63R
63C	78												
63E	56	30											
63F	71	50	60										
63G	38	44	36	60									
63H	82	55	50	45	55								
63I	50	33	18	50	18	45							
63J	43	29	22	33	22	45	11						
63M	50	38	22	44	44	30	22	13					
63N	43	25	40	30	40	45	30	30	13				
63P	63	57	67	63	67	80	67	67	63	56			
63Q	63	50	60	45	60	42	60	40	30	40	33		
63R	56	33	27	40	50	50	40	22	20	22	56	45	
63S	50	50	58	45	45	50	55	45	30	36	40	17	50

3.3.11.b Interpretation

Four of the forty-eight clinical isolates assessed were mixed or heterozygous. Thirty-eight of the remaining forty-four were within the *C. albicans* cluster with the other six isolates forming two distinct clusters of four and two isolates each, potentially belonging to other species of *Candida*. There were no clinical isolates that clustered with the *C. dubliniensis* or *C. parapsilosis* type strains.

The matrices in Table 3.38 (p., 180) are excerpts from the matrix of pairwise comparisons between isolates in this analysis contained within Appendix 3 (Table 14) and show the percentage of fixed genetic differences between isolates from the same patient. For example, isolates from patient 1 differed at 18 to 60% of loci. Isolates A, C and E were contained within the *C. albicans* cluster. Isolate B may represent a genetic extremity in the *C. albicans* species or another *Candida* species. All isolates were taken during candidiasis infection. The date isolate A was taken was not specified but was before the 9th of June 1995. Isolate B was obtained on the 9th of June, C on the 9th of November 1995 and E on the 18th of April 1996. Isolate 1A was a tongue swab, E was from a palate swab and B and C were from oral rinse samples. Each time the patient was sampled, colonization by a different *C. albicans* strain was evident. The original strain isolated from the oral hairy leukoplakia infection was not the same as that isolated from the later candidiasis infection. Isolate 1D, from an oral rinse obtained between isolates C and E, was mixed/ heterozygous.

For patient 2, isolates A and B differed at 14% of loci and represent different *C. albicans* strains based on their relative position to the Type strain. Isolate 2A was a buccal mucosal swab and 2B was an oral rinse sample, both taken when the patient had obvious candidiasis infection on an unknown date. Prior to sample collection, this patient had undergone a highly varied antifungal treatment protocol, firstly treated with itraconazole, then ketoconazole then amphotericin B, suggesting that the infection was clinically persistent and did not respond to the treatment administered. The candidiasis lesion in this patient was caused by a strain different to that isolated from the oral rinse.

Isolates 37B and C differed at 50% of the loci analysed, representing different *C. albicans* strains. Isolate B was from a tongue swab during oral hairy leukoplakia and C was an oral rinse sample both taken on the 5th of December 1995. Again, the isolate obtained from the oral rinse of this patient was not the one responsible for infection.

Isolates 54H, I and J differed at 18 to 40% of loci, representing different *C. albicans* strains. All of these isolates were from oral rinses of a patient taking part in the amphotericin B lozenge trial. The isolates were obtained on the 6th of September, the 4th of October and the 20th of December 1995, respectively. The patient was candidiasis positive when isolate J was taken. The predominant strain was constantly changing in this patient, with isolate J appearing to be clinically resistant to amphotericin B. Isolates obtained from previous samples of this patient, analysed in study 8, were all mixed/heterozygous. Perhaps the continued use of amphotericin B lozenges resulted in the selection of *C. albicans* strains with a reduced susceptibility to the antifungal.

The two isolates (B and C) from patient 58 differed at 29% of loci, representing different *C. albicans* strains. Isolate B was taken from a tongue swab on the 8th of June and C was from an oral rinse on the 4th of September 1995. Patient 58 was infected by a different strain to that isolated from their oral cavity after treatment during asymptomatic colonization.

Similarly, isolates from patient 59 had fixed genetic differences at 20 to 55% of loci with all isolates representing different *C. albicans* strains. Isolate A was collected on the 8th of June 1995, B, C and D on the 18th of January, G on the 14th of February, H on the 13th of March and I on the 8th of May 1996. Isolates A, D, G, H and I were oral rinse samples, B was a tongue swab and C was a hard palate swab during candidiasis. Isolate 59I was collected after this patient began participation in an amphotericin B lozenge trial perhaps suggesting that this strain has a decreased sensitivity to the antifungal. No single strain of *C. albicans* persisted in this patient. The candidiasis infection lesions were caused by different *C. albicans* strains and they also differed from the strains isolated by oral rinse before, during and after the infection. Isolates 59E and F, collected from an oral rinse and palate swab on the 25th of January 1996, were mixed/heterozygous.

Isolates 60A, B, C and D differed at 8 to 18% of loci representing different *C. albicans* strains. Isolate A was taken on the 9th of June 1995, B, C and D were taken on the 21st of March 1996. Isolate A was from a mouth swab, B from an oral rinse sample, C from a tongue swab and D from a palate swab. These isolates constitute closely related strains or perhaps substrains of *C. albicans*. The original infecting isolate did not persist in the patient's oral cavity and the candidiasis infection was caused by a number of different

strains. Each lesion was caused by a different strain, which differed from that isolated by oral rinse.

Numerous isolates were taken from patient 63 and most were assessed in this particular study. They had fixed genetic differences at 11 to 82% of loci. These isolates represent distinct strains or species, with isolates B, C, E, G, I, J, M, N and R belonging to *C. albicans*. Isolates Q and S formed a cluster and represent an undetermined species of *Candida*, while F and H formed a second cluster of an undetermined species. Furthermore, P constituted a third undetermined species of *Candida*. Due to the complexity of the epidemiology of this patient's isolates, a second phenogram with the relevant isolates highlighted is presented (Figure 3.15b p., 179) and the clinically-relevant details are summarised below.

- Oral rinse isolates B, M and N were collected during times of obvious candidiasis infection, R was taken during an angular chelitis infection. Isolates B, G, H, I, M, P, Q and S were from oral rinse samples; C was an oral swab, E a tongue swab, F a palate swab, J and oral swab, N a tongue edge swab and R another oral swab.
- Isolates B and C were collected on the 4th of July, E, F and G on the 30th of August, H on the 6th of September, I and J on the 20th of September, M on the 18th of October, N and P on the 25th of October, Q and R on the 15th of November and S on the 6th of December 1995.
- This patient's original oral rinse (B) and lesion swab (C) samples taken during candidiasis infection yielded different *C. albicans* strains. Approximately six weeks later, the patient's oral hairy leukoplakia infection was caused by a different *C. albicans* strain (E), the concurrent candidiasis infection was caused by a different *Candida* species (F), and the oral rinse yielded a second *C. albicans* strain (G). Within one week, all of these isolates were replaced by another strain (H) belonging the same *Candida* species as isolate F. Two weeks after, there were two different *C. albicans* strains present, one isolated from a lesion (I) and another in the oral rinse (J). Four weeks later, another different *C. albicans* strain was isolated from an oral rinse (M) and within another week there were two strains present, one *C. albicans* strain causing oral hairy leukoplakia (N) and a different *Candida* species from the oral rinse (P).
- This patient commenced participation in an amphotericin B lozenge trial prior to the collection of isolates N and P. Within three weeks another species of *Candida* was isolated from an oral rinse (Q) while another *C. albicans* strain was

obtained from an oral swab on the same day (R). The final oral rinse sample taken three weeks later (S) contained a different *Candida* strain of the species isolated in oral rinse (Q).

The use of amphotericin B may have contributed to the appearance of two alternate *Candida* species and the rapid replacement of strains observed in this patient.

Oral rinse isolate 59I was identical to lesion swab isolate 2A from patient 2. The same strain being obtained from two different HIV-positive people suggests that they may have shared a common source of infection or that *Candida* is a clonal organism. Alternatively, allozyme electrophoresis was not discriminatory enough to differentiate these closely related isolates. There were a number of unresolved loci between these two isolates in this study, which could have contributed to them appearing genetically identical.

3.3.12 Study 10

3.3.12.a Results

Isolates in study 10 (Table 3.39 p., 185-6) differed at 0 to 68.2% of the fourteen loci assessed (Table 3.40 p., 187-8), forming seven clusters differing at over 50% of loci (Figure 3.16 p., 189). Other than cluster 1, which contained most isolates assessed, the other six clusters comprised single isolates. Cluster 1 isolates varied at 0 to 38.9% of loci, and included only clinical isolates. The genetic relationships between isolates that differed at less than 33% of loci in cluster 1 were not reproducible with repeat analyses, as has been noted in previous analyses. Clusters 2, 3, 4 and 5 differed at 50.1%, 51.4%, 52.2% and 65.4% of loci, respectively, each cluster containing a single clinical isolate. Cluster 5 contained the *C. parapsilosis* type strain. Clusters 6 and 7 differed from each other at 57% of loci and from the other five clusters at 68.2% of loci. They contained the *C. albicans* and *C. dubliniensis* type strains, respectively.

Table 3.39a; Details of the fifty reference and clinical samples analysed in study 10 using allozyme electrophoresis

Isolate Code	Sex	HIV status	Site of Isolation	Candidiasis	Antifungal Resistance	Patient's Therapy	Other Notes
73A	M	+	Oral Rinse	?			
93B	M	+	Oral Rinse	?			sensitivity to amphotericin B requested
93C	M	+	Oral Rinse	?			
93D	M	+	Left Swab Floor of Mouth	Pseudomembranous candidiasis?			sensitivity to amphotericin B requested
93E	M	+	Right Floor of Mouth Swab	Pseudomembranous candidiasis?			sensitivity to amphotericin B requested
95A	M	+	Oral Rinse	?			
65A	M	+	Oral Rinse	OHL			
65B	M	+	Oral Swab	OHL			
88K	M	+	Oral Rinse	?			
96A	M	+	Cheek Swab	?			Sensitivity to fluconazole requested
97E	M	+	Oral Rinse	?			
71A	M	+	Oral Rinse	?			
84G	M	+	Oral Swab	?			Sensitivity to fluconazole, itraconazole, ketoconazole and amphotericin B requested
67A	M	+	Oral Rinse	?			
97D	M	+	Oral Swab angle of mouth				
66C	M	+	?	?			
93A	M	+	Tongue Swab	+			Pseudomembranous candidiasis, left tongue, lateral border, sensitivity to amphotericin B requested
74A	M	+	Oral Swab	?			
82A	M	+	Oral Rinse	?			
75A	M	+	Oral Rinse	?			
77A	M	+	Oral Swab	?			
88D	M	+	Oral Rinse	+			candidiasis, oral hairy leukoplakia
84E	M	+	Oral Rinse	?			
88J	M	+	Swab Buccal mucosal Left	?			Resistance to amphotericin B requested
76B	M	+	Oral Rinse	?			
88L	M	+	Oral Rinse	?			
89A	M	+	Oral Rinse	?			

Annotation as in Table 3.2 (p., 70).

Table 3.39b; Details of the fifty reference and clinical samples analysed in study 10 using allozyme electrophoresis

Isolate Code	Sex	HIV status	Site of Isolation	Candidiasis	Antifungal Resistance	Patient's Therapy	Other Notes
84F	M	+	Oral Rinse	?			Sensitivity to fluconazole, itraconazole, ketoconazole and amphotericin B requested
92A	M	+	Oral Rinse	?			
90A	M	+	Oral Rinse	?			
CBS 604			case of sprue, Puerto Rico				*C. parapsilosis* type strain
CBS 562			skin (interdigital mycosis), Uruguay				*C. albicans* type strain
CBS 7987		+	oral cavity, Ireland				*C. dubliniensis* type strain
68E	M	+	Oral Rinse	?		Amphotericin B topical therapy	Both cultures labelled 68E.1
68G	M	+	Oral Rinse	?			
68H	M	+	?	?			
68I	M	+	Oral Rinse	?			
69A	M	+	Oral Rinse	?			
72A	M	+	Oral Rinse	?			
77B	M	+	Oral Rinse	?			
78A	M	+	Oral Rinse	?			
79A	M	+	Swab - Floor of Mouth	?			
81A	M	+	Oral Rinse	?			two 81A.2 and 81A.3, no 81A.1 or 81A.4 - relabelled
83A	M	+	Oral Rinse	?			
84D	M	+	?	?		Fluconazole	
94A	M	+	Oral Rinse	?			
95B	M	+	?	?			
97A	M	+	Oral Rinse	?			
97B	M	+	Oral Swab corners of mouth	angular Chelitis			
97C	M	+	Oral Rinse	?			

Annotation as in Tables 3.2, 3.4 and 3.7 (p., 70, 78 and 85).

Table 3.40a; Allelic profiles of fifty isolates analysed in study 10 at fourteen enzyme loci

	Enzyme Locus													
Isolate Code	ALD	ENOL	EST	FUM	GDH	G6PD	GPI	HK1	HK2	NDPK	NP	PepA	PepB	PK
73A	d	-	-	-	b	c	-	e	f	b	-	c	b	c
93B	d	e	e	c	b	c	d	e	f	b	b	c	b	c
93C	d	e	e	c	b	c	d	e	f	b	b	c	b	-
93D	d	e	e	c	b	c	d	e	f	b	b	c	b	c
93E	d	e	e	c	b	c	d	e	f	b	b	c	b	c
95A	d	e	e	c	b	c	d	e	f	b	b	c	b	c
65A	d	e	e	-	-	c	c	e	f	b	b	-	b	c
65B	d	e	-	c	-	c	c	e	f	b	b	-	b	c
88K	d	e	e	c	b	c	c	e	f	b	b	c	b	c
96A	d	e	e	c	-	c	c	e	f	b	-	c	b	c
97E	d	e	-	c	b	c	c	e	f	b	b	-	b	c
71A	d	e	-	c	-	c	-	i	f	b	-	c	-	c
84G	d	e	-	c	b	c	d	e	f	b	b	c	c	c
67A	-	c	e	c	b	c	d	e	f	-	-	-	b	c
97D	b	-	e	c	b	c	c	e	f	b	b	-	b	c
66C	-	e	-	-	b	c	c	e	f	c	-	-	b	c
93A	d	e	e	c	b	c	c	e	f	b	b	c	d	a
74A	d	e	-	c	-	c	d	e	f	c	-	c	a	c
82A	d	e	e	-	b	c	-	i	f	b	-	a	f	c
75A	d	-	-	c	-	c	c	g	d	b	b	-	-	c
77A	d	e	-	c	-	c	-	g	d	b	b	-	-	c
88D	d	-	e	c	a	c	d	g	f	b	b	c	c	-
84E	d	e	e	c	b	c	c	b	b	b	-	c	c	d
88J	d	e	e	c	b	c	c	e	f	b	-	-	c	d
76B	d	f	e	-	-	-	c	e	b	b	-	c	-	c
88L	d	e	e	c	a	c	c	h	g	b	b	c	b	c
89A	d	e	-	b	a	c	c	h	a	-	b	c	b	-

Annotation as in Tables 3.3 and 3.5 (p., 71 and 79).

Table 3.40b; Allelic profiles of fifty isolates analysed in study 10 at fourteen enzyme loci

	Enzyme Locus													
Isolate Code	ALD	ENOL	EST	FUM	GDH	G6PD	GPI	HK1	HK2	NDPK	NP	PepA	PepB	PK
84F	d	f	f	-	b	c	d	g	f	-	-	-	c	-
92A	d	e	c	b	-	b	-	e	f	a	b	c	b	a
90A	d	e	c	c	b	b	c	g	g	b	-	b	d	c
CBS 604	c	b	d	-	b	b	c	e	f	-	-	-	c	-
CBS 562	-	e	e	a	-	c	d	f	g	-	-	a	c	c
CBS 7987	b	d	a	-	a	c	-	i	h	d	-	-	c	c
68E	d	e	e	c	b	c	cd	de	f	b	b	-	b	c
68G	d	e	e	c	b	-	d	e	dg	b	b	c	b	c
68H	cd	e	e	c	-	bc	-	g	f	b	b	c	b	c
68I	d	e	e	c	b	c	cd	g	f	b	b	c	b	c
69A	d	e	-	c	b	c	d	de	f	b	-	c	-	b
72A	d	e	e	c	-	bc	c	e	f	b	b	c	b	c
77B	d	e	-	c	b	c	c	d	dg	b	d	c	c	c
78A	d	ae	b	b	b	bc	a	g	d	b	ac	bc	e	-
79A	d	e	-	c	b	bc	a	g	d	b	b	c	c	c
81A	d	e	-	c	d	bc	d	e	e	b	-	c	b	c
83A	a	e	e	-	c	a	c	ac	ce	c	-	-	c	d
84D	c	e	e	c	c	c	c	b	be	b	b	c	c	d
94A	d	e	e	c	b	bc	c	e	f	b	b	c	b	d
95B	d	e	e	c	b	bc	c	e	f	b	a	-	b	c
97A	d	e	e	c	b	c	c	d	f	b	ab	c	b	c
97B	d	e	e	c	b	bc	c	e	f	-	ab	c	b	c
97C	d	e	e	c	-	bc	c	e	f	-	-	-	b	c

Annotation as in Tables 3.3, 3.5 and 3.8 (p., 71, 79 and 86).

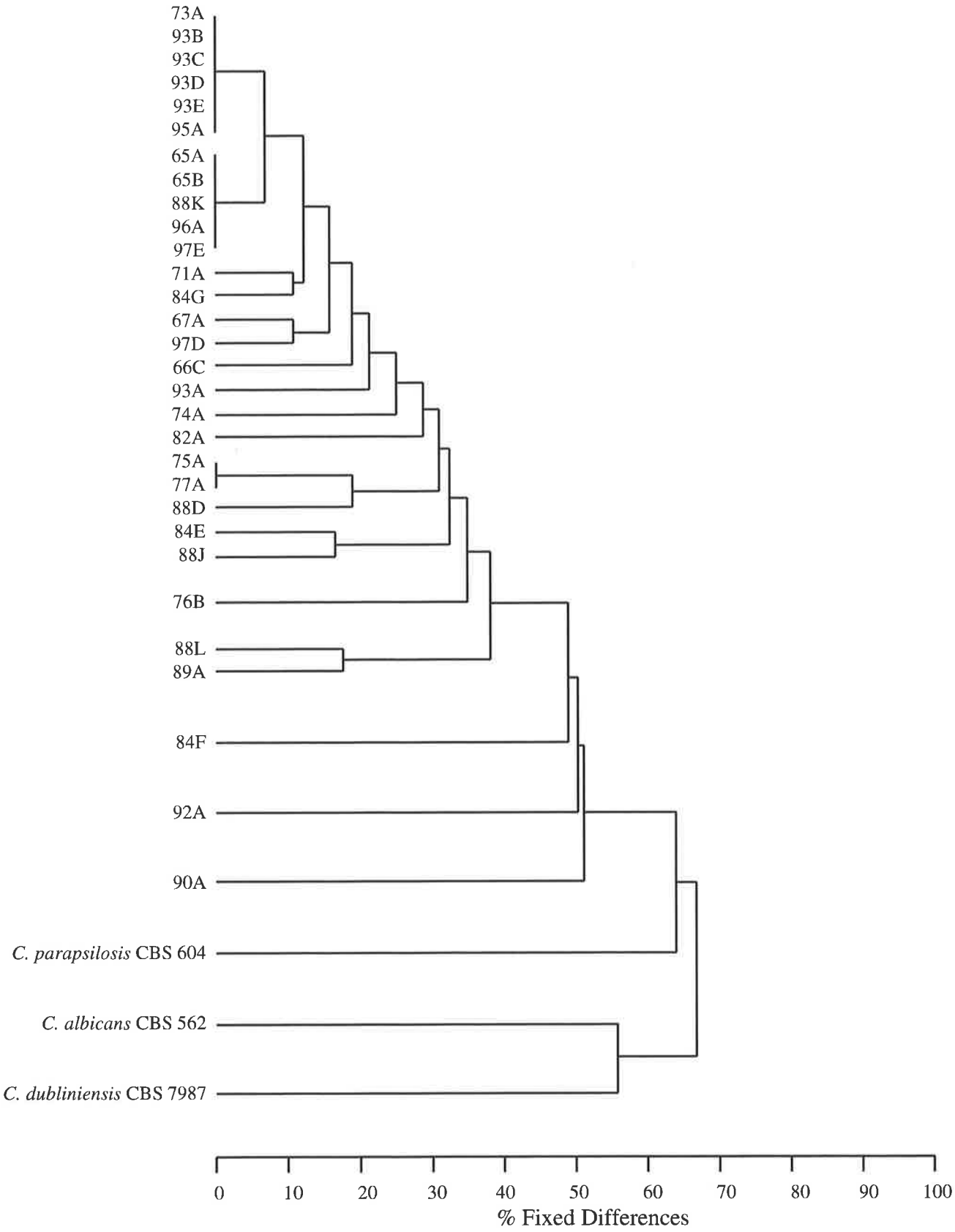


Figure 3.16; Phenogram depicting the genetic relationships between thirty-three isolates of *Candida* used in Study 10, as determined from an allozyme electrophoresis analysis using fourteen loci.

Table 3.41; Matrix of the percentage of fixed genetic difference between pairs of isolates from 5 individual patients in study 10

Isolate	65A	65B	84E	84F	84G	88D	88J	88K	88L	93A	93B	93C	93D	93E	97D
65B	**0**														
84E	40	40													
84F	63	57	**56**												
84G	20	18	**33**	**25**											
88D	33	33	36	25	18										
88J	20	20	17	44	18	**30**									
88K	0	0	31	56	15	**33**	**17**								
88L	18	18	38	78	38	**33**	**42**	**21**							
93A	18	18	31	56	23	33	17	14	36						
93B	9	9	38	44	8	25	25	7	29	**21**					
93C	10	10	33	44	8	25	18	8	31	**15**	**0**				
93D	9	9	38	44	8	25	25	7	29	**21**	**0**	**0**			
93E	9	9	38	44	8	25	25	7	29	**21**	**0**	**0**	**0**		
97D	10	10	45	63	27	45	27	8	33	25	17	18	17	17	
97E	0	0	36	50	17	40	18	0	25	17	8	9	8	8	**9**

Annotation as in Table 3.20 (p., 128).

3.3.12.b Interpretation

Seventeen of the forty-seven clinical isolates assessed were mixed or heterozygous. Twenty-seven of the remaining thirty were placed within the *C. albicans* cluster, although the *C. albicans* type strain was external to these isolates. The inclusion of isolate 71A in the first cluster, which had been analysed in clinical studies 1 and 2 previously (Sections 3.3.3 and 3.3.4 p., 111 and 118) and in both had clustered with the *C. albicans* type and reference strains, provided further evidence that this cluster represented *C. albicans*. This was also the case for isolate 88D, which was within the *C. albicans* cluster in clinical studies 2 and 4 (Sections 3.3.4 and 3.3.6 p., 118 and 130). Subsequent analysis of the species identity of the *C. albicans* type strain using CHROMagar confirmed that it had become contaminated with another species of *Candida*. The three remaining clinical isolates formed unique clusters of undetermined *Candida* species identity.

Table 3.41 (p., 190) contains excerpts from the matrix of pairwise comparisons for this analysis (Appendix 3, Table 15). This data is used below in interpreting the genetic variation between isolates obtained from a single patient.

Isolates 65A and B were genetically identical *C. albicans* strains obtained from rinse and lesions swabs during OHL. They also shared identity with isolates 88K (rinse), 96A (swab) and 97E (rinse), again suggesting that the technique was not discriminatory enough to distinguish these isolates or that *C. albicans* is clonal. Both isolates from patient 65 were taken during a candidiasis infection on the 13th of July 1995. This result suggests that the infecting strain predominated in this patient's oral cavity at the time of sample collection.

Patient 68 took part in an amphotericin B lozenge trial during the time that mixed/ heterozygous isolates D to G were collected. This analysis confirms the appearance of a mixed/heterozygous colonization in this patient noted in study 3, and suggests that this colonization continued after the patient finished participation in the lozenge trial.

Isolates 84E, F and G differed at 25 to 56% of loci with E and G within the *C. albicans* cluster and F external to the majority of isolates, possibly representing a genetic extreme of the species. Isolates E and F were from oral rinse samples and G was from an oral swab. Isolate E was taken on the 26th of June and F and G were taken on the 3rd of July 1996. The strain carried by this patient changed within one week and the infection-causing strain was not the same as that isolated from the oral rinse. This patient was probably colonized by

more than one strain during the week between sample collections. Isolate 84D was a mixed/heterozygous isolate collected on the 21st of June 1996 from an unspecified oral source. Patient 84 participated in an amphotericin B lozenge trial when isolates D to G were obtained. This trial could have led to a mixed infection and the rapid strain replacement noted.

For patient 88, the multiple isolates collected had fixed genetic differences at 17 to 42% of loci. They represent different *C. albicans* strains. Isolates 88D, K and L were obtained from oral rinse samples and J was from a buccal mucosal swab. The patient had candidiasis and oral hairy leukoplakia when isolate D was taken on the 10th of April 1996. Isolates J and K were taken on the 26th of June and isolate L was taken on the 24th of July 1996. During this patient's second infection, the strain isolated from the oral rinse was not the same as that isolated from the lesion. Additionally, the first and second infections were caused by different *C. albicans* strains.

Isolates 93 B, C, D and E were genetically identical but differed to isolate 93A at 15 to 21% of the loci. This percentage varied due to differences in resolution of some loci between individual isolates. All of these isolates were within the *C. albicans* cluster, representing two different strains. Isolate A was collected on the 3rd of April, B on the 17th of April, C, D and E on the 24th of April 1996. Isolate 93A was derived from a tongue swab, B and C were from oral rinses and D and E were from swabs of the floor of the patient's mouth. The original strain causing the oral hairy leukoplakia infection in this patient was not maintained, but was replaced by a strain that was isolated from the following oral rinse and the later candidiasis infection and oral rinse sample. The second strain from this patient was probably resistant to the treatment used for the first oral hairy leukoplakia infection. This patient participated in an amphotericin B lozenge trial when isolates A and B were collected. The lozenge use may have selected for the strain isolated from the ensuing candidiasis and oral hairy leukoplakia infections. The genetically identical isolates from patient 93 were also identical to rinse isolates 73A and 95A. This result could be due to an insufficient number of enzyme loci being compared in this study or, as was suggested in study 9, provides further evidence that *C. albicans* is a clonal organism.

Isolates 97D and E differed at 9% of loci and represent different *C. albicans* strains. Isolate D was from a swab of an angular chelitis lesion on the 26th of June 1996. Isolate E was taken from an oral rinse on the 24th of July 1996. These isolates may represent a single

strain with minor genetic variation. Isolates 97A, B and C were mixed/ heterozygous being derived from an oral rinse on the 17th of June, an angular chelitis swab and another oral rinse on the 19th of June 1996. This patient participated in an amphotericin B lozenge trial when isolates B to E were collected. This appears to have resulted in the selection of the *C. albicans* strains present when isolates D and E were obtained.

3.3.13 Study 11

3.3.13.a Results

Isolates in study 11 (Table 3.42 p., 194) having fixed genetic differences at 0 to 78.6% of the seventeen loci assessed (Table 3.43 p., 195), forming six distinct clusters with fixed differences at equal to or more than 50% of loci (Figure 3.17 p., 196). The first cluster contained the majority of isolates, with fixed differences between them at 0 to 49.9% of loci. These isolates differed from cluster 2, which contained a single clinical isolate and the *C. dubliniensis* type strain, at 60.8% of loci, and from cluster 3, the *C. albicans* type strain, at 62.6% of loci. Cluster 4 contained the *C. parapsilosis* type strain, which differed at 75.7% of loci and clusters 5 and 6 differed from each other at 64% of loci and from the other clusters at 78.6% of loci.

Table 3.42; Details of the thirty reference and clinical samples analysed in study 11 using allozyme electrophoresis

Isolate Code	Sex	HIV status	Site of Isolation	Candidiasis	Antifungal Resistance	Patient's Therapy	Other Notes
S17	F	-	Undiluted saliva	carrier			
C6	M	-	Undiluted saliva	carrier			
C3	M	-	Undiluted saliva	carrier			
S11	F	-	Undiluted saliva	carrier			
S15	M	-	Undiluted saliva	carrier			
C15	M	-	Undiluted saliva	carrier			
S5	M	-	Undiluted saliva	carrier			
C12	M	-	Undiluted saliva	carrier			
S9	F	-	Undiluted saliva	carrier			
S13	M	-	Undiluted saliva	carrier			
99A	M	+	Oral Rinse	?			
S2	M	-	Undiluted saliva	carrier			
S20	F	-	Undiluted saliva	carrier			
C1B.4, 6	M	-	Undiluted saliva	carrier			
100A	M	+	Oral Rinse	?			
CBS 7987		+	oral cavity, Ireland				*C. dubliniensis* type strain
CBS 562			skin (interdigital mycosis), Uruguay				*C. albicans* type strain
CBS 604			case of sprue, Puerto Rico				*C. parapsilosis* type strain
S25	M	-	Undiluted saliva	carrier			
C16	M	-	Undiluted saliva	carrier			
98A	M	+	Oral Rinse	?			*Candida* species, Sensitivity to fluconazole requested
101A	M	+	Oral Rinse	?			Sensitivity to amphotericin B requested
101B	M	+	?	?			
S3	F	-	Undiluted saliva	carrier			
S4	F	-	Undiluted saliva	carrier			
S14	F	-	Undiluted saliva	carrier			
S18	M	-	Undiluted saliva	carrier			
S22	M	-	Undiluted saliva	carrier			
C1B	M	-	Undiluted saliva	carrier			
C8	M	-	Undiluted saliva	carrier			

Annotation as in Tables 3.2, 3.4 and 3.7 (p., 70, 78 and 85).

Table 3.43; Allelic profiles of thirty isolates analysed in study 11 at seventeen enzyme loci

	Enzyme Locus																
Isolate Code	ALD	ENOL	EST	FDP	FUM	GDH	G6PD	GPI	HK1	HK2	NDPK	NP	PepA	PepB	PGAM	PGM	PK
S17	c	-	c	d	d	-	c	a	e	c	c	a	c	a	d	a	d
C6	-	c	c	d	-	-	c	a	e	c	c	-	c	a	d	-	d
C3	c	-	c	d	d	-	c	a	e	c	c	a	c	a	d	b	-
S11	c	c	-	d	d	-	c	a	f	c	c	a	c	a	d	a	d
S15	-	d	c	d	-	d	c	a	a	c	c	a	c	a	d	a	d
C15	c	c	c	d	d	b	c	a	c	c	d	-	c	a	-	-	d
S5	-	c	c	d	-	-	c	a	c	c	b	a	c	a	d	-	-
C12	c	c	c	-	a	e	c	a	c	c	c	a	c	a	d	a	d
S9	c	c	c	-	d	-	b	a	c	c	c	-	c	a	d	b	d
S13	d	c	c	d	d	-	c	a	c	b	c	a	c	a	c	-	-
99A	c	c	c	-	d	c	c	a	c	c	c	-	b	a	c	a	d
S2	d	c	c	c	d	-	c	a	c	c	a	-	c	b	d	-	d
S20	c	-	a	d	d	-	b	a	c	d	c	a	c	a	d	a	a
C1B.4, 6	-	e	b	-	-	e	c	a	e	c	c	b	b	a	d	a	b
100A	c	b	a	-	b	c	b	a	c	c	b	-	-	b	d	a	d
CBS 7987	b	b	a	-	b	-	b	a	c	c	e	-	-	d	c	a	d
CBS 562	a	c	c	-	b	d	c	a	d	d	e	a	b	d	c	a	-
CBS 604	e	a	c	c	-	b	a	a	g	b	a	-	-	b	d	b	c
S25	a	b	-	b	d	e	b	b	c	e	c	-	a	a	a	b	e
C16	d	b	a	a	-	c	d	b	a	a	c	-	-	a	-	-	c
98A	c	c	c	-	d	e	c	a	c	c	c	-	ac	a	d	a	d
101A	c	c	c	-	-	b	c	a	c	c	c	-	d	b	c	ab	f
101B	c	c	c	-	e	-	b	a	c	c	ac	a	-	b	d	ab	d
S3	-	-	c	c	bd	-	c	a	b	d	-	-	b	b	c	a	d
S4	d	c	a	-	d	ab	b	a	c	c	c	-	-	c	d	-	a
S14	c	cd	c	d	cd	c	c	c	a	c	c	a	c	a	d	ab	d
S18	c	c	c	d	c	b	b	b	d	c	ac	a	c	a	b	ab	d
S22	c	c	c	d	d	c	b	a	c	c	ac	-	b	a	d	b	d
C1B	-	c	c	d	-	-	c	b	c	c	ac	-	b	a	-	b	d
C8	a	-	c	d	d	e	c	a	e	c	c	b	ac	a	d	b	-

Annotation as in Tables 3.3, 3.5 and 3.8 (p., 71, 79 and 86).

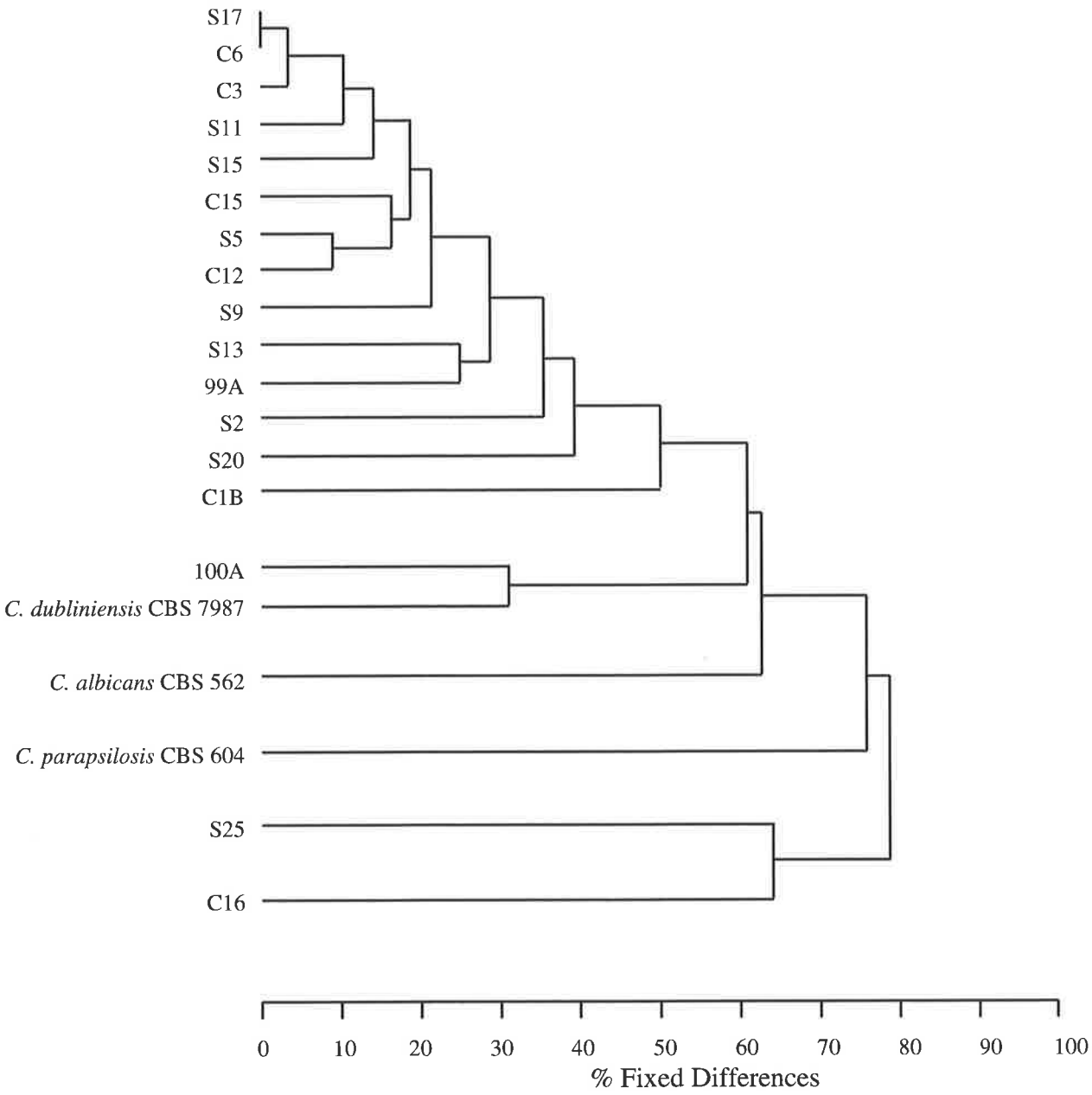


Figure 3.17; Phenogram depicting the genetic relationships between twenty isolates of *Candida* used in Study 11, as determined from an allozyme electrophoresis analysis using seventeen loci.

3.3.13.b Interpretation

The isolates within this analysis were predominantly obtained from HIV-negative oral asymptomatic carriers. As in study 10, the *C. albicans* type strain was positioned in the phenogram externally to the majority of isolates examined. It was determined, using CHROMagar, that the type strain had become contaminated with another species of *Candida*, possibly *C. tropicalis* as indicated by the presence of blue colonies.

Ten of the twenty-seven carrier and clinical isolates assessed were mixed or heterozygous. This included two clinical isolates from patient 101, who took part in an amphotericin B lozenge trial. Fourteen of the remaining seventeen were placed within the *C. albicans* cluster, one within in the *C. dubliniensis* cluster, while two were genetically unique and represent *Candida* species of undetermined identity. There was no segregation of isolates from male versus female patients, or from infection versus asymptomatic carriage, or from HIV-positive versus HIV-negative patients. As was suggested from earlier data, there is no genetic subset of *C. albicans* organisms that appear to be responsible for oral infection. This suggests that the infection by *C. albicans* is opportunistic.

Isolates S17 and C6 obtained from two unrelated asymptomatic carriers were genetically indistinguishable on the basis of the data presented in this analysis, suggesting that allozyme electrophoresis was not discriminatory enough to distinguish these two isolates, or provides further evidence that *C. albicans* is clonal.

The electrophoretic data clearly demonstrate that the predominant *Candida* species in both infection and asymptomatic carriage is *C. albicans*. The level of genetic variation between isolates obtained from either asymptomatic carriage or infection is similar suggesting that both states arise from random colonization and that there are no genetic subsets with increased virulence.

3.3.14General Results and Interpretation

The analyses in Section 3.3 comprise an epidemiological investigation of oral *Candida* colonization and infection. The complexity associated with attempting to combine the results from all analyses into a single phenogram prevents this. However, generic clusters

may be compared ie., isolates that cluster with the type strains in one study are probably closely related to isolates that cluster with the same type strain in another study. Therefore, the general results focus on clusters of isolates associated with type and reference strains.

A range of genetic diversity within defined species of *Candida* was observed but there was a predominance of *C. albicans* isolates (221 of 407 isolates assessed) in the electrophoretic analyses. The data presented in Section 3.3 indicate that genetically identical isolates can return during recurrent infections and resistance to treatment can be the result of superinfection with a different strain with a higher MIC or the same strain adapting. Additionally, it appears that the type of infection with which a patient presents is not strain-mediated because symptoms were not confined to genetically distinct subgroups of strains.

A number of clinical samples were composed of more than one type of *Candida* ie., mixed infection (120 of 407). In addition to *C. albicans* the other species that were identified in clinical samples included *C. dubliniensis* (the most predominant representing 20 of 407), a single *C. parapsilosis* isolate and a single *T. beigelii* isolate. Forty-four of the four-hundred and seven clinical isolates of *Candida* were genetically distinct from the type and reference strains in the genus used in the analyses, hence their species designation could not be established. A selection of isolates, not associated with type or reference strains or that represent the extremes of genetic variation within a cluster, were assessed in the following molecular analyses (Section 3.4 below).

3.4 Summary of allozyme electrophoresis studies

Following are the summarized results from Sections 3.1, 3.2 and 3.3. The results are generalised and trends and consistencies in the data across experiments are raised.

In general, the technique of allozyme electrophoresis can be used to discriminate between different strains and species in the genus *Candida*. The species identity of isolates can be confirmed on the basis of their allelic profile, using a panel of at least fifteen metabolic enzyme loci.

The type strain for *C. parapsilosis*, CBS604, was heterozygous for NDPK in most analyses except taxonomic study 2 and study 11, where it had a single band of activity. This strain was heterozygous for FUM in all previous analyses except taxonomic study 2, where it also had a single band. The data in taxonomic study 2 and study 11 appear to have not been as clearly resolved for CBS604, probably due to a decrease in enzyme concentration in the lysate used for analysis.

The epidemiological information gained from this study suggests that the oral manifestations of *Candida* infection of HIV-positive individuals are opportunistic and the clinical symptomology is a result of the individual's immune status. This conclusion can be drawn from the lack of evidence for genetic distinction between isolates obtained from oral infections in these patients and isolates obtained during periods of asymptomatic carriage. Additionally, there is no evidence for genetic distinction between isolates obtained from HIV-positive individuals and healthy asymptomatic carriers.

There are a number of cases of oral infection caused by more than one genetically distinct *Candida* strain, the superinfection of a patient with a new strain during the course of infection, perhaps in response to antifungal therapy, and evidence of the re-emergence of a strain over time. The infection of the oral cavity by species of *Candida* appears to be dynamic and involves the interaction of a number of diverse strains and species of *Candida*. The misuse of commonly prescribed antifungals, such as fluconazole and amphotericin B will increase the predominance of *Candida* species such as *C. lusitaniae* and *C. glabrata*, which have innate resistance to them. Additionally, transfer of resistance genes in mixed infections may lead to a number of susceptible species also becoming resistant to these treatments.

The taxonomic framework of the genus *Candida* is questionable on the basis of the genetic relationships identified between medically important *Candida* species and *S. cerevisiae*. These two genera do not form distinct genetic groups and their taxonomic distinction should therefore be reinvestigated.

3.5 *Phylogenetic analyses using 18S ribosomal RNA*

3.5.1 General Background

Comparisons of the small subunit ribosomal RNA gene sequence have been the basis of a number of systematic investigations in a range of taxa from all kingdoms of life (see Gurtler and Mayall 2001 for a discussion on the utility of current molecular methods for determining bacterial systematics). However, as has been mentioned in the introduction, the results obtained on the basis of this single gene sequence can conflict with traditional evolutionary relationships. Current sequence comparisons have questioned the accuracy of the existing phenotypically defined taxonomic structure not only of *Candida*, but of all fungi.

The following Section encompasses a comparison of these sequences between medically relevant *Candida* species, across genera, between the teleomorphs and anamorphs of species and across phyla, as have been investigated in previous Sections (eg., Section 3.2 p., 93). Previous analyses of these sequences have not included the range of medically relevant species investigated herein, nor have they analysed the phylogenetic relationships (Hendriks *et al.* 1991, Barns *et al.* 1991). Sequence alignments, phylograms obtained from the alignment of each sequence and bootstrap values for branches in the phylogram are presented within each relevant subsection. Bootstrap values in this study were determined by the percentage of times a particular branching pattern is obtained when the same data set is analysed five-hundred times using maximum likelihood analyses. When a bootstrap value exceeds 75, the branch is deemed to have strong statistical support.

3.5.2 Phylogenetic evaluation using the complete 18S rRNA sequence

The **aims** were;

- *to sequence the entire 18S rRNA gene for the C. albicans type strain, CBS 562 and compare this sequence with other relevant sequences already published on Genbank; and*
- *to analyse the phylogenetic relationships between medically relevant Candida species and related genera.*

3.5.2.a Materials and methods

A set of six primers was designed from published GenBank sequences to allow PCR amplification of the entire *Candida* 18S rRNA gene (refer to Materials and Methods Chapter 2 p., 47). The *C. albicans* type strain, CBS 562, was sequenced in its entirety and deposited on Genbank (accession number AF114470) then compared with other relevant Genbank sequences (Table 3.45 p., 202).

3.5.2.b Results

The sequence obtained for the *C. albicans* type strain CBS 562 (AF114470 Table 3.46 p., 203) aligned with 100% similarity to other Genbank *C. albicans* sequences, confirming its identity. Multiple alignments for all sequences analysed are within Appendix 3 (Table 17). The sequences were all homologous at 1055 of 1665 bases compared. The phylogenetic relationships were derived using maximum likelihood, parsimony and neighbour-joining analyses (Figure 3.18a p., 204). Bootstrap values for the branches ranged from 55 to 100% (Figure 3.18b p., 205). Ascomycetes and basidiomycetes formed two distinct lineages and the anamorphs and teleomorphs of *Candida* species were within a single clade. There are four major branches within the ascomycetes. *C. lusitaniae* and *C. krusei* formed distinct lineages, *C. kefyr*, *C. glabrata* and *S. cerevisiae* were within a single clade, as were *C. albicans*, *C. dubliniensis*, *C. viswanathii*, *C. tropicalis*, *C. guilliermondii* and *C. parapsilosis*.

Table 3.45; Details of the sequences used for an 18S rRNA gene alignment

Species	Sequence[a]	Genbank accession number	Strain[b]
C. kefyr	CkeM60303	M60303	ATCC 4135
T. glabrata	TgM60311	M60311	ATCC 2001
C. viswanathii	CvM60309	M60309	ATCC 22981
C. guilliermondii	CgM60304	M60304	ATCC 6260
C. parapsilosis	CpM60307	M60307	ATCC 22019
C. tropicalis	CtM60308	M60308	ATCC 750
C. krusei	CkrM60305	M60305	ATCC 6258
C. krusei	CkrM55528	M55528	MUCL 28949
C. lusitaniae	ClM55526	M55526	MUCL 29855
C. lusitaniae	ClM60306	M60306	ATCC 42720
C. albicans	CaM60302	M60302	ATCC 18804
C. albicans	CaAJ005123	AJ005123	Clinical[c]
C. albicans[d]	**CaAF114470**	**AF114470**	**CBS 562**
C. dubliniensis	CdX99399	X99399	CD 36
C. tropicalis	CtM55527	M55527	MUCL 30002
D. hansenii	DhX62649	X62649	NRRL Y-7426
D. hansenii	DhX58053	X58053	MUCL 28926
S. cerevisiae	ScV01335	V01335	?[e]
S. cerevisiae	ScJ01353	J01353	?
C. glabrata	CgX51831	X51831	CBS 138
K. marxianus	KmX89522	X89522	CBS 5671
K. marxianus	KmX89524	X89524	NCYC 970
K. marxianus	KmX89523	X89523	CBS 712
Cr. neoformans	CnL05428	L05428	ATCC 24067
F. neoformans	FnX60183	X60183	MUCL 30453
Cr. neoformans	CnL05427	L05427	ATCC 24065
Cr. neoformans	CnM55625	M55625	?
T. beigelii	TcX60182	X60182	MUCL 30308

a; Sequence refers to the code used for sequence analysis in this analysis and corresponds with the sequences used in the following tables and figures in this Section.
b; Strain refers to the isolate from which the sequence was obtained.
c; Clinical refers to a clinical strain not lodged in a culture collection.
d; The emboldened strain was sequenced in this thesis.
e; ? strain refers to the sequence being obtained from an unspecified strain.

Table 3.46; Complete 18S rRNA sequence for the *C. albicans* type strain CBS562 (Genbank accession number AF114470)

```
Ca CBS562   ----------------------------------------------------TCAGTTA[a]
            TCGTTTATTCATAGTACCTT-ACTACTTGG-ATAACCGTGGTAATTCTAGAGCTAATACA
            TGCTTAAAATCCCGACTGTTTGGAAGGGATGTATTTATTAGATAAAAAATCAATGCCTTC
            --GGGCTCTTTGATGATTCATAATAACTTTTCGAATCGCATGGCCTTGTGCTGGCGATGG
            TTCATTCAAATTTCTGCCCTATCAACTTTCGATGGTAGGATAGTGGCCTACCATGGTTTC
            AACGGGTAACGGGGAATAAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCAC
            ATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCCGACACGGGGAGGTAGTGACAA
            TAAATAACGATACAGGGCCCTTTTGGGTCTTGTAATTGGAATGAGTACAATGTAAATACC
            TTAACGAGGAACAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCA
            AAAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGAACCTTGGGCTTGG-CT
            GGCCGGTCCATCTTTTTG-ATGCGTACTGGAC--CCAGCCGAGCCTTTCCTTCTGGGTAG
            CCATT----------------TA-TGGCGAACCAGGACTTTTACTTTGAAAAAATTAGA
            GTGTTCAAAGCAGGCC-TTTGCTCGAATATATTAGCATGGAATAATAGAATAGGACGTTA
            TGGTTCTATTTTGTTGGTTTCTAGGACCATCGTAATGATTAATAGGGAC-GGTCGGGGGT
            ATCAGTATTCAGTTGTCAGAGGTGAAATTCTTGGATTTACTGAAGACTAACTACTGCGAA
            AGCATTTACCAAGGACGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGATGATC
            AGATACCGTCGTAGTCTTAACCATAAACTATGCCGACTAGGGATCGGTTGTTGTTCTTTT
            ATT-GACGCAATCGGCACCTTACGAGAAATCAAAGTCTTTGGGTTCTGGGGGGAGTATGG
            TCGCAAGGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGCGG
            CTTAATTTGACTCAACACGGGGAAACTCACCAGGTCCAGACACAATAAGGATTGACAGAT
            TGAGAGCTCTTTCTTGATTTTGTGGGTGGTGGTGCATGGCCGTT-CTTAGTTGGTGGAGT
            GATTTGTCTGCTTAATTGCGATAACGAACGAGACCTTAACCTACTAAATAGTGCTGCTAG
            CATT-TGCTGGTATAGTCACTTCTTAGAGGGACTATCGACTTCAAGTCGATGGAAGTTTG
            AGGCAATAACAGGTCTGTGATGCCCTTAGA-CGTTCTGGGCCGCACGCGCGCTACACTGA
            CGGAGCCAGCGAGTATAA--GCCTTGGCCGAGAGGTCTGGGAAATCTTGTGAAACTCCGT
            CGTGCTGGGGATAGAGCATTGTAATTGTTGCTCTTCAACGAGGAATTCCTAGTAAGCGCA
            AGTCATCAGCTTGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTACTAC
            CGATTGAATGGCTTAGTGAGGCCTCCGGATTGGTTTAGGAAAGGGGGCAACTNCATTCTG
            GAACCGAGAAGCTGGTCAAACTTGGTCATTTAGAGGAA----------------------
```

[a] Highlighted nucleotides indicate regions of homology between all aligned fungal 18S rRNA genes presented in Appendix 3 (Table 17).

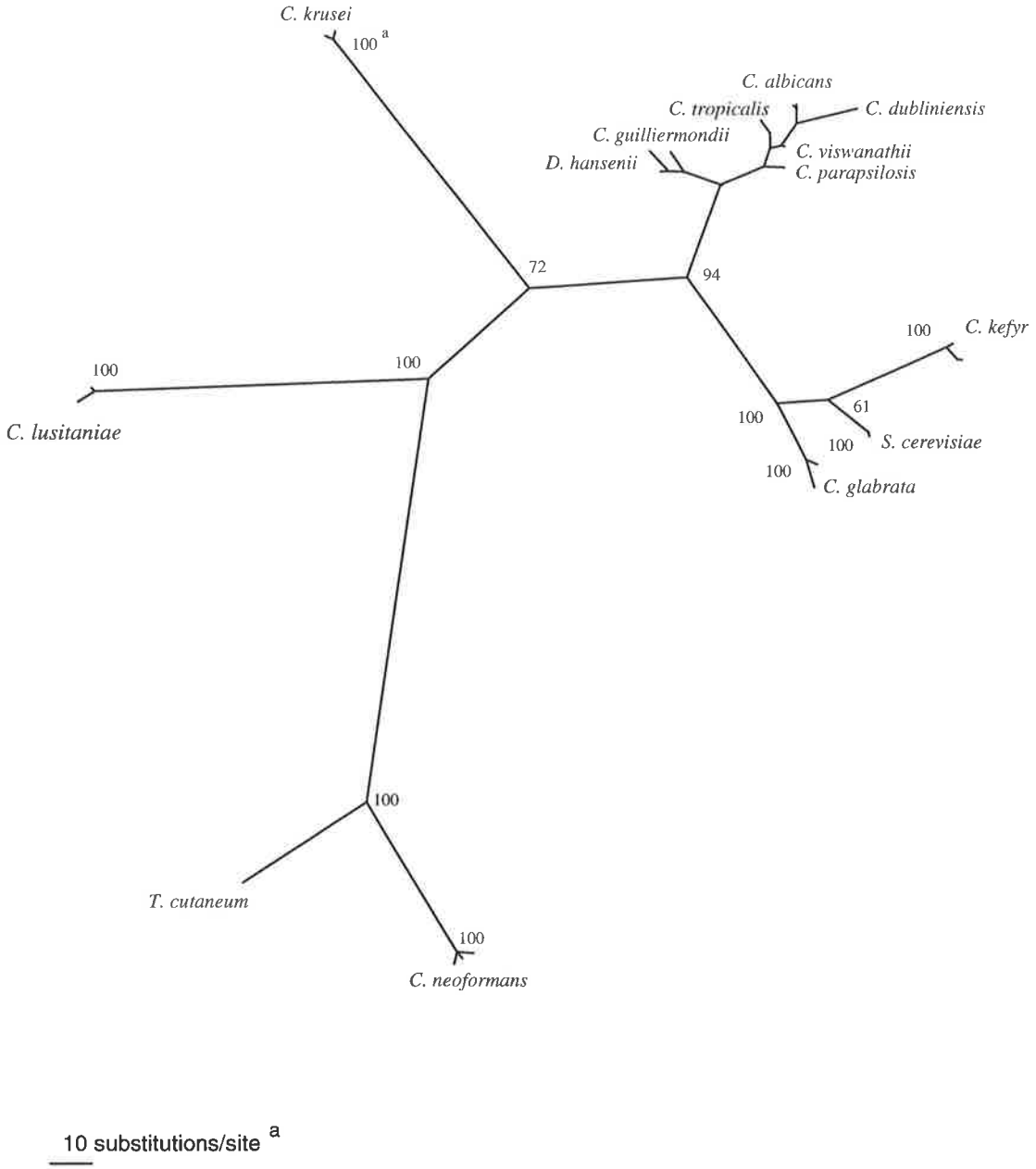


Figure 3.18a; Unrooted consensus tree of the phylogenetic relationships between medically relevant *Candida* species and related genera based on 18S rRNA gene sequences

a; the numbers at each branch indicate the bootstrap values for that branch
b; the scale bar indicates the branch length that corresponds to 10 base differences (substitutions) between two sequences

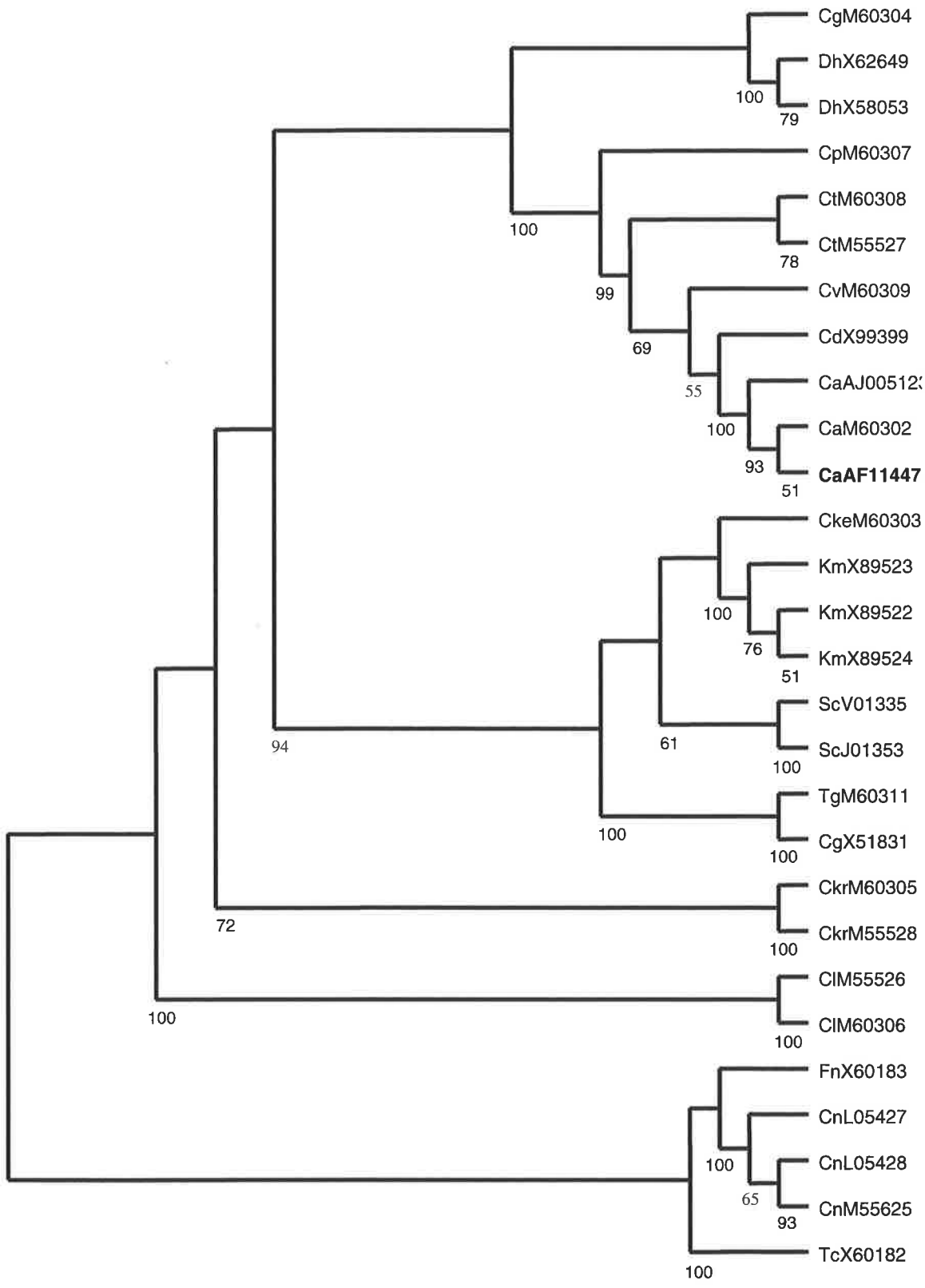


Figure 3.18b; Bootstrap values obtained by maximum likelihood analyses of five-hundred trees for branches in Figure 3.18a (p., 204).

Bootstrap values (denoted under each branch) exceeding 75 indicate that the branch has strong statistical support.

3.5.2.c Interpretation

The phylogenetic structure obtained using all of the available sequences is not in agreement with the phenelic results obtained using allozyme electrophoresis (Section 3.2 p., 93). However, this is not unexpected because each of these techniques discriminates at a different taxonomic level, allozyme electrophoresis provides a conservative estimation of genetic diversity and it is based on net charge differences in metabolic enzymes whereas 18S rRNA gene sequencing is a sequence comparison of a single gene.

By definition, species within a genus are genetically closely related, but in this case *S. cerevisiae* is in the same clade as *Candida*, questioning the current taxonomy. The basidiomycetes form a distinct lineage but anamorphs and teleomorphs of *Candida* species are genetically within a single clade together. There is strong bootstrap support for the separation of ascomycetes and basidiomycetes and for the *C. lusitaniae* branch within the ascomycetes (Figure 3.18b p., 205). There is low support for the branching of *C. krusei* from the remaining two *Candida* branches, but the support for the latter branches is high. Within the clade containing *S. cerevisiae*, there is strong support for the *C. glabrata* branch but decreased support for the association of *S. cerevisiae* with *C. kefyr*. Within the clade containing *C. albicans*, there is strong support for most of the branches except the separation of the *C. tropicalis* and *C. viswanathii* branches. The high overall bootstrap values for the maximum likelihood analysis using these sequences suggest that it is phylogenetically informative, although it again questions the validity of the genus *Candida*, as did the allozyme data in the previous Section.

3.5.3 Identification of a 490-bp species-diagnostic sequence

3.5.3.a Background

Although the accuracy of ribosomal RNA small subunit sequence has been questioned, its utility in phylogenetics has been demonstrated for many bacterial and fungal genera (eg., James *et al.* 1994, Yamada *et al.* 1995, James *et al.* 1997, Mozina *et al.* 1997). As shown in the previous Section (3.5.2, p., 201), differences in this sequence provide the basis for the differentiation of *Candida* species and related genera. However, this sequence is excessively long and therefore too laborious and costly to be effectively used for routine species diagnosis.

Alignment of the complete 18S rRNA gene sequence allowed the identification of a 490-bp segment in the middle of the sequence that was highly divergent at the species level, but conserved within species suggesting that it is potentially diagnostic. This Section is a further investigation of this segment to assess its potential application as a target for the design of a rapid PCR based diagnostic method for the clinically important species of *Candida*.

The **aim** was;

- *to assess the utility of a 490bp segment of the 18S rRNA gene sequence for species identification and phylogenetic reconstruction of Candida species and related genera.*

3.5.3.b Materials and methods

Primers M18s5 and M18s3 (Section 2.2.3.a p., 59), used to amplify the middle 490-bp region of isolate CBS 562 (Section 3.5.2 p., 201) were used to amplify type strains used in Section 3.2.1 (p., 93) for which the 18S rRNA sequence had not been previously published plus a selection of clinical isolates from potentially cryptic species (Table 3.47 p., 209). The 18S rRNA gene fragment was also extracted from the complete gene sequences in Section 3.5.2 (p., 201).

3.5.3.c Results

A Clustal X alignment of the sequences is presented in Table 3.48a-j (p., 210-19). The sequences obtained for Type and clinical strains in the present study were located within clades containing previously published sequences. All sequences were homologous at 283 of the bases compared and there was a high level of homology within species (Table 3.49 p., 220).

As with the complete 18S rRNA gene sequence, analysis of the partial fragment showed separation of the ascomycetes and basidiomycetes but the two serovars of *Cr. neoformans* were not distinguished into separate lineages (Figure 3.19a p., 221). The partial 18S rRNA gene also discriminated four distinct lineages of ascomycetes that correlated with those described using the complete sequence (Section 3.5.2 p., 201). *C. krusei* and *C. norvegensis* formed a clade, as did *C. lusitaniae* and *C. haemulonii*. Again, *S. cerevisiae* was located in the same clade as *C. kefyr* and *C. glabrata*. The final clade contained the majority of

Candida species, *C. albicans*, *C. dubliniensis*, *C. tropicalis*, *C. famata*, *C. viswanathii*, *C. guilliermondii*, *D. hansenii* and *C. parapsilosis*. Some species were very closely related using this sequence. Their relationships were reanalysed excluding the less closely related species (Figure 3.19b, p., 222). This close phylogenetic clade contained all of the *Candida* clinical isolates currently sequenced. Isolates 34A.4 and 34A.6 co-located with the *C. tropicalis* type strain, but the partial 18S sequence was too similar to allow unequivocal discrimination of many of the *Candida* species in this large clade.

The close genetic relationship between *C. famata*, *C. guilliermondii* and *D. hansenii* has been raised previously (Nishikawa *et al.* 1997). The current analyses suggest that *D. hansenii* is more closely related to *C. guilliermondii* than to *C. famata*. Following the allozyme electrophoretic analysis, clinical isolate 14130 was reidentified as *T. beigelii* using traditional characters. Partial 18S rRNA sequence comparisons of 14130 and the *T. beigelii* reference strain provided support for this identity.

Table 3.47; Isolates and Genbank sequences used for partial 18S rRNA analysis

Species [a]	Strain	Genbank accession number / Sequence code [b]
C. albicans	ATCC 18804	M60302 (Barns) / CaM60302
C. albicans	Clinical	AJ005123 (Kerkmann,M.L.) / CaAJ005123
C. kefyr	ATCC 4235	M60303 (Barns) / CkeM60303
C. dubliniensis	CD 36	X99399 (Sullivan,D.J.) / CdX99399
C. viswanathii	ATCC 22981	M60309 (Barns) / CvM60309
S. cerevisiae	baker's yeast	V01335 (Mankin,A.S.) / ScV01335
S. cerevisiae	-	J01353 M27607 (Rubtsov,P.M.) / ScJ01353
C. guilliermondii	ATCC 6260	M60304 (Barns) / CgM60304
C. parapsilosis	ATCC 22019	M60307 (Barns) / CpM60307
Cr. neoformans	ATCC 24067	L05428 (Fan,M.) / CnL05428
Cr. neoformans var. *gattii*	ATCC 24065	L05427 (Fan,M.) / CnL05427
F. neoformans var. *neoformans* (teleomorph)	MUCL 30453	X60183 (De Wachter,R.) (Van de Peer,Y.) / FnX60183
Cr. neoformans	-	M55625 (Sogin,M.L.) / CnM55625
C. glabrata	CBS 138	X51831 (Clark-Walker,G.D.) (Wong,O.C.) / CgX51831
T. glabrata	ATCC 2001	M60311 (Barns) / TgM60311
C. lusitaniae	MUCL 29855)	M55526 (Hendriks,L.) / ClM55526
C. lusitaniae	ATCC 42720	M60306 (Barns) / ClM60306
C. tropicalis	MUCL 30002	M55527 (Hendriks) / CtM55527
C. tropicalis	ATCC 750	M60308 (Barns) / CtM60308
C krusei	ATCC 6258	M60305 (Barns) / CkrM60305
C krusei	MUCL 29849	M55528 (Hendriks) / CkrM55528
T. beigelii (anamorph)	MUCL 30308	X60182 (De Wachter,R.), (Van de Peer,Y.) / TcX60182
D. hansenii (teleomorph)	NRRL Y-7426	X62649 (McNally,K.L), (Govind,N.S.), (Medlin,L.) / DhX62649
D. hansenii (teleomorph)	MUCL 29826	X58053 (Wachter,R), (Hendriks) / DhX58053
K. marxianus (teleomorph)	5671	X89522 (Cai, J.) / KmX89522
K. marxianus (teleomorph)	NCYC 970	X89524 (Cai, J.) / KmX89524
K. marxianus (teleomorph)	strain 712	X89523 (Cai, J.) / KmX89523
C. albicans	CBS 562	CalbCBS562
C. dubliniensis	CBS 7987	CdubCBS7987
C. famata	CBS 940	CfamCBS940
C. haemulonii	CBS 5149	ChaemCBS5149
C. kefyr	CBS 834	CkefyrCBS834
C. lusitaniae	CBS 6936	ClusCBS6936
Cr. neoformans var. *neoformans*	CBS 132	CneoneoCBS132
Cr. neoformans var. *gattii*	CBS 6289	Cneog6289
C. parapsilosis	CBS 604	Cpara604
C. tropicalis	CBS 94	CtropCBS94
C. viswanathii	CBS 4024	CvisCBS4024
S. cerevisiae	ATCC 2601	ScerATCC2601
	21A.3 [c]	21A.3
	27A.3-4	27A.3-4
	34B.1	34B.1
	40F.1	40F.1
	29A.1-6	29A.1-6
	40E.1	40E.1
	34A.4	34A.4
	34A.6	34A.6
	14130	14130

a; The sequences for some species include those obtained from type and reference strains and, where available, the teleomorphic species.
b; Sequence code used in the following Tables and Figures in this section.
c; Clinical strains obtained for the current study.

Table 3.48a; CLUSTAL X (1.64b) multiple sequence alignment of a 490-bp fragment of the 18S rRNA gene in *Candida* and related genera. The nucleotides in bold represent the forward primer binding site and highlighted areas are homologous regions across all aligned sequences. The identities of the isolates assessed is within Table 3.47 p., 209

Table 3.48b; CLUSTAL X (1.64b) multiple sequence alignment of a 490-bp fragment of the 18S rRNA gene in *Candida* and related genera. The nucleotides in bold represent the forward primer binding site and highlighted areas are homologous regions across all aligned sequences. The identities of the isolates assessed is within Table 3.47 p., 209

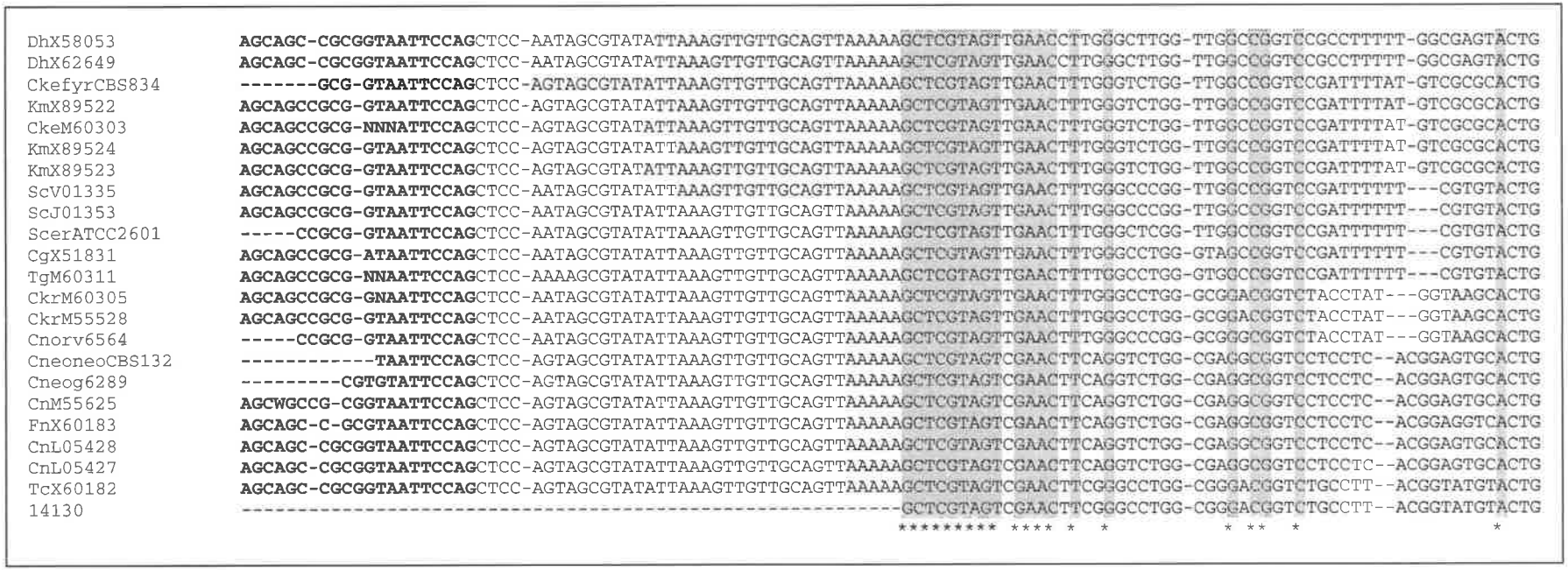


Table 3.48c; CLUSTAL X (1.64b) multiple sequence alignment of a 490-bp fragment of the 18S rRNA gene in *Candida* and related genera. Highlighted areas are homologous regions across all aligned sequences. The identities of the isolates assessed is within Table 3.47 p., 209

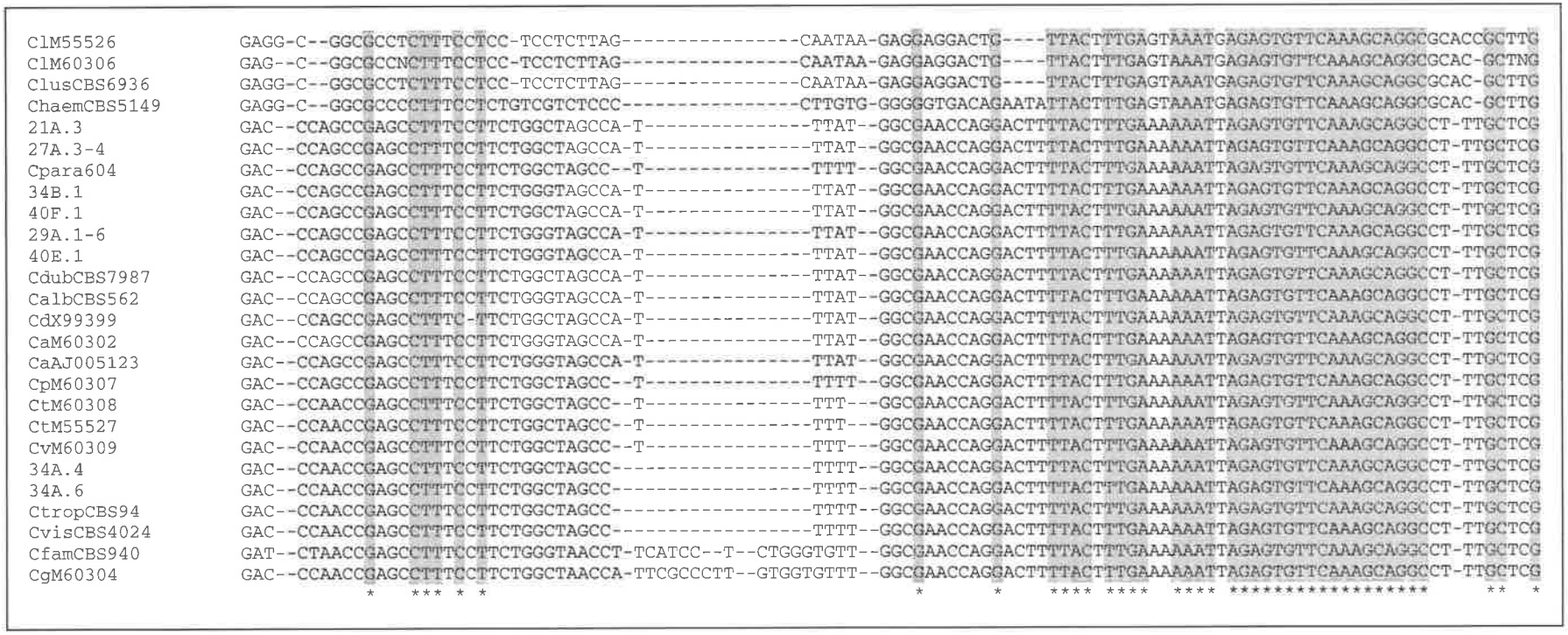


Table 3.48d; CLUSTAL X (1.64b) multiple sequence alignment of a 490-bp fragment of the 18S rRNA gene in *Candida* and related genera. Highlighted areas are homologous regions across all aligned sequences. The identities of the isolates assessed is within Table 3.47 p., 209

Table 3.48e; CLUSTAL X (1.64b) multiple sequence alignment of a 490-bp fragment of the 18S rRNA gene in *Candida* and related genera. Highlighted areas are homologous regions across all aligned sequences. The identities of the isolates assessed is within Table 3.47 p., 209

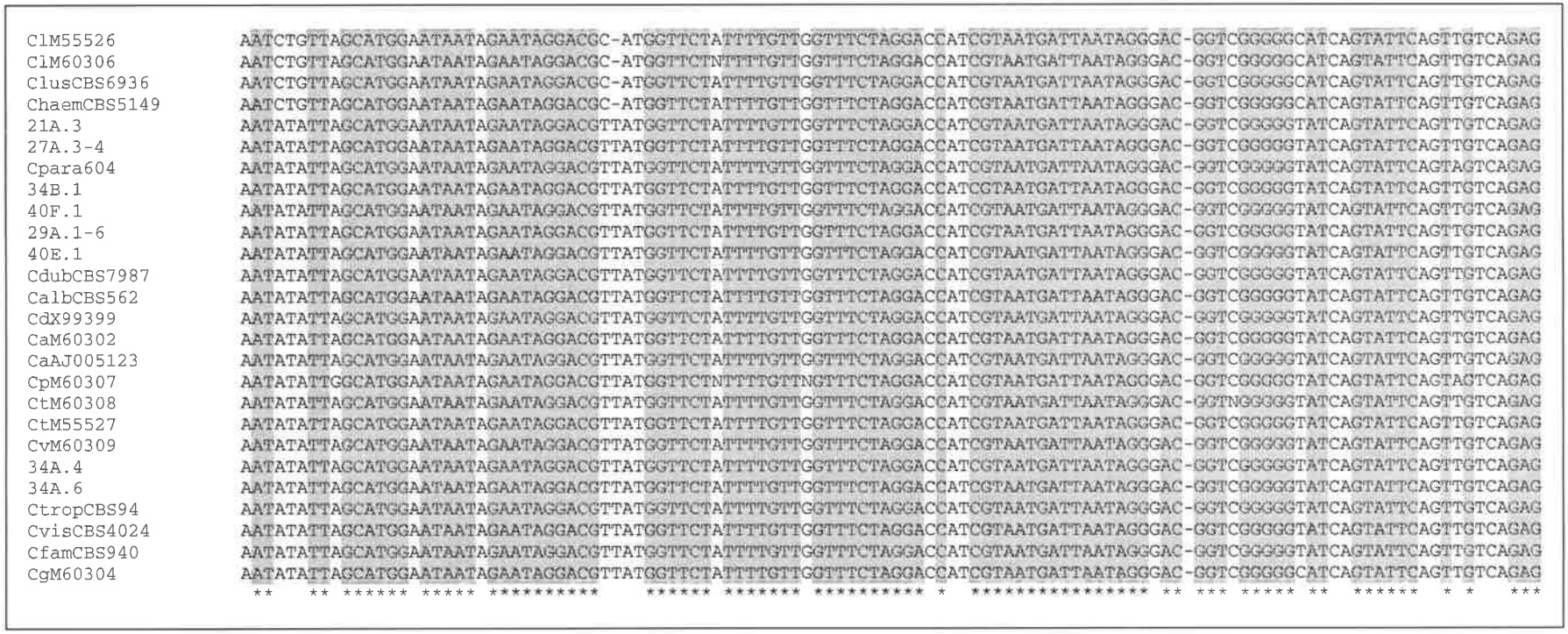


Table 3.48f; CLUSTAL X (1.64b) multiple sequence alignment of a 490-bp fragment of the 18S rRNA gene in *Candida* and related genera. Highlighted areas are homologous regions across all aligned sequences. The identities of the isolates assessed is within Table 3.47 p., 209

Table 3.48g; CLUSTAL X (1.64b) multiple sequence alignment of a 490-bp fragment of the 18S rRNA gene in *Candida* and related genera. Highlighted areas are homologous regions across all aligned sequences. The identities of the isolates assessed is within Table 3.47 p., 209

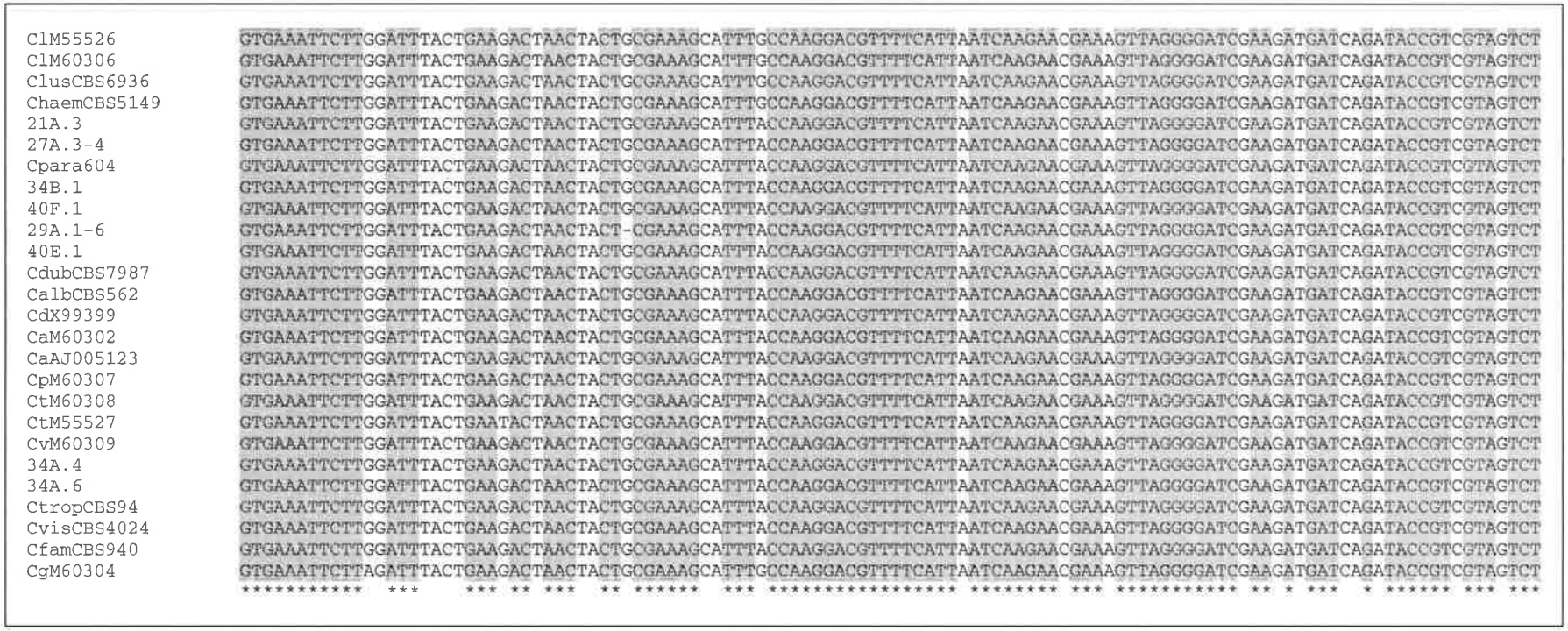


Table 3.48h; CLUSTAL X (1.64b) multiple sequence alignment of a 490-bp fragment of the 18S rRNA gene in *Candida* and related genera. Highlighted areas are homologous regions across all aligned sequences. The identities of the isolates assessed is within Table 3.47 p., 209

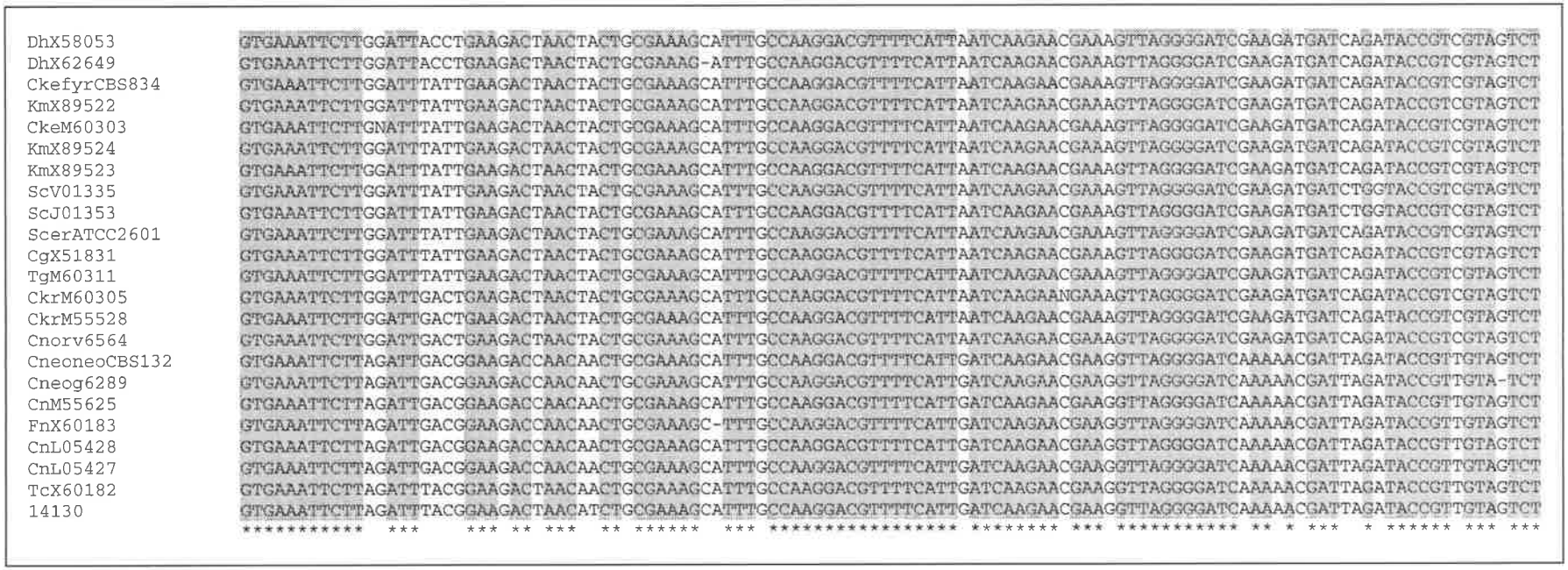


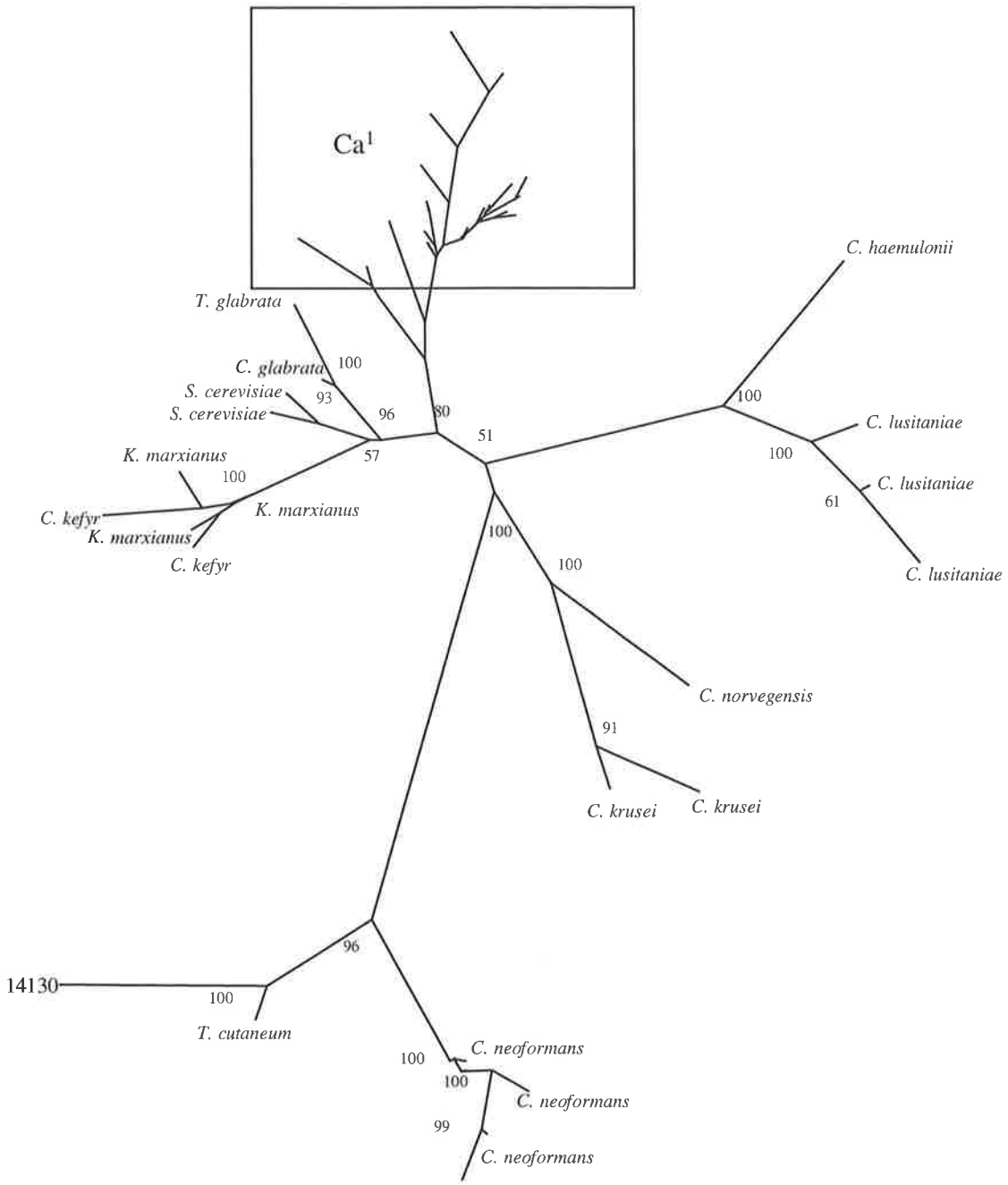
Table 3.48i; CLUSTAL X (1.64b) multiple sequence alignment of a 490-bp fragment of the 18S rRNA gene in *Candida* and related genera. The nucleotides in bold represent the reverse primer binding site and the highlighted areas are homologous regions across all aligned sequences. The identities of the isolates assessed is within Table 3.47 p., 209

```
ClM55526          TAACCATAAACTATGCCGACTAGGGATCGGGCGGCGTTCATTTAGT-GACGCGCTCGGCACCTTACGAGAAATCAAAGTCTTT-GGGTTCTGGGGGGAGTAT-GGTCG-
ClM60306          TAACCATAAACTATGCCGACTAGGGATCGGGCGGCGTTCATTTAGT-GACGCGCTCGGCACCTTACGAGAAATCAAAGTCTTT-GGGT----------------------
ClusCBS6936       TAACCATAAACTATGCCGACTAGGGATCGGGCGGCGTTCATTTAGT-GACGCGCTCGGCACCTTACGAGAAATCAAAGTCTTT-GGGTTCTGGGGGGAGTAT-GGTC-C
ChaemCBS5149      TAACCATAAACTATGCCGACTAGGGATCGGGCGGCGTTCATCTAGT-GACGCGCTCGGCACCTTACGAGAAATCAAAGT-TTT-GGGTTCTGGGGGGA-T-TT------
21A.3             TAACCATAAACTATGCCGACTAGGGATCGGTTGTTGTTCTTTTATT-GACGCAATCGGCACCTTACGAGAAATCAAAGTCTTT-GGGTTCTGGGGGGAGTAT-GGTCGC
27A.3-4           TAACCATAAACTATGCCGACTAGGGATCGGTTGTTGTTCTTTTATT-GACGCAATCGGCACCTTACGAGAAATCAAAGTCTTT-GGGTTCTGGGGGGAGTAT-GGTC--
Cpara604          TAACCATAAACTATGCCGACTAGGGATCGGTTGTTGTTCTTTTATT-GACGCAATCGGCACCTTACGAGAAATCAAA-TCTTT-GGGTTCTGGGGGGAGTAT-GGTCGC
34B.1             TAACCATAAACTATGCCGACTAGGGATCGGTTGTTGTTCTTTTATT-GACGCAATCGGCACCTTACGAGAAATCAAA-TCTCC-GGGTTCTGGGGGGA-TAT-GGTC-C
40F.1             TAACCATAAACTATGCCGACTAGGGATCGGTTGTTGTTCTTTTATT-GACGCAATCGGCACCTTACGAGAAATCAAA-TCTYY-GGGTTCTGGGGGGA-TAT-GGTC-C
29A.1-6           TAACCATAAACTATGCCGACTAGGGATCGGTTGTTGTTCTTTTATT-GACGCAATCGGCACCTTACGAGAAATCAAA-TCTTT-GGGTTCTGGGGGGAGTAT-GGTCGC
40E.1             TAACCATAAACTATGCCGACTAGGGATCGGTTGTTGTTCTTTTATT-GACGCAATCGGCACCTTACGAGAAATCAAA-TCTTT GGGGTTCTGGGGGGAGTAT-GGTCG
CdubCBS7987       TAACCATAAACTATGCCGACTAGGGATCGGTTGTTGTTCTTTTATT-GACGCAATCGGCACCTTACGAGAAATCAAAGTCTTT-GGGTTCTGGGGGGAGTAT-GGTCG-
CalbCBS562        TAACCATAAACTATGCCGACTAGGGATCGGTTGTTGTTCTTTTATT-GACGCAATCGGCACCTTACGAGAAATCAAAGTCTTT-GGGTTCTGGGGGGAGTAT-GGTCG-
CdX99399          TAACCATAAACTATGCCGACTAGGGATCGGTTGTTGTTCTTTTATT-GACGCAATCGGCACCTTACGAGAAATCAAAGTCTTT-GGGTTCTGGGGGGAGTAT-GGTCG-
CaM60302          TAACCATAAACTATGCCGACTAGGGATCGGTTGTTGTTCTTTTATT-GACGCAATCGGCACCTTACGAGAAATCAAAGTCTTT-GGGTTCTGGGGGGAGTAT-GGTCG-
CaAJ005123        TAACCATAAACTATGCCGACTAGGGATCGGTTGTTGTTCTTTTATT-GACGCAATCGGCACCTTACGAGAAATCAAAGTCTTT-GGGTTCTGGGGGGAGTAT-GGTCG-
CpM60307          TAACCATAAACTATGCCGACTAGGGATCGGTTGTTGTTCTTTTATT-GACGCAATCGGCACCTTACGAGAAATCAAAGTCT----------------------------
CtM60308          TAACCATAAACTATGCCGACTAGGGATCGGTTGTTGTTCTTTTATT-GACGCAATCGGCACCTTACGAGAAATCAAAGTCTTT-GGG-----------------------
CtM55527          TAACCATAAACTATGCCGACTAGGGATCGGTTGTTGTTCTTTTATT-GACGCAATCGGCACCTTACGAGAAATCAAAGTCTTT-GGGTTCTGGGGGGAGTAT-GGTCG-
CvM60309          TAACCATAAACTATGCCGACTAGGGATCGGTTGTTGTTCTTTTATT-GACGCAATCGGCACCTTACGAGAAATCAAAGTC----------------------------
34A.4             TAACCATAAACTATGCCGACTAGGGATCGGTTGTTGTTTTTTTTATT-GACGCAATCGGCACCTTACGAGAAATCAAA-TCTTT-GGGTTCTGGGGGGAGTAT-GGTC-C
34A.6             TAACCATAAACTATGCCGACTAGGGATCGGTTGTTGTTTTTTTTATT-GACGCAATCGGCACCTTACGAGAAATCAAA-TCTCT-GGGTTCTGGGGGGA-TAT-GGTC-C
CtropCBS94        TAACCATAAACTATGCCGACTAGGGATCGGTTGTTGTTCTTTTATT-GACGCAATCGGCACCTTACGAGAAATCAAAGTCTTT-GGGTTCTGGGGGGA-TTT-GGTC--
CvisCBS4024       TAACCATAAACTATGCCGACTAGGGATCGGTTGTTGTTCTTTTATT-GACGCAATCGGCACCTTACGAGAAATCAAAGTCTTT-GGGTTCTGGGGGGAATAT-GGTC-C
CfamCBS940        TAACCATAAACTATGCCGACTAGGGATCGGGTGTCGTTCTTTTTTT-GACGCACTCGGCACCTTACGAGAAATCAAAGTCTTT-GGGTTCTGGGGGGAGTAT-GGTCG-
CgM60304          TAACCATAAACTATGCCGACTAGGGATCGGGTGTTGTTCTKTTTTT-GACGCACTCGGCACCTTACGAGAAATCAAAGTCNTT-GGG-----------------------
                  **** ***** ********** ***** *  **** **** ******** *
```

Table 3.48j; CLUSTAL X (1.64b) multiple sequence alignment of a 490-bp fragment of the 18S rRNA gene in *Candida* and related genera. Nucleotides in bold represent the reverse primer binding site and the highlighted areas are homologous regions across all aligned sequences. The identities of the isolates assessed is within Table 3.47 p., 209

Table 3.49; Details of the sequence variation at the 490-bp fragment of the 18S rRNA gene within medically important *Candida* species and related genera

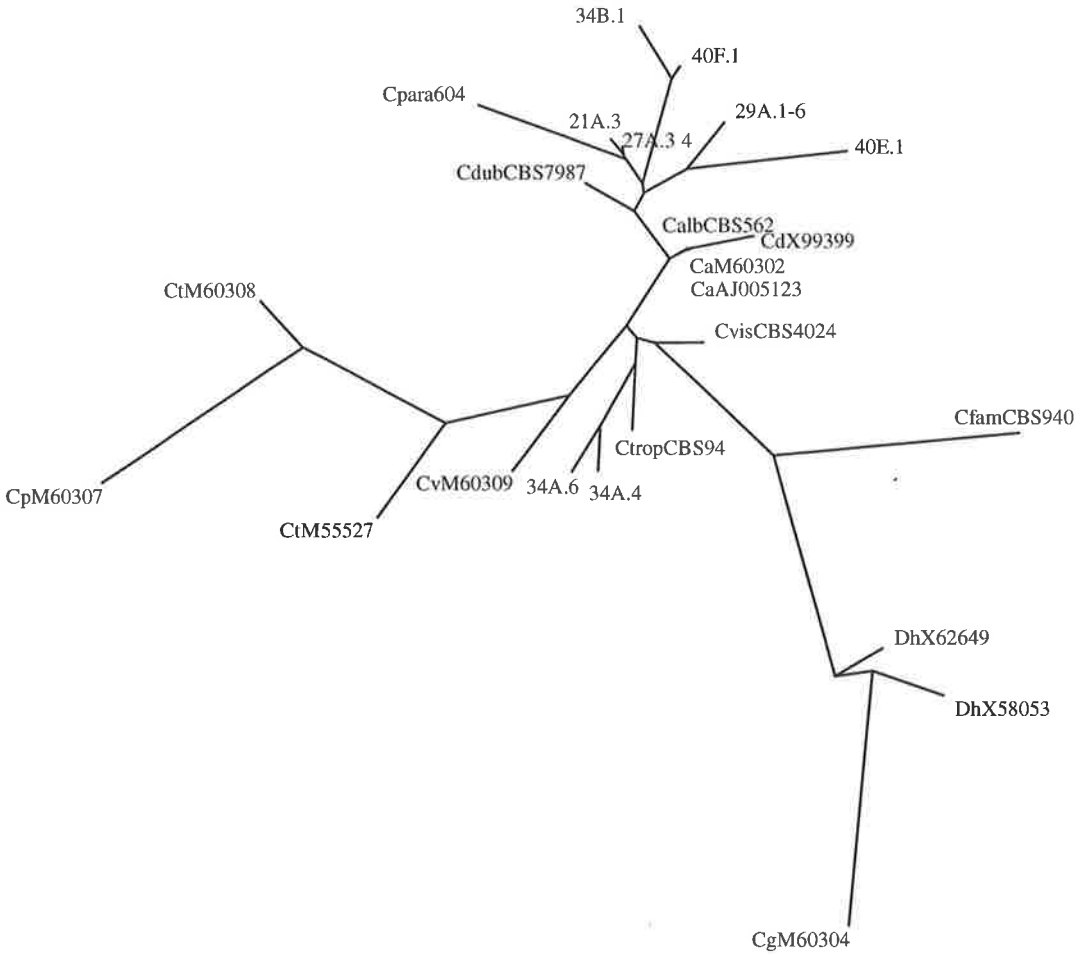
Species	Number of sequences analysed	Number of mismatches and insertions/ deletions or indels	Number of ambiguous sites	Sequence lengths (bp)
C. albicans	3	0	0	513
C. lusitaniae	3	8	5	505
C. parapsilosis	2	3	3	515
C. dubliniensis	2	2	0	514
C. tropicalis	3	1	1	512
C. viswanathii	2	0	0	507
C. kefyr	5	3	5	528
S. cerevisiae	3	7	0	525
C. glabrata	2	3	6	525
C. krusei	2	2	6	443
Cr. neoformans	6	3	0	515
T. beigelii/ 14130	2	8	0	476
C. famata/ *D. hansenii*	3	15	0	529
C. guilliermondii/ *D. hansenii*	3	7	1	523



0.1 substitutions/site

Figure 3.19a; Unrooted consensus tree of the phylogenetic relationships derived from 18S rRNA sequence comparisons for medically relevant *Candida* species and related genera

1; This clade contains all of the *C. albicans*, *C. dubliniensis*, *C. tropicalis*, *C. famata*, *C. viswanathii*, *C. guilliermondii*, *C. parapsilosis* and *D. hansenii* sequences assessed. These isolates were assessed independently to clarify their close genetic relationships, which is represented in Figure 3.19b (p., 229).



0.01 substitutions/site

Figure 3.19b; Phylogram from the reanalysis of the partial 18S rRNA gene sequence of closely related isolates within the clade of Figure 3.19a (p., 221)

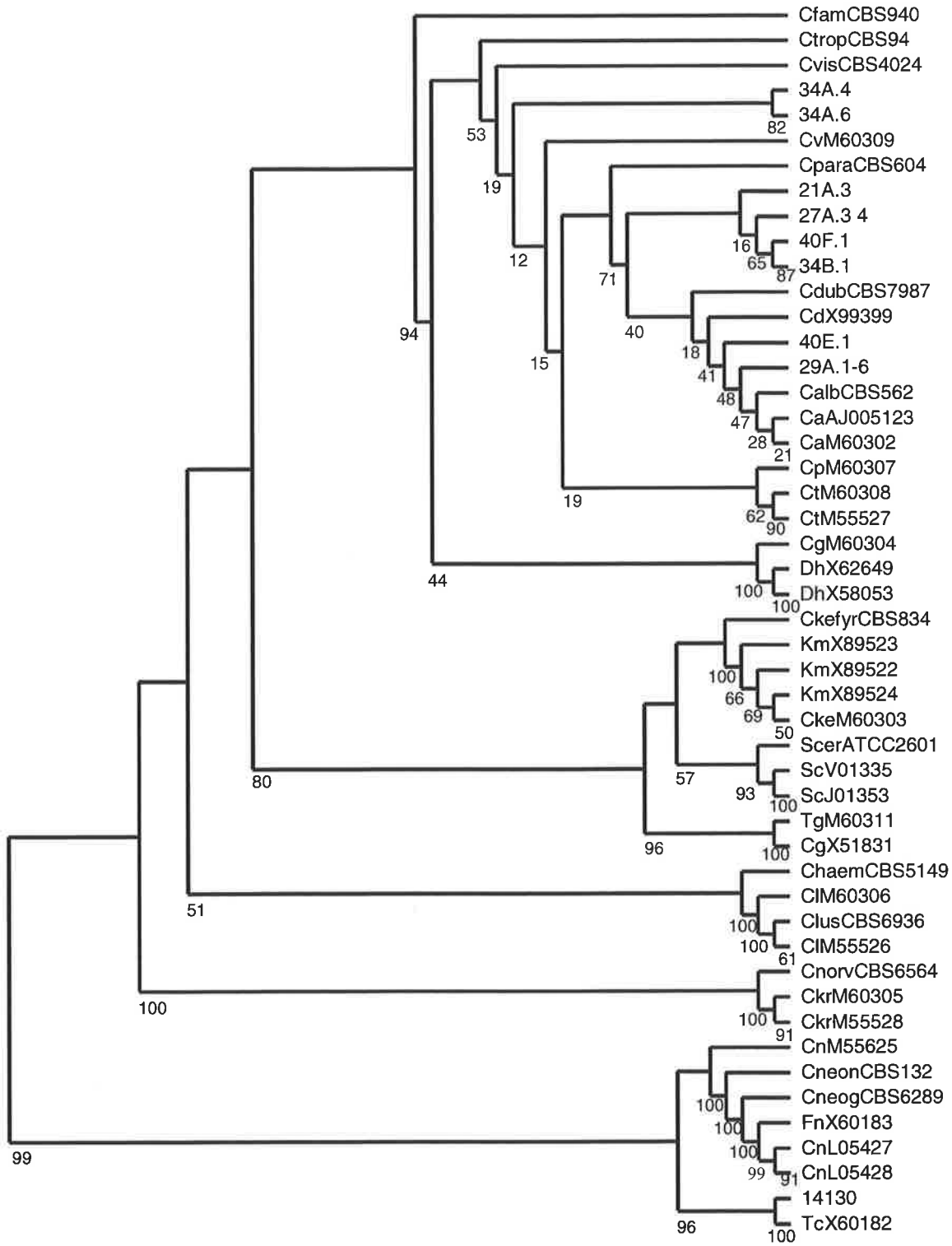


Figure 3.19c; Bootstrap values for the phylogenetic relationships based on a fragment of the 18SrRNA gene obtained by maximum likelihood analyses from five-hundred replicates

Bootstrap values (denoted under each branch) exceeding 75 indicate that the branch has strong statistical support.

3.5.3.d Interpretation

The 490-bp fragment of the 18S rRNA gene is relatively conserved within a species but variable between species, as was the case for the complete 18S rRNA gene sequences in Section 3.5.2 (p., 201). The phylogenetic analysis included a number of sequences for many of the species examined and an increased number of species in comparison with the sequence data of the entire 18S rRNA gene. It is important to note that the inclusion of additional taxa did not alter the general genetic relationships between *Candida* species nor related genera; the ascomycetes and basidiomycetes were easily differentiated and the four major ascomycete lineages were maintained. These results suggest that it may not be necessary to sequence the entire 18S rRNA gene to obtain phylogenetic information for *Candida* species. However, using only the 490bp fragment of the 18S rRNA gene sequences resulted in the relationships of some *Candida* species being unresolved, such as of *C. tropicalis*, *C. parapsilosis* and *C. viswanathii*; *C. albicans*, *C. dubliniensis* and *C. parapsilosis*. The bootstrap values for this analysis were again strong for the separation of ascomycetes and basidiomycetes and for the branches within the basidiomycetes (Figure 3.19c p., 223). The support was also strong for the association of isolates 14130 and *T. beigelii*, an identity corroborated by the use of allozyme electrophoresis and traditional diagnostic characters. The relative positions of *C. krusei* and *C. lusitaniae* were different when analysing the complete and partial 18S sequence data. In both analyses, the most basal branch had the strongest support, but the relationship between the two species and the rest of the *Candida* genus was less supported. The branching order may have been affected by the addition of other *Candida* species associated with these branches in this analysis. The two clades containing *S. cerevisiae* and *C. albicans* had good support in this analysis, as with the analysis using the complete 18S gene. However, the bootstrap values between species within clades in this analysis were not as high. This result suggests that the 490bp fragment of the 18S rRNA gene is not as phylogenetically informative within the four major *Candida* branches.

As has been shown in previous subsections, the relationships between the species currently examined conflict with traditional taxonomy. This may indicate that the 18S rRNA gene used in isolation is not discriminatory enough for accurately assessing genetic relationships between closely related species of *Candida*.

3.6 Genetic analyses using ITS1, 5.8S rRNA, ITS2 sequences

The following subsection is an analysis of sequence variation in the ITS1 and 2 regions of the rRNA gene region within *Candida* and related genera. This method was arbitrarily chosen because it had been suggested as epidemiologically informative. However, since the present evaluation, a number of other methods have been suggested as potentially more discriminatory within *Candida* species. Nevertheless, the general approach used in this Section could be applied for the evaluation of any of the traditional or contemporary epidemiological methods available.

3.6.1 Epidemiological utility of 5.8S rRNA, ITS1 and ITS2 sequences

3.6.1.a Background

According to the literature, the internal transcribed sequences of the rRNA gene region are more variable than the coding regions. Therefore they have been suggested as potential targets for the design of molecular epidemiological tools (eg., Lin *et al.* 1995). The 5.8S rRNA gene situated between the ITS regions is coding unlike the two ITS flanking regions. This means that sequence variation within the 5.8S gene region is more strictly controlled. In this study it is used as an anchor for aligning the more variable flanking ITS regions.

The **aim** was;

- *to assess the utility of the ITS regions of the ribosomal RNA gene region for detecting intraspecies variation and its potential for use in epidemiological investigations.*

3.6.1.b Materials and methods

The specific protocol for this analysis is outlined in Chapter 2 (p., 47). The two primers of White *et al.* (1990) were used to assess the variability of these regions across the range of type and reference strains that had been used previously in the allozyme electrophoretic analyses plus an additional four *C. albicans* strains and one *C. dubliniensis* clinical strain (Table 3.50 p., 227).

3.6.1.c Results

Alignments of the sequences using Clustal X are presented in Table 3.51a-g (p., 228-34). The sequences were homologous at 50 of 456 bases compared, indicating a high degree of variability between species of *Candida* and related genera. Unfortunately, as is evident from the diverse branching pattern within *Candida* species in Figure 3.20a (p., 236), the sequences are too variable to be used for phylogenetic investigations. However, as was observed from the 18S rRNA gene sequence analysis, there is a high degree of sequence conservation in this region within *Candida* species (Table 3.52 p., 235).

As with the 18S rRNA analyses, *Candida* species and clinical strains were located in the same clade. Of clinical significance, the isolates from patients were positioned in clades comprising either *C. albicans* or *C. dubliniensis*. The ascomycetes and basidiomycetes were genetically different, but they did not form distinct lineages. As had been noted using the 18S rRNA sequence data, there were four main lineages within the ascomycetes; *C. krusei* and *C. norvegensis*, *C. lusitaniae* and *C. haemulonii*, *C. kefyr, C. glabrata* and *S. cerevisiae* and a large clade containing *C. albicans*, *C. dubliniensis*, *C. viswanathii*, *C. tropicalis*, *C. stellatoidea*, *C. famata*, *C. guilliermondii* and *C. parapsilosis*. The close genetic relationships within this large clade were then analysed independently to clarify them (Figure 3.20b p., 237). This allowed the distinction of each of the *Candida* species within the clade and the more evident association of the clinical strains with the *C. albicans* and *C. dubliniensis* clades. This sequence appears species diagnostic allowing grouping of *Candida* species, but there is too little variation within *Candida* species for it to be epidemiologically informative. Conversely, there is too much genetic variation between *Candida* species and related genera for the 5.8S rRNA and flanking ITS gene sequences to be used independently for phylogenetic investigations as is evidenced by the number of branches with lower bootstrap values.

Table 3.50; Details of the sequences and isolates used to assess the phylogenetic utility of the 5.8S rRNA gene and flanking ITS1 and 2 regions of the ribosomal RNA gene

Species	Genbank Accession number	Sequence [a]	Strain [b]
C. albicans	-[c]	17484	clinical[d]
C. albicans	-	12716	clinical
C. albicans	-	18527	clinical
C. albicans	-	17640	clinical
C. albicans	-	Cal	CBS562
C. dubliniensis	-	15526	clinical
C. dubliniensis	-	Cdu	CBS7987
C. stellatoidea	L47114	1Cst	?[e]
C. tropicalis	L47112	1Ctr	?
C. tropicalis	-	Ctr	CBS94
C. viswanathii	-	Cvi	CBS4024
C. guilliermondii	-	Cgu	CBS566
C. guilliermondii	L47110	1Cgu	?
C. famata	-	Cfa	CBS940
C. glabrata	-	Cgl	CBS138
C. glabrata	L47108	1Cgl	?
C. kefyr	-	Cke	CBS834
C. kefyr	L47107	1Cke	?
C. haemulonii	-	Cha	CBS5149
C. lusitaniae	-	Clu	CBS6936
T. beigelii	-	Tbe	CBS2466
Cr. neoformans var. *neoformans*	-	Crnn	CBS132
Cr. neoformans var. *gattii*	-	Crng	CBS6289
C. norvegensis	-	Cno	CBS6564
C. krusei	-	Ckr	CBS573
C. krusei	L47113	1Ckr	?
S. cerevisiae	-	Sce	ATCC2610
C. parapsilosis	-	Cpa	CBS604
C. parapsilosis	L47109	1Cpa	?

a; Sequence refers to the code used for this sequence analysis and corresponds to the codes used in Table 3.51 (p., 228-34) and Figures 3.20a-c (p., 236-8).
b; Strain refers to the isolate from which the sequence was obtained.
c; - refers to sequence obtained in this thesis and not lodged on Genbank.
d; Clinical refers to a clinical strain not lodged in a culture collection.
e; ? strain refers to the sequence being obtained from an unspecified strain.

Table 3.51a; CLUSTAL X (1.64b) multiple sequence alignment of the 5.8S rRNA gene and flanking ITS1 and ITS2 regions of the ribosomal RNA gene in *Candida* and related genera. Nucleotides in bold represent the forward primer binding site and highlighted areas are homologous regions across all aligned sequences. The identities of the isolates assessed is within Table 3.50 p., 227

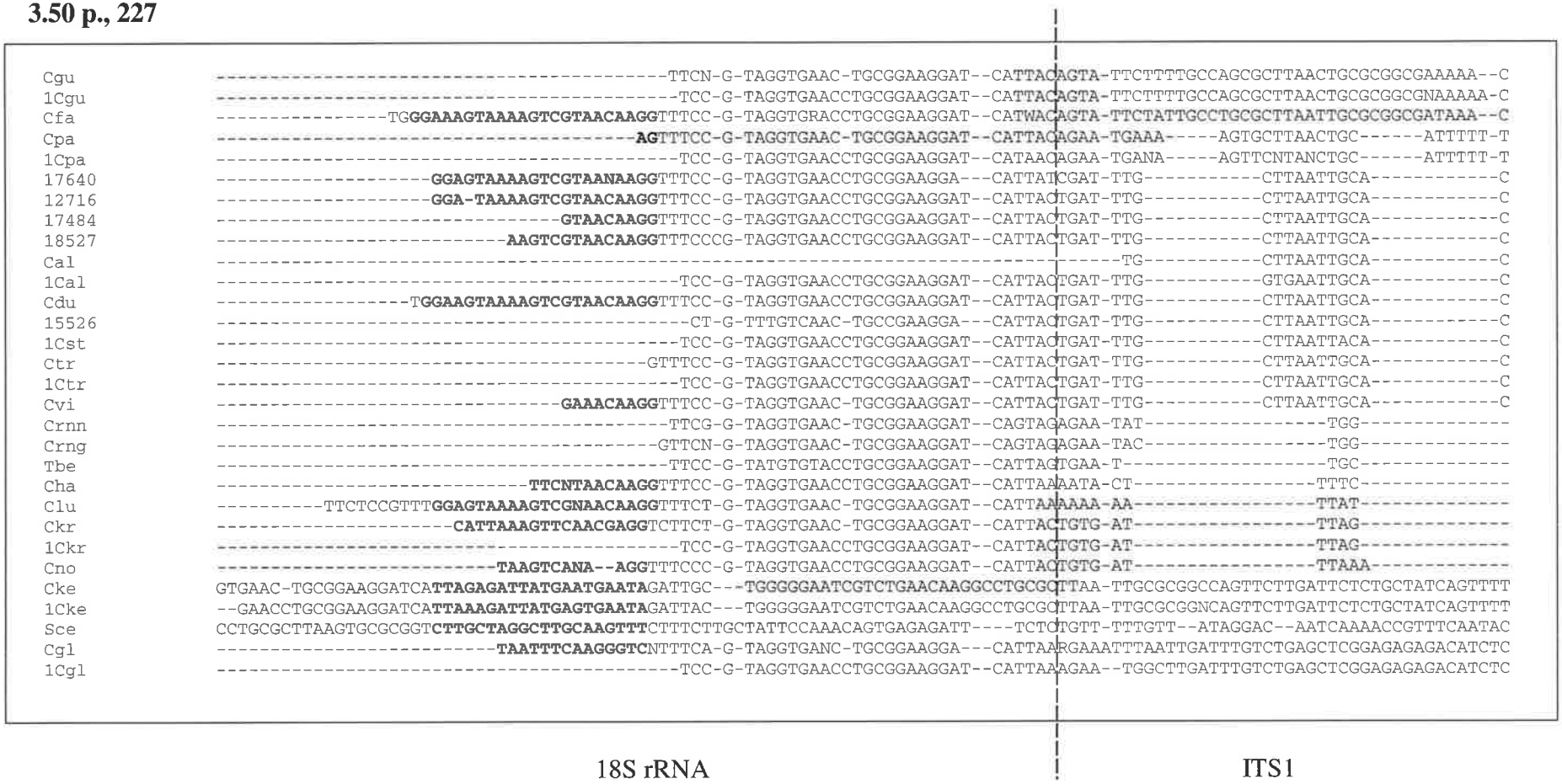
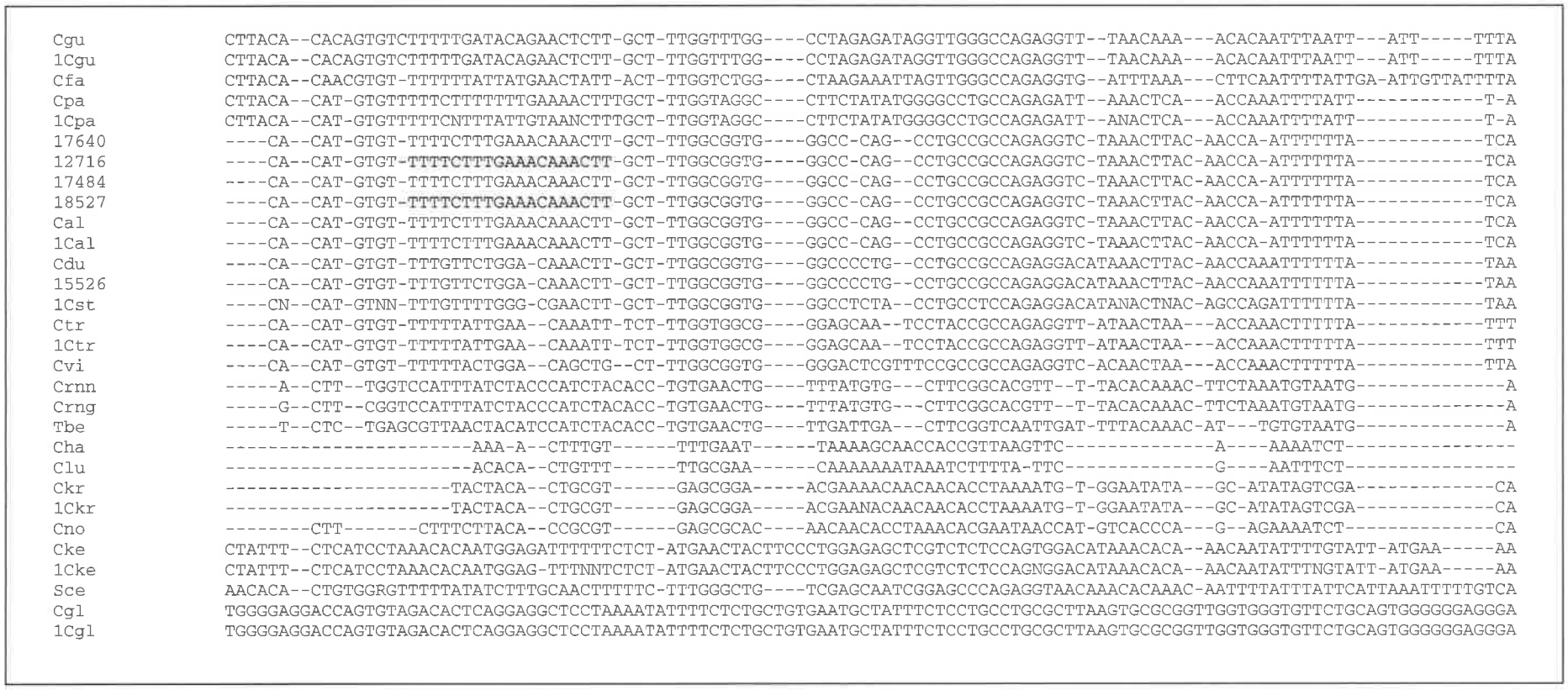


Table 3.51b; CLUSTAL X (1.64b) multiple sequence alignment of the 5.8S rRNA gene and flanking ITS1 and ITS2 regions of the ribosomal RNA gene in *Candida* and related genera. Highlighted areas are homologous regions across all assessed sequences. The identities of the isolates aligned is within Table 3.50 p., 227



ITS1

Table 3.51c; CLUSTAL X (1.64b) multiple sequence alignment of the 5.8S rRNA gene and flanking ITS1 and ITS2 regions of the ribosomal RNA gene in *Candida* and related genera. Highlighted areas are homologous regions across all aligned sequences. The identities of the isolates assessed is within Table 3.50 p., 227

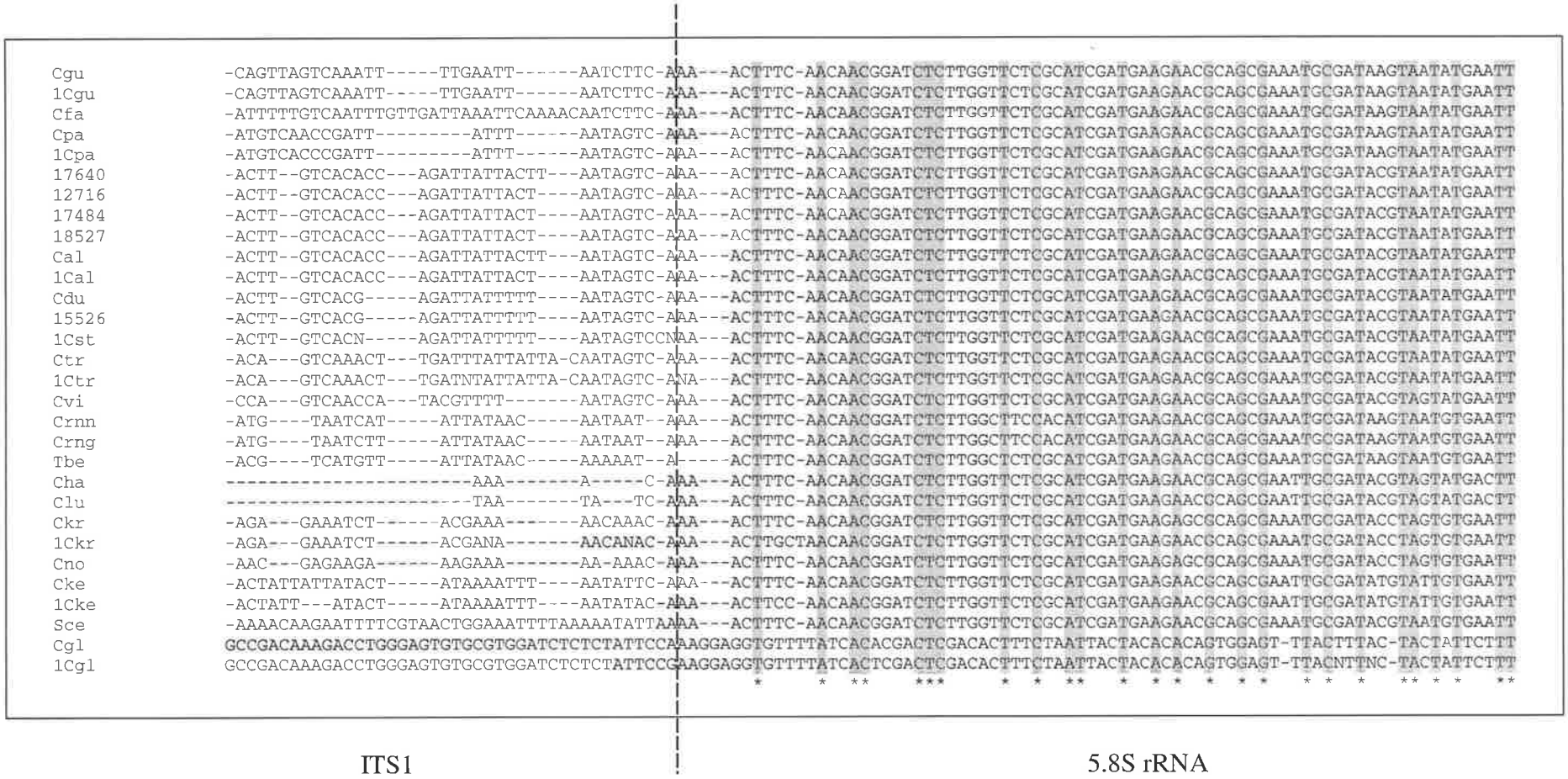


Table 3.51d; CLUSTAL X (1.64b) multiple sequence alignment of the 5.8S rRNA gene and flanking ITS1 and ITS2 regions of the ribosomal RNA gene in *Candida* and related genera. Highlighted areas are homologous regions across all aligned sequences. The identities of the isolates assessed is within Table 3.50 p., 227

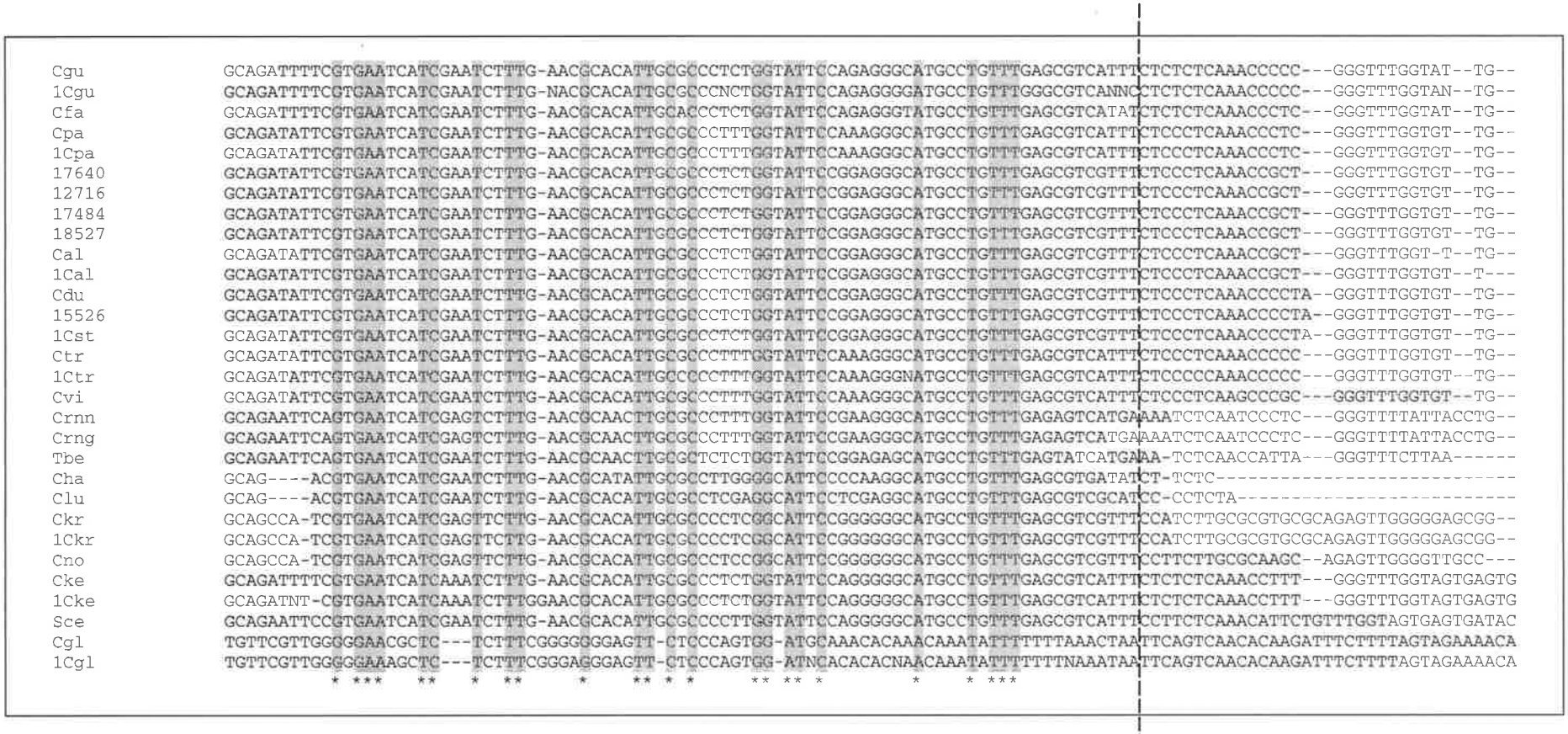


Table 3.51e; CLUSTAL X (1.64b) multiple sequence alignment of the 5.8S rRNA gene and flanking ITS1 and ITS2 regions of the ribosomal RNA gene in *Candida* and related genera. Highlighted areas are homologous regions across all aligned sequences. The identities of the isolates assessed is within Table 3.50 p., 227

```
Cgu         --A--GTGATACTCTTAGTC-GGACTAGGCGTTTGCTTGAAAA--GTATTGGCATGGGTAGTACTG-GATAGTGCTGTC--GACCT------CTC-AATGTATTAGGTTTATCCAACT-C
1Cgu        --A--GTGANACTCTTAGTC-GGACTAGGCGTTTGCTTGAAAA--NTATTGTCATGGGTAGTACTA-GATAGTGCTGTC--GACCT------CTC-AATGTATTAGGTTTATCCAACT-C
Cfa         --A--GTGATACTCTTAGTC-GAACTAGGCGTTTGCTTGAAAT--ATATTGGCACGAGTAGTGTTG-AACAGTGTTGTC--TGAAC------ATC-AATGTATTAGGTTTATCCAACT-C
Cpa         --A--GCGATACGCT-------------GGGTTTGCTTGAAA--------GAAAGGCGGAGTAT----AAACT----------------------AATGGAT-AGGTTTTTTCCACT-C
1Cpa        --A--GCGATACGCT-------------GGGTTTGCTTGAAA--------GAAAGGCGGAGTAT----AAACT----------------------AATGGAT-AGGTTTTTTCCACT-C
17640       --AA-GCAATACGACT-----------TGGGTTTGCTTGAAAG--AC---GGTAGTGGTA--AGGC-GGGATCGCTTT---GA-----------C-AATGGCTTAGGTCTAACCAAAAAC
12716       --A--GCAATACGACT-----------TGGGTTTGCTTGAAAG--AC---GGTAGTGGTA--AGGC-GGGATCGCTTT---GAA----------C-AATGGCTTAGGTCTAACCAAAAAC
17484       --A--GCAATACGACT-----------TGGGTTTGCTTGAAAG--AC---GGTAGTGGTA--AGGC-GGGATCGCTTT---GA-----------C-AATGGCTTAGGTCTAACCAAAAAC
18527       --A--GCAATACGACT-----------TGGGTTTGCTTGAAAG--AC---GGTAGTGGTA--AGGC-GGGATCGCTTT---GA-----------C-AATGGCTTAGGTCTAACCAAAAAC
Cal         --A--GCAATACGACT-----------TGGGTTTGCTTGAAAG--AC---GGTAGTGGTA--AGGC-GGGATCGCTTT---GA-----------C-AATGG-TTAGGTCTAACCAAAAAC
1Cal        --A--GCAATACGACT-----------TGGGTTTGCTTGAAAG--AC---GGTAGTGGTA--AGGC-GGGATCGCTTT---GA-----------C-AATGGCTTAGGTCTAACCAAAAAC
Cdu         --A--GCAATACGACT-----------TGGGTTTGCTTGAAAG--AT---GATAGTGGTATAAGGC-GGAGATGCTT----GA-----------C-AATGGCTTAGGTGTAACCAAAAAC
15526       --A--GCAATACGACT-----------TGGGTTTGCTTGAAAG--AT---GATAGTGGTATAAGGC-GGAGATGCTT----GA-----------C-AATGGCTTAGGTGTAACCAAAAAC
1Cst        --A--GCAATACGACT-----------TGGGTTTGCTTGAAAG--AT---GATAGTGGTA--AGGC-GGAGATGCTT----GA-----------C-AATGGCTTAGGTGTAACCAAAAAC
Ctr         --A--GCAATACGC-------------TAGGTTTGTTTGAAAG--AA---TTTAA-CGT----G---GAAACTTATTT---TA-------------AGCGACTTAGGTTTATCCAAAAAC
1Ctr        --A--GCAANACCC-------------TAGGTTTGTTTGAAAG--AA---TTTA--CGT----G---GAAACTTATTT---TA-------------AGCGACTTAGGTTTANCCAAAA-C
Cvi         --A--GCAATACGC-------------CAGGTTTGTTTGAAAG--AC---GT-A--CGT----G---GAGACT-ATAT---TA-------------GCGACTTAGGTTCTACCAAAA-C
Crnn        --TT-GGACTTGGATTTG---GGTGTTTGCCGCGACCTGCAAAGGACGTCGGCTCGCCTTAAATGT-GTTAGTGGGAAGGTGATTA------CCT-GTCAGCCC-GGCGTAATAAGTTTC
Crng        --TT-GGACTTGGATTTG---GGTGTTTGCCGCGACCTGCAAAGGACGTCGGCTCGCCTTAAATGT-GTTAGTGGGAAGGTGATTA------CCT-GTCAGCCC-GGCGTAATAAGTTTC
Tbe         ---T-GGA-TTGGATTTG---GGCGCTGCCAGTAGCCTG------------GCTCGCCTTAAAAGA-GTTAGCGTGTT-----TAA------CTT-GTCTTATCTGGCGTAATAAGTTTC
Cha         -------ACCGTTGGT------------GGATTTGTTTCT-----------AAAT----------------ATCATGCCA---------------C-AGTGAAGTCTAC------------
Clu         -------ACCCCCGGTTA----------GGCGTTGCTCCG-----------AAAT---------------ATCAA-CCG---------------C-GCTGTCAAACAC------------
Ckr         --AGCGGACGACGTGTAAAGAGCGTCGGAGCTGCGACTCGCCT-------GAAAGGGAG------C-GA-AGCTGGCC----------------G-AGCGAACTAGACTTTTTTTCAGGG
1Ckr        --AGCGGACGACGTGTAAAGAGCGTCGGAGCTGCGACTCGCCT-------GAAAGGGAG------C-GA-AGCTGGCC----------------G-AGCGAACTAGACTTTTTTTCAGGG
Cno         -------ACGGCCCGT-----GCGGCCTGTGTGTGGCTCCCCC-------GAAACGGAA------C-GGCAGCGGGACT---------------G-AGCGAAGTACACAA-----CACTC
Cke         ATACTCGTCTCGGGTTAACTTGAAAGTGGCTAGCCGTTGCCATCTGCGTGAGCAGGGCTGCGTGTC-AAGTCTATGGACTCGACTCTTGCACATC-TACGTCTTAGGTTTGCGCCAATTC
1Cke        ATACTCGTCTCGGGTTNACTTGAAAGTGGCTAGCCGTTGCCATCTGCGTGAGCAGGGCTGCGTGTC-GAGTCTATGGACTCGACTCTTGCACATC-TACGTCTTAGGTTTGCGCCAATTC
Sce         TCTTTGGAGTTAACTTGAAA-TTGCTGGCCTTTTCATTGGATGTTTTTTTTCCAAAGAGAGGTTTCTCTGCGTGCTTGAGGTATAATGCAAGTACGGTCGTATTAGGTTTTACCAACTGC
Cgl         --ACTTCAAAACTTTCAACAATGGATCTCTTGGTTCTCGCATC--GATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATTGCAGAATTCCGTGAATCATCGAATCTTTGAACGCAC
1Cgl        --ACTTCAAAACTTTCAACAATGGATCTCTTGG--CTCGCATC--GATGAAGAACGCAGC----------------------------------------------------------
```

ITS2

Table 3.51f; CLUSTAL X (1.64b) multiple sequence alignment of the 5.8S rRNA gene and flanking ITS1 and ITS2 regions of the ribosomal RNA gene in *Candida* and related genera. Highlighted areas are homologous regions across all aligned sequences. The identities of the isolates assessed is within Table 3.50 p., 227

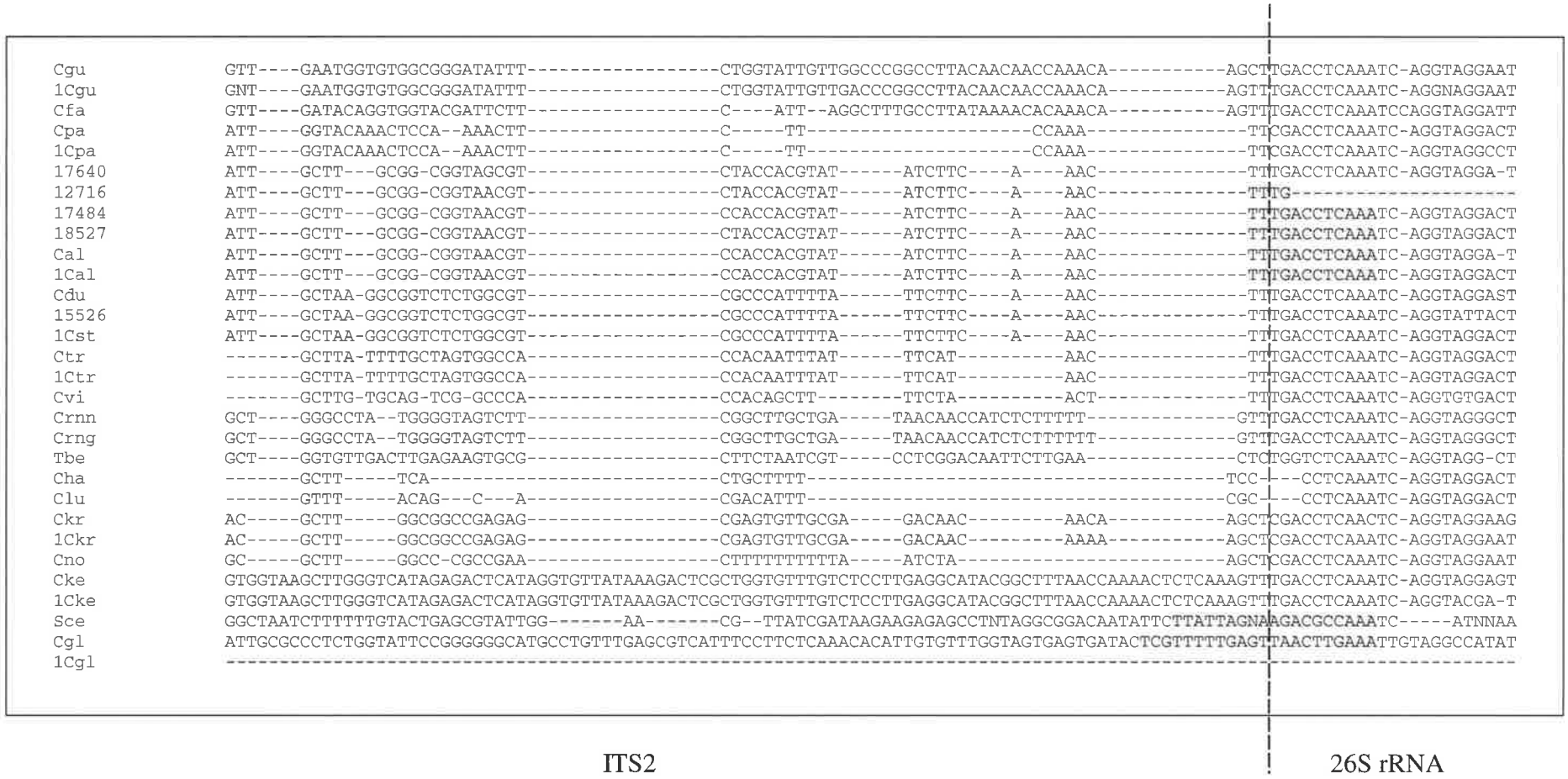


Table 3.51g; CLUSTAL X (1.64b) multiple sequence alignment of the 5.8S rRNA gene and flanking ITS1 and ITS2 regions of the ribosomal RNA gene in *Candida* and related genera. Nucleotides in bold represent the reverse primer binding site and highlighted areas are homologous regions across all aligned sequences. The identities of the isolates assessed is within Table 3.50 p., 227

26S rRNA

Table 3.52; Details of the sequence variation within species for the 5.8S rRNA gene and flanking ITS1 and ITS2 regions within medically important *Candida* species and related genera

Isolate 1	Isolate 2	Mismatches	Ambiguous bases	Sequence length compared
Cr. neoformans var *neoformans*	*Cr. neoformans* var *gattii*	5	1	536
C. kefyr	*C. kefyr*	12	9	706
C. guilliermondii	*C. guilliermondii*	8	11	588
C. parapsilosis	*C. parapsilosis*	7	6	499
C. albicans	*C. albicans* 17484	6	0	519
C. tropicalis	*C. tropicalis*	3	5	506
C. glabrata	*C. glabrata*	16	7	482
C. krusei	*C. krusei*	12	1	510
C. dubliniensis	*C. dubliniensis* 15526	14	2	527
C. albicans	*C. dubliniensis*	47	2	544

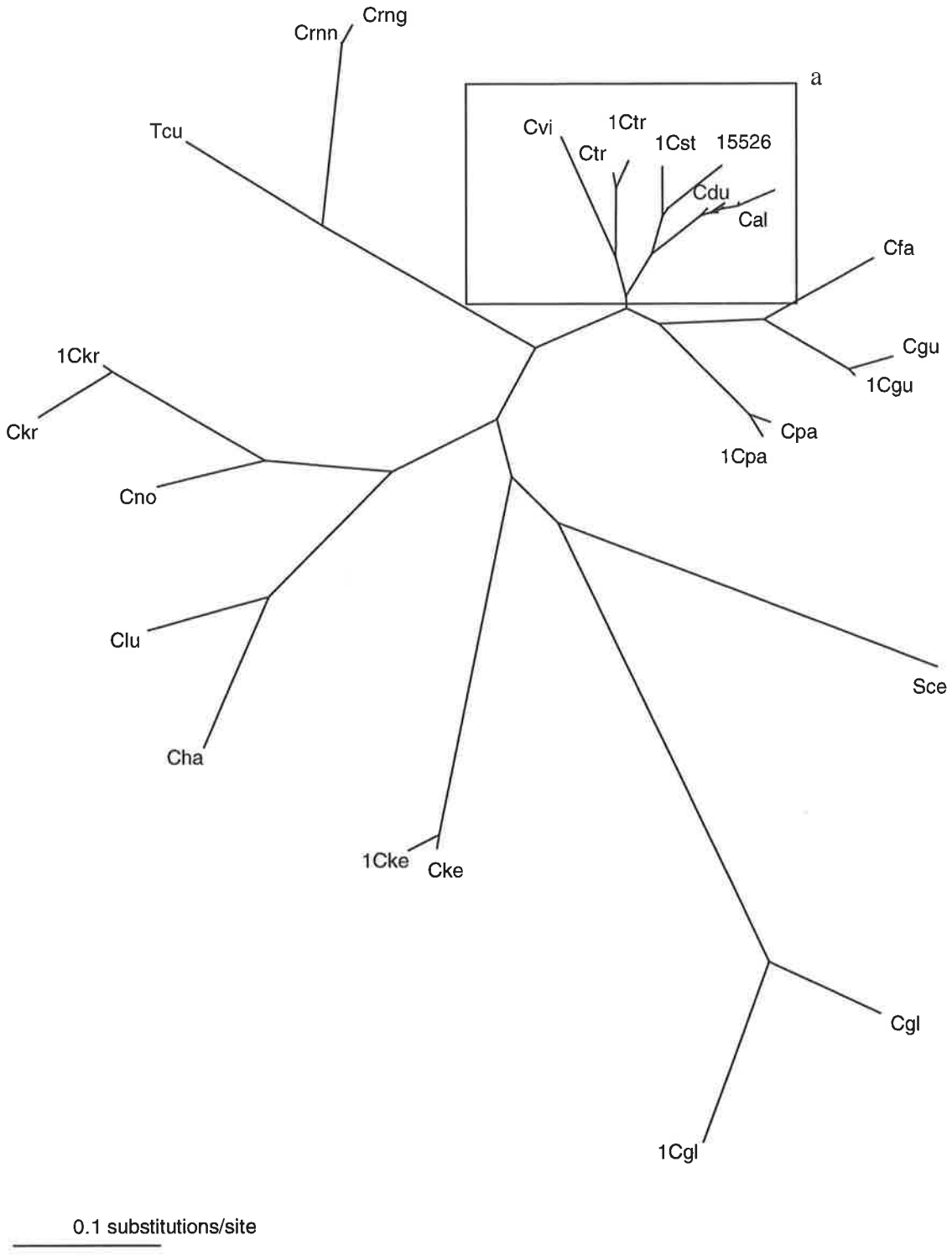


Figure 3.20a; Unrooted consensus tree of the phylogenetic relationships derived from 5.8S rRNA gene and ITS1 and 2 flanking region sequence comparisons for medically relevant *Candida* species and related genera

a; Clade of closely related *Candida* species whose genetic relationships were reanalysed independently (Fig 3.20b p., 237).

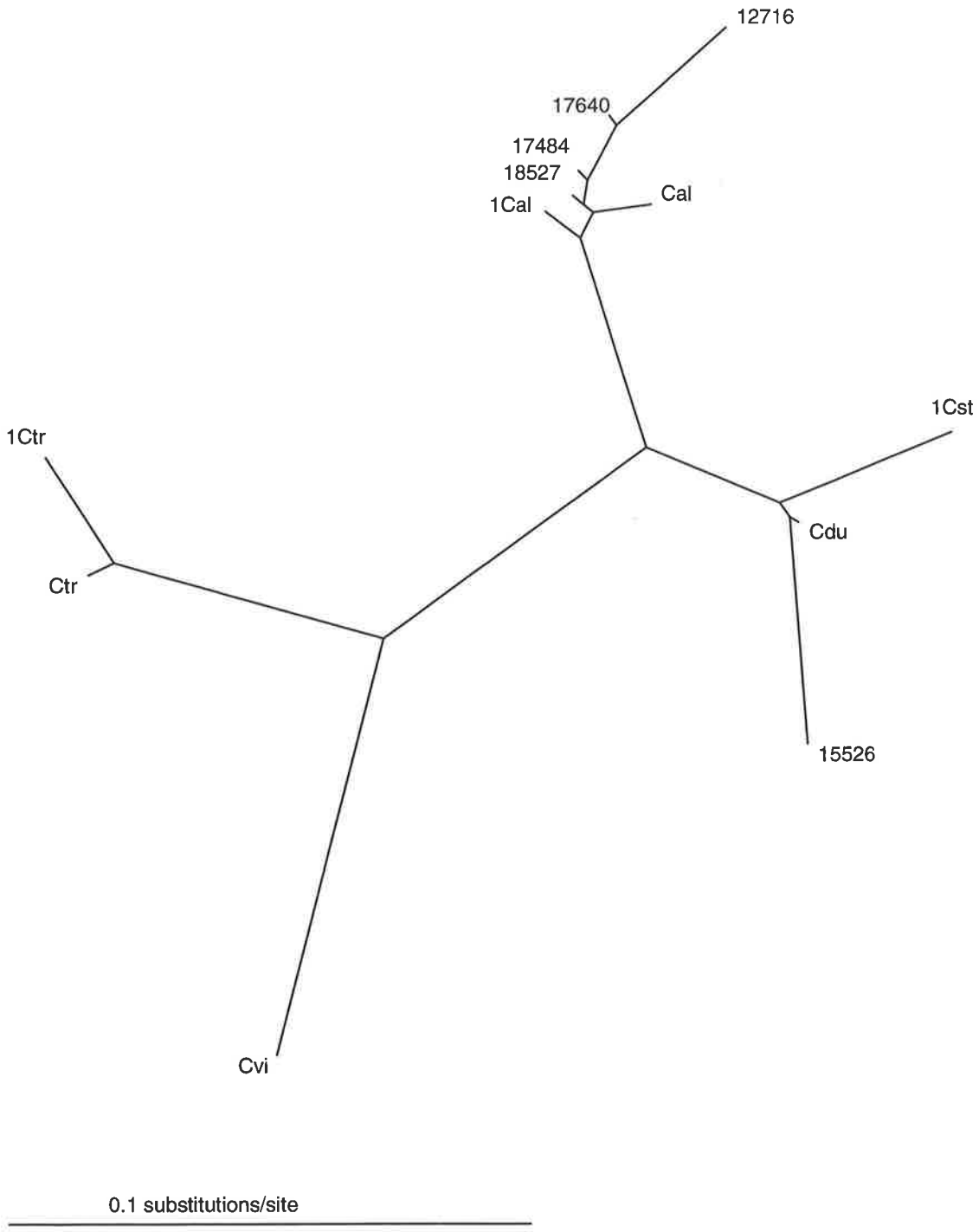


Figure 3.20b; Phylogram of the reanalysis of the 5.8S rRNA gene and ITS1 and 2 flanking region sequences of closely related *Candida* isolates highlighted in Figure 3.20a (p., 236)

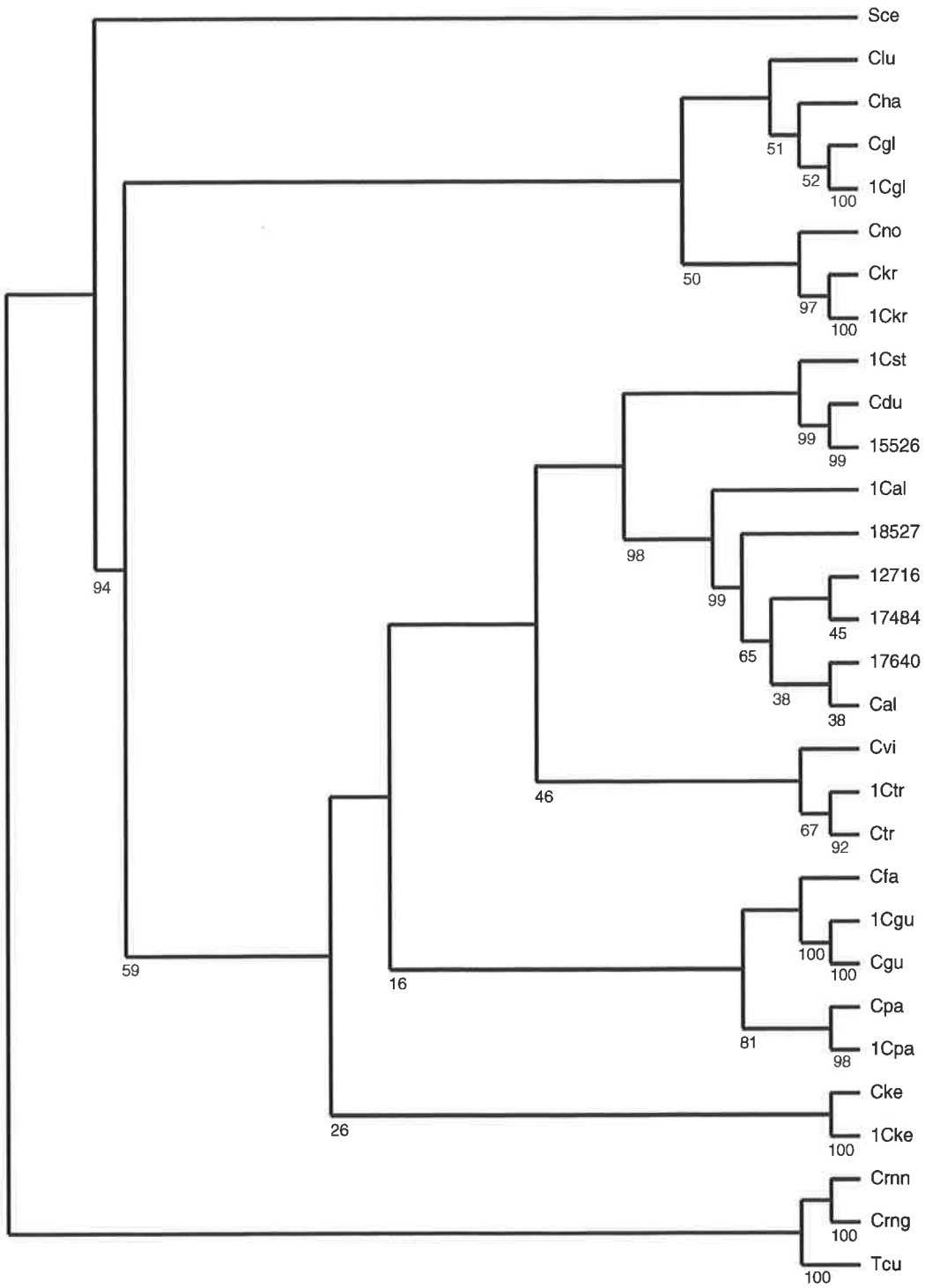


Figure 3.20c; Bootstrap values for phylogenetic relationships based on ITS1, 5.8S rRNA and ITS2 sequences obtained by maximum likelihood analyses from five-hundred replicates

Bootstrap values (denoted under each branch) exceeding 75 indicate that the branch has strong statistical support.

3.6.1.d Interpretation

There was separation of basidiomycetes and ascomycetes based on analysis of the ITS1, 5.8S rRNA and ITS2 sequences. However, unlike the previous 18S rRNA sequence analysis, there was strong support for the genus *Candida* forming a separate clade to *S. cerevisiae* (Figure 3.20c p., 238). This has arisen because the 5.8S and two flanking ITS regions are highly conserved within the species analysed, indicating that this region has a slow rate of evolution at this taxonomic level. In contrast, there was poor support for many of the branches within the genus *Candida*. Additionally, the high level of sequence conservation within the genus limits the epidemiological value of the ITS regions within *Candida*.

3.6.2 Phylogenetic utility of the ITS2 region

At a recent conference (International Symposium on Yeasts, Sydney, Australia, 1999) it was suggested that the ITS2 region of ribosomal RNA gene region may be phylogenetically informative for the genus *Candida* than 18S sequence data. Additionally, a recent paper by Chen *et al.* (2000) suggested the diagnostic utility of the ITS2 region. In the following subsection, this region has been assessed independently, using the sequences from Section 3.6.1 with the addition of currently available Genbank ITS2 sequences for *Candida* (Table 3.53a-b p., 241-2).

3.6.2.a Results

The Clustal X alignment of sequences at the ITS2 region is presented in Table 3.54a-h (p., 243-50). Some of the previously published Genbank sequences are only small segments of the ITS2 region, which limited the number of nucleotides that could be used for a complete alignment across *Candida* species. Therefore, areas of homology across all aligned sequences were not identified. There was extensive sequence variation between *Candida* species and related genera. Most sequences grouped into species, with the exception of sequence Ca17, a very short sequence that aligns more closely with basidiomycete sequences than with *C. albicans* sequences. As with the comparisons of ITS1, ITS2 and the 5.8S rRNA gene in the previous subsection (Section 3.6.1, p., 225), the ascomycetes and basidiomycetes do not form distinct lineages (Figure 3.21a p., 251). Although each species had a unique ITS2 sequence, this rRNA region did not provide a

sufficient number of phylogenetically informative characters to infer relationships within the genus *Candida*. However, a subsequent analysis including species with similar sequences (ie., *C. albicans*, *C. dubliniensis* and *C. stellatoidea*) showed that all 12 isolates of *C. albicans* formed a discrete clade to the exclusion of the 7 isolates of *C. dubliniensis* and the single isolate of *C. stellatoidea*.

3.6.2.b Interpretation

Bootstrap values obtained using maximum likelihood analysis on five-hundred replicates provided very low support for most branches (Figure 3.21c p., 253). These data indicate that the ITS2 sequence is of limited epidemiological or phylogenetic utility.

Table 3.53a; Details of the sequences and isolates used to assess the phylogenetic utility of the ITS2 region

Species	Accession number [a]	Sequence [b]	Strain [c]
C. dubliniensis	ABO35589	Cdu89	CBS7987
C. dubliniensis	AB035590	Cdu90	CBS7988
C. glabrata	ABO32177	Cgl77	IFO 0622
P. guilliermondii	ABO 32176	Pgu76	JCM1539
C. albicans	ABO32174	Cal74	NIHB792
C. albicans	ABO32173	Cal73	NIHA207
C. albicans	ABO32172	Cal72	CBS562
C. albicans	AJ249486	Cal86	GDH2346
C. dubliniensis	AJ249485	Cdu85	CD36
C. dubliniensis	AJ249484	Cdu84	M334a
C. albicans	ABO18038	Cal38	CBS1905, 1918
C. albicans	ABO18037	Cal37	CBS562, NIHB792, NIHA207, IFO 1061, 1389, 0589, 0579
C. glabrata	AF167993	Cgl93	ATCC15545
C. dubliniensis	U96719	Cdu19	CBS7987
P. norvegensis	U70509	Pno09	ATCC22977
P. guilliermondii	U70499	Pgu99	ATCC6260
K. marxianus	U70502	Kma02	ATCC46764
D. hansenii	U70500	Dha00	ATCC62894
C. viswanathii	U70510	Cvi10	ATCC22981
C. haemulonii	U70501	Cha01	ATCC22991
C. glabrata	U70498	Cgl98	Y-65
C. lusitaniae	AF009215	Clu15	ATCC34449
C. parapsilosis	U10989	Cpa89	MCO429
C. parapsilosis	U10988	Cpa88	MCO457
C. parapsilosis	U10987	Cpa87	ATCC22019
C. albicans	L28817	Cal17	WO1
C. albicans	-	17484	clinical [d]
C. albicans	-	12716	clinical
C. albicans	-	18527	clinical
C. albicans	-	17640	clinical
C. albicans	-	Cal	CBS562
C. dubliniensis	-	15526	clinical
C. dubliniensis	-	Cdu	CBS7987
C. stellatoidea	L47114	1Cst	? [e]
C. tropicalis	L47112	1Ctr	?
C. tropicalis	-	Ctr	CBS94
C. viswanathii	-	Cvi	CBS4024

a; Accession number refers to the Genebank accession number.
b; Sequence refers to the code used for sequence analysis and corresponds to the codes in Table 3.54 and Figures 3.21a-c (p., 243-53).
c; Strain refers to the isolate from which the sequence was obtained.
d; Clinical refers to a clinical strain not lodged in a culture collection.
e; ? strain refers to the sequence being obtained from an unspecified strain.

Table 3.53b; Details of the sequences and isolates used to assess the phylogenetic utility of the ITS2 region

Species	Accession number [a]	Sequence [b]	Strain [c]
C. guilliermondii	-	Cgu	CBS566
C. guilliermondii	L47110	1Cgu	? [d]
C. famata	-	Cfa	CBS940
C. glabrata	-	Cgl	CBS138
C. glabrata	L47108	1Cgl	?
C. kefyr	-	Cke	CBS834
C. kefyr	L47107	1Cke	?
C. haemulonii	-	Cha	CBS5149
C. lusitaniae	-	Clu	CBS6936
T. beigelii	-	Tbe	CBS2466
Cr. neoformans var. *neoformans*	-	Crnn	CBS132
Cr. neoformans var. *gattii*	-	Crng	CBS6289
C. norvegensis	-	Cno	CBS6564
C. krusei	-	Ckr	CBS573
C. krusei	L47113	1Ckr	?
S. cerevisiae	-	Sce	ATCC2610
C. parapsilosis	-	Cpa	CBS604
C. parapsilosis	L47109	1Cpa	?

a; Accession number refers to the Genebank accession number.
b; Sequence refers to the code used for sequence analysis and corresponds to the codes in Table 3.54 and Figures 3.21a-c (p., 243-53).
c; Strain refers to the isolate from which the sequence was obtained.
d; ? strain refers to the sequence being obtained from an unspecified strain.

Table 3.54a; CLUSTAL X (1.64b) multiple sequence alignment of the ITS2 region in *Candida* and related genera. Highlighted areas are homologous regions across all aligned sequences. The identities of the isolates assessed is within Table 3.53 p., 241-2

```
17484           ----------------------------------------------------------------------------------------------------
Cal74           ----------------------------------------------------------------------------------------------------
Cal72           ----------------------------------------------------------------------------------------------------
Cal37           ----------------------------------------------------------------------------------------------------
Cal73           ----------------------------------------------------------------------------------------------------
Cal38           ----------------------------------------------------------------------------------------------------
12716           ----------------------------------------------------------------------------------------------------
18527           ----------------------------------------------------------------------------------------------------
1Cal            ----------------------------------------------------------------------------------------------------
17640           ----------------------------------------------------------------------------------------------------
Cal             ----------------------------------------------------------------------------------------------------
Cdu89           ----------------------------------------------------------------------------------------------------
Cdu85           ----------------------------------------------------------------------------------------------------
Cdu19           ----------------------------------------------------------------------------------------------------
15526           ----------------------------------------------------------------------------------------------------
Cdu84           ----------------------------------------------------------------------------------------------------
Cdu             ----------------------------------------------------------------------------------------------------
Cdu90           ----------------------------------------------------------------------------------------------------
1Cst            ----------------------------------------------------------------------------------------------------
1Ctr            ----TATTATTACAATAGTCANAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGATACGTAATATGAATTGCAGATATTCGTGAATCATCGAATC
Ctr             ----TATTATTACAATAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGATACGTAATATGAATTGCAGATATTCGTGAATCATCGAATC
Cvi10           ----------------------------------------------------------------------------------------------------
Cvi             ----------------------------------------------------------------------------------------------------
Pgu76           ----------------------------------------------------------------------------------------------------
Pgu99           ----------------------------------------------------------------------------------------------------
Dha00           ----------------------------------------------------------------------------------------------------
Cgu             -AAATTTTGAATTAATCTTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATATGAATTGCAGATTTTCGTGAATCATCGAATC
1Cgu            -AAATTTTGAATTAATCTTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATATGAATTGCAGATTTTCGTGAATCATCGAATC
```

Table 3.54b; CLUSTAL X (1.64b) multiple sequence alignment of the ITS2 region in *Candida* and related genera. Highlighted areas are homologous regions across all aligned sequences. The identities of the isolates assessed is within Table 3.53 p., 241-2

```
Cfa         -TAAATTCAAAACAATCTTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATATGAATTGCAGATTTTCGTGAATCATCGAATC
Cgl77       ------------------------------------------------------------------------------------------------------------------------
Cgl98       ------------------------------------------------------------------------------------------------------------------------
Cgl93       ------------------------------------------------------------------------------------------------------------------------
Cgl         ------------------------------------------------------------------------------------------------------------------------
1Cgl        ------------------------------------------------------------------------------------------------------------------------
Kma02       ------------------------------------------------------------------------------------------------------------------------
Cke         -ACTATAAAATTTAATATTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAATTGCGATATGTATTGTGAATTGCAGATTTTCGTGAATCATCAAATC
1Cke        -ACTATAAAATTTAATATACAAAACTTCCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAATTGCGATATGTATTGTGAATTGCAGATNTCGTGAATCATCAAATCT
Cha01       ------------------------------------------------------------------------------------------------------------------------
Cha         ---------------AAAACAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAATTGCGATACGTAGTATGACTTGCAGACGTG----AATCATCGAATC
Clu15       ------------------------------------------------------------------------------------------------------CCCCTCTAACC
Clu         -------------TAATATCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAATTGCGATACGTAGTATGACTTGCAGACGTG----AATCATCGAATC
Cal17       GATTATTACTAATAGTCAAAA--CTTTC-AACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGATACGTAATATGAATTGCAGATATTCGTGAATCATCGAATC
Tbe         TGTTATTA-TAACAAAAATAA--CTTTC-AACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATC
Crnn        TCATATTA-TAACAATAATAAAACTTTC-AACAACGGATCTCTTGGCTTCCACATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTC
Crng        TCTTATTA-TAACAATAATAAAACTTTC-AACAACGGATCTCTTGGCTTCCACATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTC
Pno09       ------------------------------------------------------------------------------------------------------------------------
Cno         --AGAAGAAAGAAAAAAAACAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAGCGCAGCGAAATGCGATACCTAGTGTGAATTGCAGCCATC-GTGAATCATCGAGTT
Ckr         --ATCTACGAAAAACAAACAAAACTTTC-AACAACGGATCTCTTGGTTCTCGCATCGATGAAGAGCGCAGCGAAATGCGATACCTAGTGTGAATTGCAGCCATC-GTGAATCATCGAGTT
1Ckr        --ATCTACGANAAACANACAAAACTTGCTAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGATACCTAGTGTGAATTGCAGCCATC-GTGAATCATCGAGTT
Sce         --AAATTTTAAAAATATTAAAAACTTTC-AACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATTGCAGAATTCCGTGAATCATCGAATC
Cpa89       ------------------------------------------------------------------------------------------------------------------------
Cpa88       ------------------------------------------------------------------------------------------------------------------------
Cpa87       ------------------------------------------------------------------------------------------------------------------------
Cpa         ---AACCGATTATTTAATAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATATGAATTGCAGATATTCGTGAATCATCGAA
1Cpa        ---ACCCGATTATTTAATAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATATGAATTGCAGATATTCGTGAATCATCGAA
```

Table 3.54c; CLUSTAL X (1.64b) multiple sequence alignment of the ITS2 region in *Candida* and related genera. Highlighted areas are homologous regions across all aligned sequences. The identities of the isolates assessed is within Table 3.53 p., 241-2

```
17484   ----------------------------------G-GGCATGCCTGTTT-GAGCGTCGTTTCTCCCTCAAACCGCT----GGGTTTGGTGTTG-AGCAATACGACTT----------GGG
Cal74   ------------------------------------------------------------CTCCCTCAAACCGCT----GGGTTTGGTGTTG-AGCAATACGACTT----------GGG
Cal72   ------------------------------------------------------------CTCCCTCAAACCGCT----GGGTTTGGTGTTG-AGCAATACGACTT----------GGG
Cal37   ------------------------------------------------------------CTCCCTCAAACCGCT----GGGTTTGGTGTTG-AGCAATACGACTT----------GGG
Cal73   ------------------------------------------------------------CTCCCTCAAACCGCT----GGGTTTGGTGTTG-AGCAATACGACTT----------GGG
Cal38   ------------------------------------------------------------CTCCCTCAAACCGCT----GGGTTTGGTGTTG-AGCAATACGACTT----------GGG
12716   ----------------------------------G-GGCATGCCTGTTT-GAGCGTCGTTTCTCCCTCAAACCGCT----GGGTTTGGTGTTG-AGCAATACGACTT----------GGG
18527   ----------------------------------G-GGCATGCCTGTTT-GAGCGTCGTTTCTCCCTCAAACCGCT----GGGTTTGGTGTTG-AGCAATACGACTT----------GGG
1Cal    ----------------------------------G-GGCATGCCTGTTT-GAGCGTCGTTTCTCCCTCAAACCGCT----GGGTTTGGTGTT--AGCAATACGACTT----------GGG
17640   --------------------------------G-GGCATGCCTGTTT-GAGCGTCGTTTCTCCCTCAAACCGCT----GGGTTTGGT-TTG-AGCAATACGACTT----------GGG
Cal     --------------------------------G-GGCATGCCTGTTT-GAGCGTCGTTTCTCCCTCAAACCGCT----GGGTTTGGTGTTGAAGCAATACGACTT----------GGG
Cdu89   ------------------------------------------------------------TCCCTCAAACCCCTA---GGGTTTGGTGTTG-AGCAATACGACTT----------GGG
Cdu85   ------------------------------------------------------------TCCCTCAAACCCCTA---GGGTTTGGTGTTG-AGCAATACGACTT----------GGG
Cdu19   ------------------------------------------------------------CTCCCTCAAACCCCTA---GGGTTTGGTGTTG-AGCAATACGACTT----------GGG
15526   ----------------------------------G-GGCATGCCTGTTT-GAGCGTCGTTTCTCCCTCAAACCCCTA---GGGTTTGGTGTTG-AGCAATACGACTT----------GGG
Cdu84   ------------------------------------------------------------TCCCTCAAACCCCTA---GGGTTTGGTGTTG-AGCAATACGACTT----------GGG
Cdu     ----------------------------------G-GGCATGCCTGTTT-GAGCGTCGTTTCTCCCTCAAACCCCTA---GGGTTTGGTGTTG-AGCAATACGACTT----------GGG
Cdu90   ------------------------------------------------------------------------------------------------------------------------
1Cst    ----------------------------------G-GGCATGCCTGTTT-GAGCGTCGTTTCTCCCTCAAACCCCTA---GGGTTTGGTGTTG-AGCAATACGACTT----------GGG
1Ctr    TTTGAACGCACATTGCCCCCTTTGGTATTCCAAAG-GGNATGCCTGTTT-GAGCGTCATTTCTCCCCCAAACCCCC----GGGTTTGGTGTTG-AGCAANACCCT------------AGG
Ctr     TTTGAACGCACATTGCGCCCTTTGGTATTCCAAAG-GGCATGCCTGTTT-GAGCGTCATTTCTCCCTCAAACCCCC----GGGTTTGGTGTTG-AGCAATACGCT------------AGG
Cvi10   ------------------------------------------------------------CTCCCTCAACCCCGC----GGGTTTGGTGTTG-AGCAATACGCC------------AGG
Cvi     ----------------------------------G-GGCATGCCTGTTT-GAGCGTCATTTCTCCCTCAAGCCCGC----GGGTTTGGTGTTG-AGCAATACGCC------------AGG
Pgu76   ------------------------------------------------------------CTCTCTCAAACCCCC----GGGTTTGGTATTG-AGTGATACTCTTAGTCGGACTAGGCG
Pgu99   ------------------------------------------------------------CTCTCTCAAACCCCC----GGGTTTGGTATTG-AGTGATACTCTTAGTCGGACTAGGCG
Dha00   ------------------------------------------------------------CTCTCTCTC-CCCTT----GGGATTGGTATTG-AGAGATACTCTTAGTCGAACTAGGCG
Cgu     TTTGAACGCACATTGCGCCCTCTGGTATTCCAGAG-GGCATGCCTGTTT-GAGCGTCATTTCTCTCTCAAACCCCC----GGGTTTGGTATTG-AGTGATACTCTTAGTCGGACTAGGCG
1Cgu    TTTGNACGCACATTGCGCCCNCTGGTATTCCAGAG-GGGATGCCTGTTT-GGGCGTCANNCCTCTCTCAAACCCCC----GGGTTTGGTANTG-AGTGANACTCTTAGTCGGACTAGGCG
```

Table 3.54d; CLUSTAL X (1.64b) multiple sequence alignment of the ITS2 region in *Candida* and related genera. Highlighted areas are homologous regions across all aligned sequences. The identities of the isolates assessed is within Table 3.53 p., 241-2

```
Cfa         TTTGAACGCACATTGCACCCTCTGGTATTCCAGAG-GGTATGCCTGTTT-GAGCGTCATATCTCTCTCAAACCCTC----GGGTTTGGTATTG-AGTGATACTCTTAGTCGAACTAGGCG
Cgl77       ------------------------------------------------------------TTCTCAAACACAT-----TGTGTTTGGTAGTG-AGTGATACTC-------GTTTTTGAG
Cgl98       ------------------------------------------------------CCTTCTCAAACACAT-----TGTGGTTGGTAGTG-AGTGATACCC-------CGTTTTGAG
Cgl93       ----------------------------------------------------------TTCTCAAACACGT-----TGTGTTTGGTAGTG-AGTGATACTCTC-----GTTTTTGAG
Cgl         ------------------------------------------------------GCCGACAAAGACCTGGGAGTGTGCGTGGATCTC-TCT-ATTCCAAA-----GGAGGTGTT
1Cgl        ------------------------------------------------------GCCGACAAAGACCTGGGAGTGTGCGTGGATCTC-TCT-ATTCCGAA-----GGAGGTGTT
Kma02       ----------------------------------------------------------CTCTCTCAAACCT----TTGGGTTTGGTAGTG-AGTGATACTC-------GTCTCGGGC
Cke         TTTGAACGCACATTGCGCCCTCTGGTATTCCAGGG-GGCATGCCTGTTT-GAGCGTCATTTCTCTCTCAAACCT----TTGGGTTTGGTAGTG-AGTGATACTC-------GTCTCGGG-
1Cke        TTGGAACGCACATTGCGCCCTCTGGTATTCCAGGG-GGCATGCCTGTTT-GAGCGTCATTTCTCTCTCAAACCT----TTGGGTTTGGTAGTG-AGTGATACTC-------GTCTCGGG-
Cha01       ------------------------------------------------------GAGCGTGATATCT-TCTC--ACCGTT----GGT---GGATTTG-TTTCTAAATATCATG------CCACA
Cha         TTTGAACGCATATTGCGCCTTGGGGCATTCCCCAA-GGCATGCCTGTTT-GAGCGTGATATCT-TCTC--ACCGTT----GGT---GGATTTG-TTTCTAAATATCATG------CCACA
Clu15       CCCGGTTAGGCGTTGCTCCGAAATATCAACCGCGCTGTCAAACACGTTTAGAGCGTCGCATCC-CCTCTAACCCCC----GGTTA-GGCGTTG-CTCCGAAATATCAA-------CCGCG
Clu         TTTGAACGCACATTGCGCCTCGAGGCATTCCTCGA-GGCATGCCTGTTT-GAGCGTCGCATCC-CCTCTAACCCCC----GGTTA-GGCGTTG-CTCCGAAATATCAA-------CCGCG
Cal17       TTTGAACGCACATTGCGCCCTCTGGTATTCCGGA----------------------------------------------------------------------------------
Tbe         TTTGAACGCAACTTGCGCTCTCTGGTATTCCGGAG-AGCATGCCTGTTT-GAGTATCATGAAA-TCTCAACCATTA----GGGTT--TCTTAA------TGGA-TTGGA------TTTGG
Crnn        TTTGAACGCAACTTGCGCCCTTTGGTATTCCGAAG-GGCATGCCTGTTT-GAGAGTCATGAAAATCTCAATCCCTC----GGGTT--TTATTA-CCTGTTGGACTTGGA------TTTGG
Crng        TTTGAACGCAACTTGCGCCCTTTGGTATTCCGAAG-GGCATGCCTGTTT-GAGAGTCATGAAAATCTCAATCCCTC----GGGTT--TTATTA-CCTGTTGGACTTGGA------TTTGG
Pno09       ------------------------------------------------------------CCTTCTTGCGCAAGC----AGAA---GTTGGG----GTTGCC------------ACGGC
Cno         CTTGAACGCACATTGCGCCCTCCGGCATTCCGGGG-GGCATGCCTGTTT-GAGCGTCGTTTCCTTCTTGCGCAAGC----AGA----GTTGGG----GTTGCC------------ACGGC
Ckr         CTTGAACGCACATTGCGCCCCTCGGCATTCCGGGG-GGCATGCCTGTTT-GAGCGTCGTTTCCATCTTGCGCGTGC----GCAGA--GTTGGG----GGAGCGGAGCGG------ACGAC
1Ckr        CTTGAACGCACATTGCGCCCCTCGGCATTCCGGGG-GGCATGCCTGTTT-GAGCGTCGTTTCCATCTTGCGCGTGC----GCAGA--GTTGGG----GGAGCGGAGCGG------ACGAC
Sce         TTTGAACGCACATTGCGCCCCTTGGTATTCCAGGG-GGCATGCCTGTTT-GAGCGTCATTTCCTTCTCAAACATTC----TGTTT-GGTAGTG-AGTGATACTCTTTGGA----GTTAAC
Cpa89       ----------------------------------------------------------CTCCCTCAAACCTTC----GGGTTTGGTGTTG-AGCGATAC-CTGGG-------TTTGC
Cpa88       ----------------------------------------------------------CTCCCTCAAACCTTC----GGGTTTGGTGTTG-AGCGATAC-CTGGG-------TTTGC
Cpa87       ----------------------------------------------------------TCCCTCAAACCTTC----GGGTTTGGTGTTG-AGCGATAC-CTGGG-------TTTGC
Cpa         TCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTTGAGCGTCATTTCTCCCTCAAACCTC----GGGTTTGGTGTTG-AGCGATACGCTGGG-------TTTGC
1Cpa        TCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTTGAGCGTCATTTCTCCCTCAAACCCTC----GGGTTTGGTGTTG-AGCGATACGCTGGG-------TTTGC
```

Table 3.54e; CLUSTAL X (1.64b) multiple sequence alignment of the ITS2 region in *Candida* and related genera. Highlighted areas are homologous regions across all aligned sequences. The identities of the isolates assessed is within Table 3.53 p., 241-2

```
17484       TTTGCTTGAAAGACGGT------AGTGGTA--AGGCGGGATCGCTTTG-------A-CAATGGCTTAGGT-CTAACCAAAAACATT--GCTT---GCGG-CGGTAACGTCCACCACGTAT
Cal74       TTTGCTTGAAAGACGGT------AGTGGTA--AGGCGGGATCGCTTTG-------A-CAATGGCTTAGGT-CTAACCAAAAACATT--GCTT---GCGG-CGGTAACGTCCACCACGTAT
Cal72       TTTGCTTGAAAGACGGT------AGTGGTA--AGGCGGGATCGCTTTG-------A-CAATGGCTTAGGT-CTAACCAAAAACATT--GCTT---GCGG-CGGTAACGTCCACCACGTAT
Cal37       TTTGCTTGAAAGACGGT------AGTGGTA--AGGCGGGATCGCTTTG-------A-CAATGGCTTAGGT-CTAACCAAAAACATT--GCTT---GCGG-CGGTAACGTCCACCACGTAT
Cal73       TTTGCTTGAAAGACGGT------AGTGGTA--AGGCGGGATCGCTTTG-------A-CAATGGCTTAGGT-CTAACCAAAAACATT--GCTT---GCGG-CGGTAACGTCCACCACGTAT
Cal38       TTTGCTTGAAAGACGGT------AGTGGTA--AGGCGGGATCGCTTTG-------A-CAATGGCTTAGGT-CTAACCAAAAACATT--GCTT---GCGG-CGGTAACGTCTACCACGTAT
12716       TTTGCTTGAAAGACGGT------AGTGGTA--AGGCGGGATCGCTTTG-------AACAATGGCTTAGGT-CTAACCAAAAACATT--GCTT---GCGG-CGGTAACGTCTACCACGTAT
18527       TTTGCTTGAAAGACGGT------AGTGGTA--AGGCGGGATCGCTTTG-------A-CAATGGCTTAGGT-CTAACCAAAAACATT--GCTT---GCGG-CGGTAACGTCTACCACGTAT
1Cal        TTTGCTTGAAAGACGGT------AGTGGTA--AGGCGGGATCGCTTTG-------A-CAATGGCTTAGGT-CTAACCAAAAACATT--GCTT---GCGG-CGGTAACGTCCACCACGTAT
17640       TTTGCTTGAAAGACGGT------AGTGGTA--AGGCGGGATCGCTTTG-------A-CAATGG-TTAGGT-CTAACCAAAAACATT--GCTT---GCGG-CGGTAACGTCCACCACGTAT
Cal         TTTGCTTGAAAGACGGT------AGTGGTA--AGGCGGGATCGCTTTG-------A-CAATGGCTTAGGT-CTAACCAAAAACATT--GCTT---GCGG-CGGTAGCGTCTACCACGTAT
Cdu89       TTTGCTTGAAAGATGAT------AGTGGTATAAGGCGGAGATGCTT-G-------A-CAATGGCTTAGGT-GTAACCAAAAACATT--GCTAA-GGCGGTCTCTGGCGTCGCC-ATTTTA
Cdu85       TTTGCTTGAAAGATGAT------AGTGGTATAAGGCGGAGATGCTT-G-------A-CAATGGCTTAGGT-GTAACCAAAAACATT--GCTAA-GGCGGTCTCTGGCGTCGCCCATTTTA
Cdu19       TTTGCTTGAAAGATGAT------AGTGGTATAAGGCGGAGATGCTT-G-------A-CAATGGCTTAGGT-GTAACCAAAAACATT--GCTAA-GGCGGTCTCTGGCGTCGCCCATTTTA
15526       TTTGCTTGAAAGATGAT------AGTGGTATAAGGCGGAGATGCTT-G-------A-CAATGGCTTAGGT-GTAACCAAAAACATT--GCTAA-GGCGGTCTCTGGCGTCGCCCATTTTA
Cdu84       TTTGCTTGAAAGATGAT------AGTGGTA--AGGCGGAGATGCTT-G-------A-CAATGGCTTAGGT-GTAACCAAAAACATT--GCTAA-GGCGGTCTCTGGCGTCGCCCATTTTA
Cdu         TTTGCTTGAAAGATGAT------AGTGGTATAAGGCGGAGATGCTT-G-------A-CAATGGCTTAGGT-GTAACCAAAAACATT--GCTAA-GGCGGTCTCTGGCGTCGCCCATTTTA
Cdu90       ----------------------------------------------------------------------------------------------------------------------------
1Cst        TTTGCTTGAAAGATGAT------AGTGGTA--AGGCGGAGATGCTT-G-------A-CAATGGCTTAGGT-GTAACCAAAAACATT--GCTAA-GGCGGTCTCTGGCGTCGCCCATTTTA
1Ctr        TTTGTTTGAAAGAATTT------A--CGTG----G--AAACTTATTTT-------A--AGCGACTTAGGT-TTANCCAAAA-C-----GCTTA-TTTTGCTAGTGGCCACCACAATTTA-
Ctr         TTTGTTTGAAAGAATTT------AA-CGTG----G--AAACTTATTTT-------A--AGCGACTTAGGT-TTATCCAAAAAC-----GCTTA-TTTTGCTAGTGGCCACCACAATTTA-
Cvi10       TTTGTTTGAAAGACGT-------A--CGTG----G--AGACA-ATATT-------A---GCGACTTAGGT-TCTACCAAAA-C-----GCTTG-TGCAGTCGGTCCCACACACAGTGTAA
Cvi         TTTGTTTGAAAGACGT-------A--CGTG----G--AGACT-ATATT-------A---GCGACTTAGGT-TCTACCAAAA-C-----GCTTG-TGCAGTCGGCCCACCACAGCTTTT--
Pgu76       TTTGCTTGAAAAGTATTGGC---ATGGGTAGTACTGGATAGTGCTGTC--GACCTCTCAATGTATTAGGT--TTATCCAACTCGTT--GAATGGTGT-GGCGGGATATTTCTGGTATTGT
Pgu99       TTTGCTTGAAAAGTATTGGC---ATGGGTAGTA-TGGATAGTGCTGTC--GACCTCTCAATGTATTAGGT--TTATCCAACTCGTT--GAATGGTGT-GGCGGGATATTTCTGGTATTGT
Dha00       TTTGCTTGAAATTTATTGGC---ATGAGTGACGCTGAGAAGTGCATTCAGGAAATATCAATGTATTAGGTAGTTATCCAACTCGTT--GACAATTCTTGGTTGTGAATTTTTGGTGTTA-
Cgu         TTTGCTTGAAAAGTATTGGC---ATGGGTAGTACTGGATAGTGCTGTC--GACCTCTCAATGTATTAGGT--TTATCCAACTCGTT--GAATGGTGT-GGCGGGATATTTCTGGTATTGT
1Cgu        TTTGCTTGAAAANTATTGTC---ATGGGTAGTACTAGATAGTGCTGTC--GACCTCTCAATGTATTAGGT--TTATCCAACTCGNT--GAATGGTGT-GGCGGGATATTTCTGGTATTGT
```

Table 3.54f; CLUSTAL X (1.64b) multiple sequence alignment of the ITS2 region in *Candida* and related genera. Highlighted areas are homologous regions across all aligned sequences. The identities of the isolates assessed is within Table 3.53 p., 241-2

```
Cfa         TTTGCTTGAAATATATTGGC---ACGAGTAGTGTTGAACAGTGTTGTCT-GAAC-ATCAATGTATTAGGT--TTATCCAACTCGTT--GA----TACAGGTGGTACGATTCTTC-ATTA-
Cgl77       TTAACTTGAAATTGTAGG-------CCATATCAGTATGTGGGACACGAGCGCAAGCTTCTCTATTAATCTGCTGCTCGTTTGCGCGA-GCGG-----CGGGGGTTAATACTGTATTAGGT
Cgl98       CTAACTTGAAATTGTAGG-------CCATATCAGTATGTGGGACACGAGCGCAAGCTTCTCTATTAATCTGCTGCTCGTTTGCGCGA-GCGG-----CGGGGGTTAATACTGTATTAGGT
Cgl93       TTAACTTGAAATTGTAGG-------CCATATCAGTATGTGGGACACGAGCGCAAGCTTCTCTATTAATCTGCTGCTCGTTTGCGCGA-GCGG-----CGGGGGTTAATACTGTATTAGGT
Cgl         TTATCACACGACTCGACA-------CTTTCTAATTACTACACACAGTGGAGTTTACTTTACTACTATTCTTTTGTTCGTTGGGGGAACGCTCTCTTTCGGGGGGGAGTTCTCCCAGTGGA
1Cgl        TTATCACTCGACTCGACA-------CTTTCTAATTACTACACACAGTGGAGTTTACNTTNCTACTATTCTTTTGTTCGTTGGGGGAAAGCTCTCTTTCGGGAGGGAGTTCTCCCAGTGGA
Kma02       TTAACTTGAAAGTGGCTAACCGTTGCCATCTGCGTGAGCAGG-CTGCGTGTCAAGTCTATGGACTCGACTCTTGCACATCTACGTCTTAAGTA--TGCGCCAATTCGTGGTAAGCTTGGG
Cke         TTAACTTGAAAGTGGCTAGCCGTTGCCATCTGCGTGAGCAGGGCTGCGTGTCAAGTCTATGGACTCGACTCTTGCACATCTACGTCTTAGGTT--TGCGCCAATTCGTGGTAAGCTTGGG
1Cke        TTNACTTGAAAGTGGCTAGCCGTTGCCATCTGCGTGAGCAGGGCTGCGTGTCGAGTCTATGGACTCGACTCTTGCACATCTACGTCTTAGGTT--TGCGCCAATTCGTGGTAAGCTTGGG
Cha01       GTGAAGTCTACGC-----------------------------------------------------------------------------------------------------------
Cha         GTGAAGTCTACGC---TTTCACTGCTTTTTCCCCTCAAATCAGGTAGG--ACTACCCGCTGAACTTAAGCATATCAATA-----------------------------------------
Clu15       CTGTCAAATACG----CAGCACCACATTTCGCCCTCAAATCAGGTAGG--ACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA--------------------------------
Clu         CTGTCAAACACGTTTACAGCACGACATTTCGCCCTCAAATCAGGTAGG--ACTACCCGCTGAACTTAAGCATATCAATAAGCGGAG-----------------------------------
Cal17       -----------------------------------------------------------------------------------------------------------------------
Tbe         GCGCTGCCAGTAGCCTG------------GCTCGCCTTAAAAGA---G--TTAGCGTGTTTAACTTGTCTTATCTG----GCGTAATAAGTTTCGCTGGTGTTGACTTGAGAAGTGCGCT
Crnn        GTGTTTGCCGCGACCTGCAAAGGACGTCGGCTCGCCTTAAATGT---G--TTAGTGGGAAGGTGATTACCTGTCAGCCCGGCGTAATAAGTTTCGCTGGGCCT--ATGGGGTAGTCTTCG
Crng        GTGTTTGCCGCGACCTGCAAAGGACGTCGGCTCGCCTTAAATGT---G--TTAGTGGGAAGGTGATTACCTGTCAGCCCGGCGTAATAAGTTTCGCTGGGCCT--ATGGGGTAGTCTTCG
Pno09       CCGT-----GCGGCCTG------TGTGTGGCTCCCC-GAAACGGAACG--GCAGCGGGACTGAGCGAAGTACACAA-----------CACTCGCGCTTGGCCCGCC-GAACTTTTTTTT-
Cno         CCGT-----GCGGCCTG------TGTGTGGCTCCCCCGAAACGGAACG--GCAGCGGGACTGAGCGAAGTACACAA-----------CACTCGCGCTTGGCCCGCC-GAACTTTTTTTT
Ckr         GTGTAAAGAGCGTCGGA------GCTGCGACTCGCCTGAAAGGGAGCG--A-AGCTGG-CCGAGCGAACTAGACTTT------TTTTCAGGGACGCTTGGCGGCCGAGAGCGAGTGTTGC
1Ckr        GTGTAAAGAGCGTCGGA------GCTGCGACTCGCCTGAAAGGGAGCG--A-AGCTGG-CCGAGCGAACTAGACTTT------TTTTCAGGGACGCTTGGCGGCCGAGAGCGAGTGTTGC
Sce         TTGAAATTGCTGGCCTTTTCAT-TGGATGTTTTTTTTCCAAAGAGAGG--TTTCTCTGCGTGCTTGAGGTATAATGCAAGTACGGTCGTATTAGGTTTTACCAACTGCGGCTAATCTTT-
Cpa89       TTGAAAGAAAGGCGGAGTATAAACTAATGGATAGGTTTTTTTTT------TCCACTCATTGGTACAAACTCCAAAC---ATTCTTCCAAATT-------------------------
Cpa88       TTGAAAGAAAGGCGGAGTATAAACTAATGGATAGGTTTTTTT---------CCACTCATTGGTACAAACTCCAAA----ATTCTTCCAAATT-------------------------
Cpa87       TTGAAAGAAAGGCGGAGTATAAACTAATGGATAGGTTTTTTT---------CCACTCATTGGTACAAACTCCAAAA---CTTCTTCCAAATT-------------------------
Cpa         TTGAAAGAAAGGCGGAGTATAAACTAATGGATAGGTTTTTTT----------CCACTCATTGGTACAAACTCCAAAA---CTTCTTCCAAATTCGACCTCAAATCAGGTAGGACTACCCGC
1Cpa        TTGAAAGAAAGGCGGAGTATAAACTAATGGATAGGTTTTTTT----------CCACTCATTGGTACAAACTCCAAAA---CTTCTTCCAAATTCGACCTCAAATCAGGTAGGCCTACCCGC
```

Table 3.54g; CLUSTAL X (1.64b) multiple sequence alignment of the ITS2 region in *Candida* and related genera. Highlighted areas are homologous regions across all aligned sequences. The identities of the isolates assessed is within Table 3.53 p., 241-2

```
17484           ATCTTCAAACTTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTACTT-----------------------------------------
Cal74           ATCTTCAAACT-----------------------------------------------------------------------------------
Cal72           ATCTTCAAACT-----------------------------------------------------------------------------------
Cal37           ATCTTCAAACT-----------------------------------------------------------------------------------
Cal73           ATCTTCAAACT-----------------------------------------------------------------------------------
Cal38           ATCTTCAAACT-----------------------------------------------------------------------------------
12716           ATCTTCAAACTTTG--------------------------------------------------------------------------------
18527           ATCTTCAAACTTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAAG-----------------------
1Cal            ATCTTCAAACTTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA-----------------------
17640           ATCTTCAAACTTTGACCTCAAATCAGGTAGGA-TACCCGCTGAACTTAAGCATATCAATAAGCAGAA-------------------------
Cal             ATCTTCAAACTTTGACCTCAAATCAGGTAGGA-TACCCGCTGAACTTAA---------------------------------------------
Cdu89           TTCTTCAAACT-----------------------------------------------------------------------------------
Cdu85           TTCTTCAAACTTT---------------------------------------------------------------------------------
Cdu19           TTCTTCAAAC------------------------------------------------------------------------------------
15526           TTCTTCAAACTTTGACCTCAAATCAGGTATTACTAGCCCCTGAACTTACACCTC----------------------------------------
Cdu84           TTCTTCAAACTTT---------------------------------------------------------------------------------
Cdu             TTCTTCAAACTTTGACCTCAAATCAGGTAGGASTACCCGYTGAACTTAAGCATATCAATAAGCGGAAAGA---------------------
Cdu90           ----------TTGACCTCAAATCAGGTAG----------------------------------------------------------------
1Cst            TTCTTCAAACTTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA-----------------------
1Ctr            ------------------------------------------------------------------------------------------------
Ctr             ------------------------------------------------------------------------------------------------
Cvi10           GCTAACA----------------------------------------------------------------------------------------
Cvi             -CTAACTTTTGACCTCAAATCAGGTGTGACTACCCGCTGAACTTAANCAT---------------------------------------------
Pgu76           TGGCCCGGCCTTACAACA-ACCAAACAAG-----------------------------------------------------------------
Pgu99           TGGCCCGGCCTTACAACA-ACCAAACAAGC----------------------------------------------------------------
Dha00           -GGCTTTGCCTTAAAAAACAACAAACAA------------------------------------------------------------------
Cgu             TGGCCCGGCCTTACAACA-ACCAAACAAGCTTGACCTCAAATCAGGTAGGAATACCCGCTGAACTTAA-----------------------
1Cgu            TGACCCGGCCTTACAACA-ACCAAACAAGTTTGACCTCAAATCAGGNAGGAATANCCGCTGAACTTAAGCATATCAATAAGC---------
```

Table 3.54h; CLUSTAL X (1.64b) multiple sequence alignment of the ITS2 region in *Candida* and related genera. Highlighted areas are homologous regions across all aligned sequences. The identities of the isolates assessed is within Table 3.53 p., 241-2

```
Cfa             -GGCTTTGCCTTATAAAA-CACAAACAAGTTTGACCTCAAATCCAGGTAGGATTACCCGCTGAACTTTATCCTATCCCAATA---------
Cgl77           T-TTACCAACTCGGTGTTGATCTAGGGAGGGATAAGTGAGTGTTTT-GTGCGT-GCTG-GGCAGACAGACGTCTTTAAGTTT---------
Cgl98           T-TTACCAACTCGGTGTTGATCTAGGGAGGGATAAGTGAGTGTTTT-GTGCGT-GCTG-GGCAGACAGACGTCTTTAAG-----------
Cgl93           T-TTACCAACTCGGTGTTGATCTAGGAAGGGATAAGTGAGTGTTCTCGTGCGTCGCTGAGGCAAACAGACGTCTTTAAGTTT---------
Cgl             T-GCAAACACAAACAAATATTTTTTTAAA--CTAATTCAGTCAACACAAGATTTCTTTTAGTAGAAA-ACAACTTCAAAACT---------
1Cgl            T-NCACACACNAACAAATATTTTTTTNAA--ATAATTCAGTCAACACAAGATTTCTTTTAGTAGAAA-ACAACTTCAAAACT---------
Kma02           TCATAGAGACTCATAGGTGTTATAAAGACTCGCTGGTGTTTGTCTCCTTGAGGCATACGGCTTTAC----AACTCTCAAG-----------
Cke             TCATAGAGACTCATAGGTGTTATAAAGACTCGCTGGTGTTTGTCTCCTTGAGGCATACGGCTTTAACCAAAACTCTCAAAGTTTGACCTCA
1Cke            TCATAGAGACTCATAGGTGTTATAAAGACTCGCTGGTGTTTGTCTCCTTGAGGCATACGGCTTTAACCAAAACTCTCAAAGTTTGACCTCA
Cha01           ----------------------------------------------------------------------------------------
Cha             ----------------------------------------------------------------------------------------
Clu15           ----------------------------------------------------------------------------------------
Clu             ----------------------------------------------------------------------------------------
Cal17           ----------------------------------------------------------------------------------------
Tbe             TCTAATCGTCCTCGGACAATTCTTGAA-CTCTGGTCTCAAATCAGGTAGG-CTACCCGCTGAACTTAA-------------------------
Crnn            GCTTGCTGATAACAACCATCTCTTTTT-GTTTGACCTCAAATCAGGTAGGGCTACCCGCTGAACTTAA-------------------------
Crng            GCTTGCTGATAACAACCATCTCTTTTTTGTTTGACCTCAAATCAGGTAGGGCTACCCGCTGAACTTA-------------------------
Pno09           ------AATCTA-----AG----------------------------------------------------------------------
Cno             T-----AATCTA-----AGCTC----------GACCTCAAATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGC---------
Ckr             G-----AGACAACAACAAGCTC----------GACCTCAACTCAGGTAGGAAGACCCGCTGAACTTAAGCATATCAATAAGC---------
1Ckr            G-----AGACAACAAAAAGCTC----------GACCTCAAATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGC---------
Sce             ------TTTGTACTG--AGCGTATTGGAACGTTATCGATAAGAAGAGAGC-CTNTAGGCGGACAATATTCTTATTAGNAAGA---------
Cpa89           ----------------------------------------------------------------------------------------
Cpa88           ----------------------------------------------------------------------------------------
Cpa87           ----------------------------------------------------------------------------------------
Cpa             TGAACTTA---------------------------------------------------------------------------------
1Cpa            TGAACTTAAGCATATCAATAAGCGGAGGA------------------------------------------------------------
```

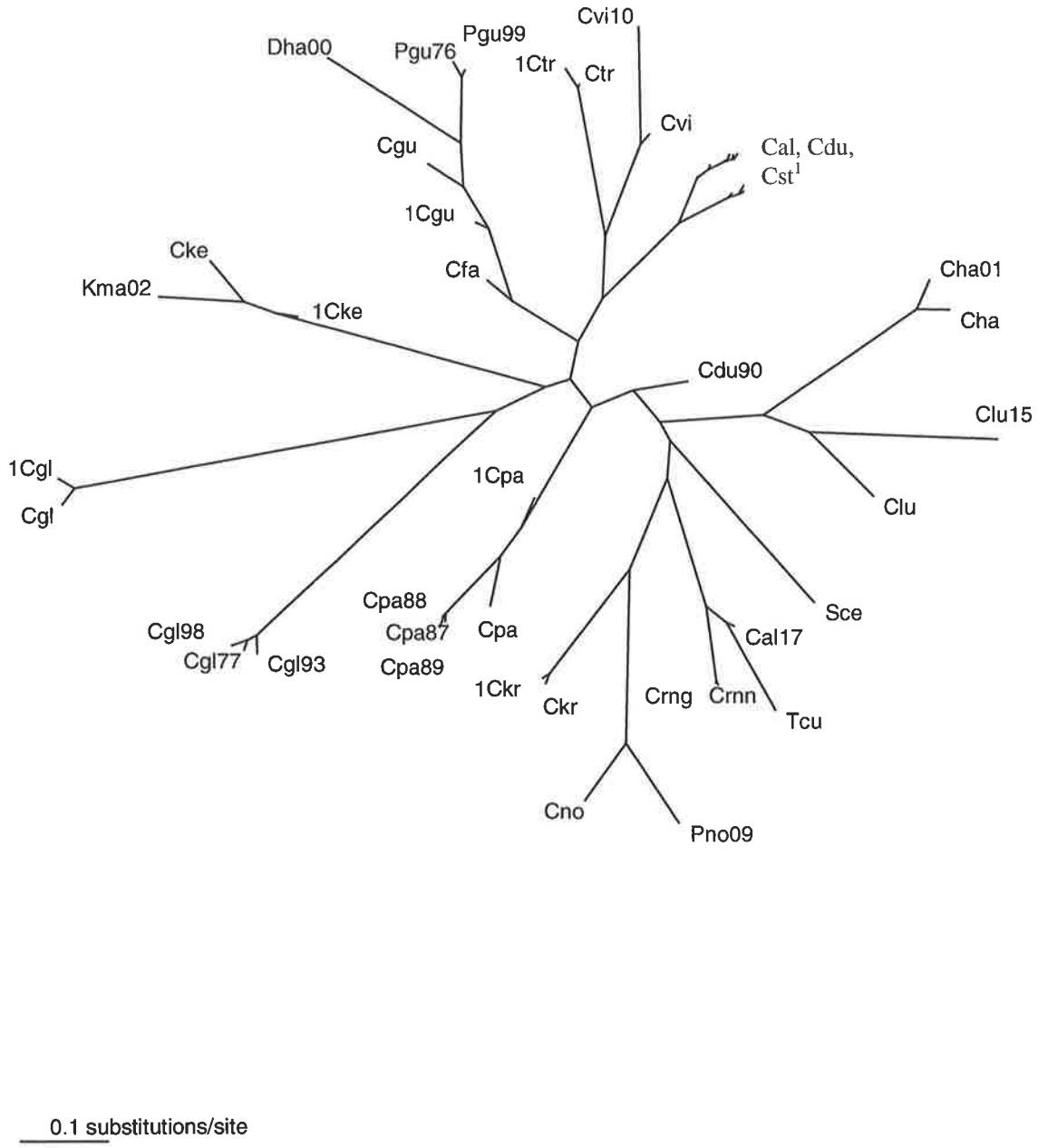


Figure 3.21a; Consensus tree of the phylogenetic relationships derived from ITS2 sequence comparisons for medically relevant *Candida* species and related genera

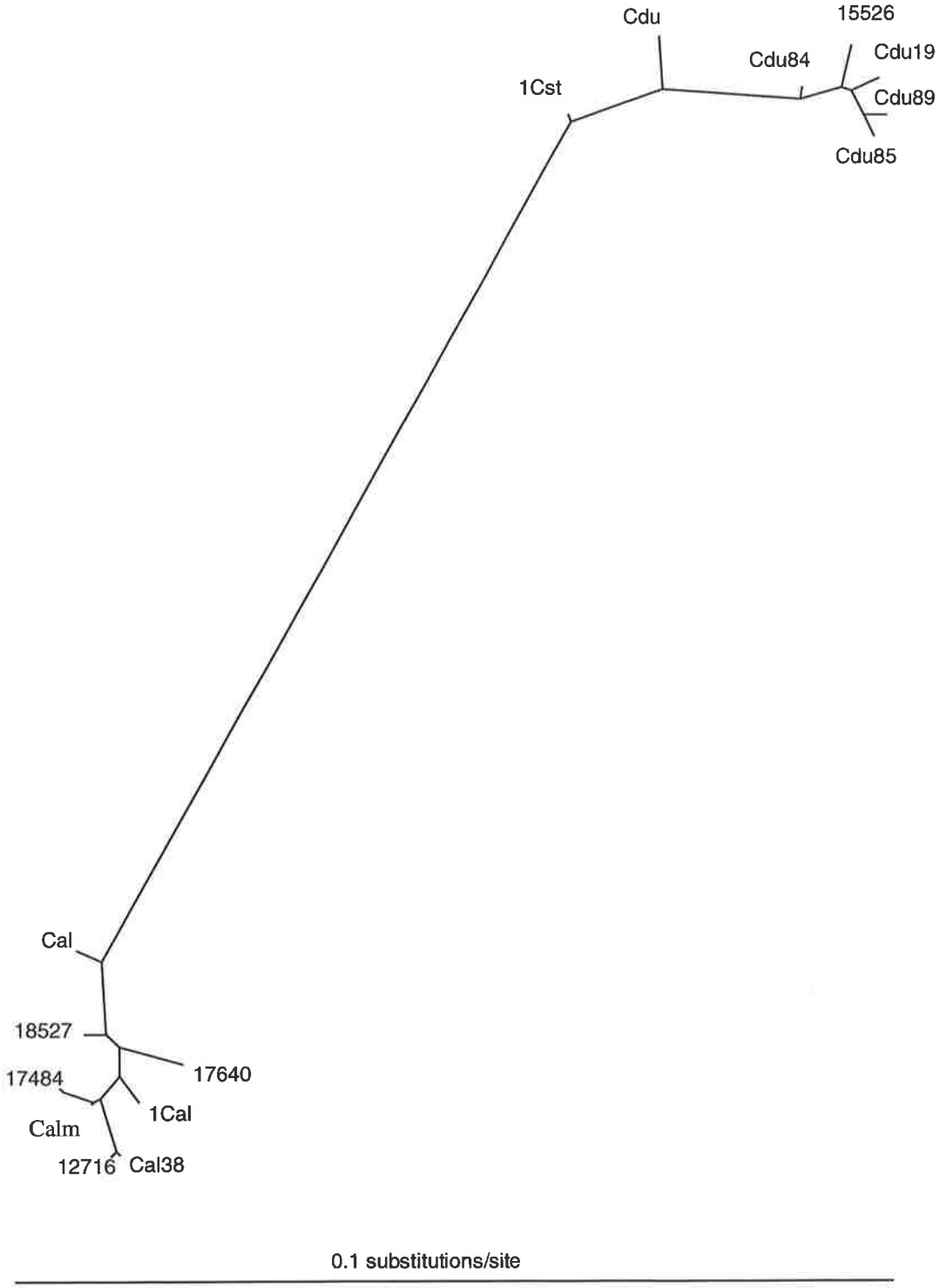


Figure 3.21b; Phylogram of the reanalysis of the ITS2 sequences of the closely related isolates from Figure 3.21a (p., 251)

a; Calm contains identical *C. albicans* sequences Cal37, 72, 73 and 74

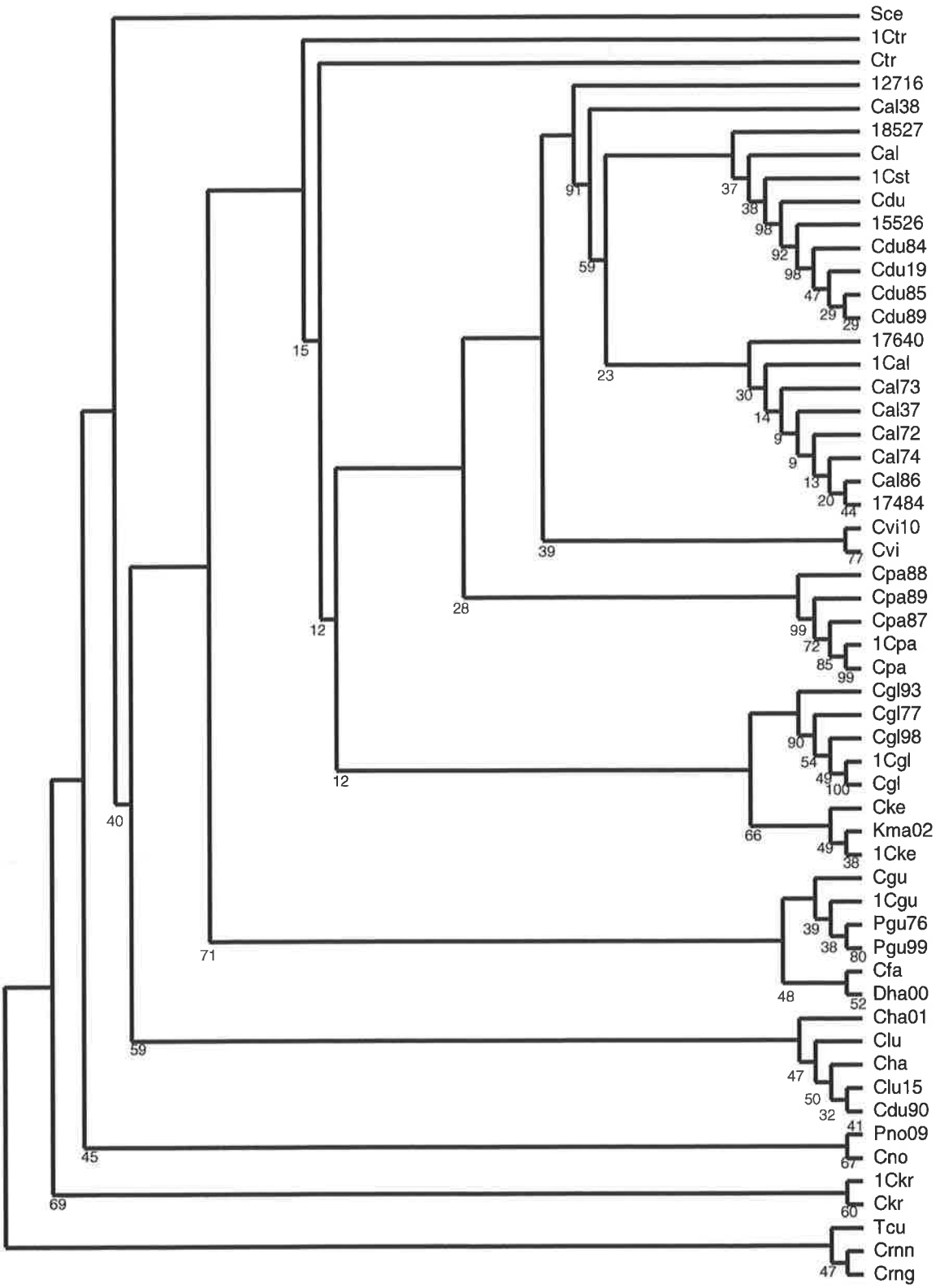


Figure 3.21c; Bootstrap values for phylogenetic relationships based on ITS2 sequences obtained by maximum likelihood analyses from five-hundred replicates

Bootstrap values (denoted under each branch) exceeding 75 indicate that the branch has strong statistical support.

4 DISCUSSION

4.1 *Restatement of Aim*

The specific aim of this study was;

> *To examine the systematics of the genus Candida using traditional and contemporary methodologies and to assess their diagnostic potential to unequivocally identify and characterise species and strains of this medically and dentally important yeast genus.*

The results from each study have been discussed independently in the interpretation sections of the Results Chapter. The following sections address the general observations from these studies in the context of the aim.

4.2 *Epidemiology of oral Candida colonisation*

Of the four-hundred and seven isolates obtained from 101 HIV-positive and 20 HIV-negative individuals in this study, one-hundred and twenty of them (29.5%) comprised mixed infections or isolates heterozygous for one or more enzyme loci. The incidence of mixed or heterozygous colonisation detected in HIV-positive individuals appears high. Mixed infections may indicate a transitional stage of strain turnover, or co-colonisation with a number of strains or species. This observation has clinical implications for the acquisition of antifungal resistant infections, sources of reinfection and the rapid rate of strain turnover seen as a consequence of fluconazole use observed in this thesis, as are discussed in the relevant sections below. Alternatively, heterozygosity has important biological relevance, suggesting that *Candida* may have a sexual life cycle. This is also discussed below.

Two hundred and twenty one of the remaining isolates were classified, based on electrophoretic data, as *C. albicans* (77%), twenty were *C. dubliniensis* (7%), one was *C. parapsilosis* (0.3%). However, forty-five isolates were genetically different and represent a number of other undetermined *Candida* species (15.7%). Genetic studies have led to the discovery of a number of cryptic species (eg., McCullough *et al.* 1995, Sullivan *et al.* 1995),

some with increased virulence and decreased antifungal susceptibility (eg., Anthony *et al.* 1995). Due to the range of species of clinical importance that were included in this thesis, not all of them could be assessed concurrently with each clinical strain. This prevented the accurate identification of isolates from species other than *C. albicans*, *C. dubliniensis* or *C. parapsilosis* whose type strains were included in all allozyme electrophoretic analyses. A selection of the isolates from other *Candida* species were assessed using 18S rRNA sequencing. These isolates were placed in clades with the Type strains of other *Candida* species, but their relationships were not well supported. The possible identity of the forty-five "indeterminate" strains is discussed in the taxonomy section below (Chapter 4.3 p., 266).

Epidemiological investigations are used to discriminate isolates in order to identify common sources of infection and biologically relevant populations that are, for example, antifungal resistant or more pathogenic subspecies. Based on the results in this thesis, it might be argued that allozyme electrophoresis is too discriminatory for epidemiological analyses since almost all isolates appeared genetically distinct. Conversely, the results preclude the existence of clonal populations of *Candida* in the isolates assessed and support the theory that *Candida* is not entirely asexual. The conservative estimation of genetic diversity obtained using allozyme electrophoresis in other Kingdoms should also be taken into account when interpreting the results obtained herein.

Allozyme electrophoresis is not only used to discriminate among closely related isolates but the electrophoretic data provides information on the genetic relationships of isolates. Based on the limited patient information that was available at the time when the clinical oral samples were collected, strain associations with HIV status, clinical presentation and the acquisition of antifungal resistance are discussed below. Although this information was not available for all of the clinical strains assessed, it is possible to infer a number of clinically important conclusions.

4.2.1 Strain variability

Nearly all of the isolates assessed were genetically distinct within each study based on their allelic profiles. There were four exceptions to this. They were; a strain isolated from both an HIV-positive patient with oral hairy leukoplakia (58A) and an HIV-negative individual who had just undergone radiotherapy and was a *Candida* oral carrier (56A) (taxonomic study 2 p., 100); another strain isolated from an oral rinse sample from a patient with oropharyngeal candidiasis (59I) and from a throat swab of another patient with candidiasis (2A) (study 9 p.,

173); a rinse and swab isolate from one patient (65A and B) with rinses from two other patients (88K and 97E) and a lesion swab from another (96A) (study 10 p., 184); and two rinse and two lesions swabs from one patient (93B, C, D and E) with rinses from two other patients (73A and 95A) (study 10 p., 184). In all cases, the identical isolates may have actually been distinct strains that were not distinguishable with the panel of enzymes utilised or may be single clones transmitted between these individuals. Considering the degree of genetic diversity observed between all other isolates, the strain would need to have been transmitted between the patients. It is more likely that the strains are very closely related, but not genetically identical, especially considering that allozyme electrophoretic data provide a conservative estimation of the overall degree of genetic variability. Using the frameworks of genetic relationships obtained in this thesis, it is possible to assess the relative discriminatory power of a range of epidemiological and diagnostic techniques. For example, investigations can reveal the techniques that can discriminate the strains with identical allelic profiles and those that are less discriminatory than allozyme electrophoresis. However, probably the most important conclusion is that these few isolates were the only ones that could not be distinguished, suggesting that there is a lot more genetic variation within *Candida* species than has been reported previously.

Many authors have suggested that *C. albicans* is a clonal species due to the appearance of the same genetic type in multiple samples from different patients (eg., Lockhart *et al.* 1995, Graeser *et al.* 1996). The electrophoretically-identical isolates in this study support this theory. However, the extreme genetic variation observed between all other isolates examined suggests that *Candida* species may possess a sexual recombination growth phase, as has also been suggested previously (eg., Graeser *et al.* 1996). This observation would support the concept that holomorphs arise from a single genetic strain, and represent different life cycle stages of "*Candida*" species.

It has been suggested that microevolution is responsible for changes in pathogenicity, antifungal susceptibility and morphology and biochemistry of yeast strains (eg., Fries and Casadevall 1998). The present results suggest that strain turnover is more important clinically, because the infecting strain is rarely maintained by a patient and the replacement strain is often very genetically distinct, either representing a different strain of *C. albicans* or a different species of *Candida*.

The electrophoretic studies 4, 6 and 9 of this thesis described the existence of subclusters with tied fixed genetic differences between isolates of *C. albicans*, which may represent the

genetically homogeneous subcluster of *C. albicans* that has been described previously as a general-purpose genotype (eg., Schmid *et al.* 1999). Comparisons between isolates from these studies is required and may lead to the description of an internationally-distributed genotype or genotypes having arisen from different geographical regions.

Extreme genetic variation within successive strains from an individual patient has been reported previously (eg., Anthony *et al.* 1995). This was evident in the present study where there was little evidence of the maintenance of a single strain within any patient. Multiple isolates obtained during one visit or over successive appointments were compared within single studies for 38 patients. The number of patients where multiple strains were compared was limited by the inability to pool data across gels from different electrophoretic studies and limitations of resolution of some isolates that were mixed or were heterozygous at one or more loci. Such studies require repeat analyses of multiple isolates from a patient, including separate analyses of individual colonies for isolates identified as a mixed or heterozygous genotype.

Identical banding patterns from multiple isolates from the same patient were evident in electrophoretic studies 3, 6, 7, 8 and 10. In study 7 (p., 155), patient 9 had the same strain in an initial rinse and two subsequent lesions despite fluconazole treatment suggesting persistence of a fluconazole resistant strain. Also in study 7, the same strain was isolated from two rinses from patient 22, but the strain was not isolated from a tongue lesion also present at the same time. However, the lesion swab strain was very similar to the rinse strain, perhaps representing a more virulent substrain that was clinically resistant to fluconazole. In study 8 (p., 164), patient 46 had the same strain isolated from two oral rinses but the subsequent oral swab isolate was different, suggesting superinfection with a more virulent strain. In study 10 (p., 184), patient 65 had the same strain isolated in an oral rinse and lesion swab on the same day. Isolates from three other patients also shared genetic identity with the isolates from patient 65 (88K, 96A and 97E), suggesting either the existence of a clonal population or that allozyme electrophoresis was not discriminatory enough to separate them. Patient 88 had the same strain in a preliminary oral rinse and from a subsequent lesion in study 6 (p., 145). The immune status of this patient may have changed during the time between isolate collection allowing an asymptomatically colonising strain to cause an infection. Alternatively, the strain became virulent through phenotypic switching or micro-evolution as has been described by Odds (1997). Patient 93 had the same strain isolated in four different samples in study 10 (p., 184), suggesting strain maintenance. The first isolate was from an oral rinse and the other three were obtained one week later from a second oral rinse and swabs of two lesions on the floor of the

patient's mouth. The same strain persisted and caused infection in this patient, again perhaps due to a decrease in the patient's immune status. As with patient 65, this patient's strains were identical to oral rinse isolates from two other patients (73A and 95A).

In an electrophoretic study by Reynes *et al.* (1996), 10 polymorphic loci were used to show that there is one predominant strain of *C. albicans* in mixed infections and that it is present for the entire infection. The data obtained herein did not support their observation, instead suggesting that there are a number of different strains present in mixed and single strain infection and that often the same strain was not recovered twice within a patient. The difference in results is probably due to the increased number of polymorphic loci assessed in this thesis. In the present study, for 31 of the 38 patients where data from multiple isolates could be compared, each isolate was a unique strain. (See patient 1 A, B, C and E (study 9); patient 2 A and B (study 9); patient 17 A and B (pilot 1), A, C, D and E (study 4) and C, D, F, G and H (study 6); patient 19 A, B, C and D, isolate D may have belonged to a different species (studies 4 and 6); patient 21 A and B, isolate A may be *C. dubliniensis* (study 6); patient 22 A and B (study 4); patient 23 A, B, C and D, isolates A and D may be *C. dubliniensis* (studies 4 and 6); patient 27 A, B and C, all strains may be *C. dubliniensis* (studies 5 and 6); patient 28 A and B (studies 5 and 6); patient 29 A and B (study 7); patient 31 A, B, C and E (studies 4 and 6); patient 32 A and B (study 6); patient 34 A and B, may both belong to different *Candida* species (pilot 2, study 5 and study 6); patient 40 B, D, F, H and J (studies 3 and 4); patient 42 A, B, C, D and E, strains D and E may belong to different *Candida* species (study 7); patient 44 G and H, strains may belong to different *Candida* species (study 8); patient 46 A and B (study 3); patient 50 A, B and C (study 8); patient 52 A, B and C (study 8); patient 54 G, H, I and J (study 9); patient 58 B and C (study 9); patient 59 A, B, C, D, G, H and I (study 9); patient 60 A, B, C and D (study 9); patient 63 B, C, E, F, G, H, I, J, M, N, P, Q, R, S (study 9); patient 71 A and B (study 4); patient 84 E, F and G (study 10); patient 88 D, K, L and J (study 10); and patient 97 E and D (study 10). These observations hint at an enormous range of undiscovered genetic diversity within patients, especially considering the conservative nature of allozyme electrophoresis as an epidemiological tool.

Barchiesi *et al.* (1997) found that fluconazole resistance could be acquired by a single strain over time. The present data suggest that superinfection by a resistant strain is more often responsible for the acquisition of a clinically resistant infection. As Takasuka *et al.* (1998) found, there is a huge variety of strains isolated within a single patient within a short time despite antifungal treatment. Cowen *et al.* (1999, 2000) found that fluconazole resistance was

rapidly gained and lost by strains of *C. albicans* and that, as a consequence, genotyping methods were unable to track or predict the resistance pattern of a strain. The data obtained in this thesis suggest that fluconazole and amphotericin B therapy increase strain diversity by weakening the predominant strain and allowing opportunistic colonisation by a number of *Candida* strains. By chance, some of these strains are fluconazole resistant and able to survive and cause infection. Additionally, these strains may not be detected when therapy is ceased but may return with repeat therapy, being derived from a common source.

One-hundred and twenty isolates, from 46 HIV-positive patients and 9 HIV-negative control individuals, were comprised of heterozygous strains or contained more than one strain in a mixed colonisation or infection. For 6 samples, each of the six individual colonies collected was analysed independently. In patient 17, isolates A, B, C and D all appeared mixed upon preliminary investigation. When independent colonies were assessed, isolates A and B were found to comprise 6 different strains (pilot 1) and isolates C and D were comprised of at least two strains (study 6). For patient 40, isolate G comprised two distinct strains (pilot 2) and patient 42 isolate D was also a combination of 2 strains (study 7). The cause of multiple bands in the other 114 isolates was not pursued in the current study.

The ITS regions were suggested as discriminatory at the strain level by Lin *et al.* (1995) based on the detection of distinct genetic types within *C. parapsilosis*. The data presented herein did not support their observation; there was no difference in the sequence of these regions within the *Candida* species assessed, a result supported in a number of other studies (eg., Shin *et al.* 1996, Lott *et al.* 1998, Reiss *et al.* 1998). Instead there was extensive variation between *Candida* species, suggesting that the genetic groups observed by Lin *et al.* (1995) may in fact be distinct cryptic species within *C. parapsilosis*. This finding illustrates the dangers of inadequately assessing the resolving power of diagnostic techniques prior to interpreting the results obtained using them.

4.2.2 Phenotypic observations

An association between the colony morphology on cornmeal agar and biotype of a strain has been correlated with the infections lichen planus and leukoplakia (Lipperheide *et al.* 1996). The phenotypic variability seen as adhesiveness and rough appearance of colonies on Sabouraud's agar plates in the present study is not restricted to a genetic subset of strains nor a patient's clinical presentation. The colony morphology may instead be influenced by the host's

oral environment. Alternatively, it may not represent a morphological switch, but may reflect growth rate differences.

As is the case for *Candida albicans*, the phenotypically diagnostic characteristic of germ-tube formation was found to be variable in *C. dubliniensis* isolates. This observation precludes the sole use of this character for diagnosis of either species. Since both species have the ability to form germ-tubes, the character may be assumed to have evolved prior to the divergence *C. albicans* and *C. dubliniensis*, suggesting that it is a recently evolved character.

Candida dimorphism induced by changes in the environmental growth conditions (i.e., temperature, oxygenation, growth medium) does not affect metabolic enzyme expression or vary the genetic profile observed using allozyme electrophoresis. This means that switches between blastospore and hyphal growth is a phenotypic/gene expression change that does not affect expression of the strains basic "housekeeping" metabolic enzymes.

4.2.3 HIV status

The present study confirms findings in other studies that 40% of the HIV-negative population are asymptomatic *Candida* oral carriers (eg., Abu Elteen and Abu Alteen 1998).

There has been some evidence of clustering of isolates from HIV-positive or HIV-negative individuals with oral candidiasis versus asymptomatic *Candida* carriers (eg., Boerlin *et al.* 1995, Boerlin *et al.* 1996). There was no evidence of such clustering in the present study.

The pilot screen in this thesis supported the phylogenetic separation of isolates from oral versus extraoral infection into distinct clusters. However, with the addition of more isolates, these clusters dissociated. Overall there was no evidence of clustering with respect to *Candida* strain and HIV status, clinical presentation or antifungal resistance. Additionally, a single genetically-indistinguishable strain was obtained from two unrelated individuals, one HIV-positive with OHL and the other HIV-negative undergoing radiotherapy treatment (58A and 56A, p., 100). These observations suggest that oral candidiasis is an opportunistic infection in HIV-positive individuals and that HIV-negative individuals are a likely reservoir for infection.

The number of distinct genetic types identified in a patient at any one time is predominantly dependent on the discriminatory power of the technique used to differentiate them. For this reason, some investigators suggest that there is a limited subset of strains associated with HIV-

negative individuals (eg., Mathaba *et al.* 1995) or that there are pathogenic types associated with HIV-positive patients with candidiasis (eg., Challacombe *et al.* 1995, Wu *et al.* 1996). Reynes *et al.* (1996) used allozyme electrophoresis comparisons of 10 polymorphic loci to show that during infection, even through periods of mixed colonisation, a single strain predominated. Challacombe *et al.* (1995) identified an increased genetic diversity of *Candida* isolates in HIV-positive individuals. The present results show that each strain can be differentiated and an equivalent degree of genetic diversity was observed in HIV-positive and HIV-negative individuals. Also, it was not always the case that the same strain was carried throughout the course of isolate collection (eg., patient 63 study 9 p., 173 had distinct *C. albicans* strains in all samples analysed). Using allozyme electrophoresis, Boerlin *et al.* (1996) similarly found that there was no genetic distinction between strains isolated from HIV-positive and HIV-negative individuals with or without oral candidiasis, nor were there clusters of isolates resistant to fluconazole. An increase in the number of genetic types within the oral cavity of an HIV-positive individual may be more dependent upon their current immune status, as is also discussed in the context of the acquisition of an antifungal resistant infection below (Section 4.2.5 p., 263). Also, the prevalence of species other than *C. albicans* appeared equivalent in both populations, unlike the study of Schoofs *et al.* (1998) who reported an increased incidence in the HIV-positive population. This may be due to the small number of HIV-negative individuals included in the present study inadequately representing the population.

In the present study, of the 101 patients sampled, only patient 21 (study 6 p., 145) was female. The clustering of the strains isolated from her with those isolated from the many male patients examined suggest that there is no genetic distinction within *Candida* strains causing infection based on the sex of the patient. However, the data set is obviously limited.

4.2.4 Clinical presentation

There were no genetic clusters of strains associated with the type of oral infection ie., candidiasis, oral hairy leukoplakia. In agreement with a previous observation by Boerlin *et al.* (1996), there was no clustering associated with fluconazole susceptibility or clinical symptoms. Reichart *et al.* (2000) have suggested that a breakdown in local mucosal immunity leads to pseudomembranous candidiasis and that hypersensitivity against *C. albicans* results in erythematous candidiasis. Therefore, it appears that the infection is opportunistic and the clinical presentation is not strain mediated, instead being a consequence of the host immune response.

Strain persistence in a single patient over time was noted. In one patient, the same strain was isolated from an oral hairy leukoplakia lesion during obvious *Candida* infection and from an oral rinse sample during asymptomatic carriage. Mixed colonization with a number of different strains and/or species of *Candida* occurred both in asymptomatic carriage and infection thus, the presence of a mixed infection does not suggest selective pressure for strain turnover within the oral cavity. In some cases, mixed isolates were obtained from a single lesion swab, indicating that a number of different strains may cause one lesion.

Also of clinical importance, this study shows that a swab of an oral hairy leukoplakia lesion and a concurrent oral rinse sample can yield different strains. This means that there may be more than one strain within the oral cavity of a patient with oral hairy leukoplakia, and that the predominant strain is not necessarily the one causing the lesions.

Strain and species replacement occurred as often as weekly in some patients. This rapid turnover of strains suggests a number of different sources of infection must exist. *Candida* colonisation and infection is not static. Instead it is an ecological interaction between the *Candida* strains present and probably the host immune status and other microorganisms in the patient's oral cavity.

In some cases, a number of genetically closely related strains (substrains) were isolated from a patient. The genetic relatedness of these strains suggests that they may have arisen from microevolution of a single strain, a theory that has previously been proposed to explain the acquisition of antifungal resistance and the subsequent persistence of an infection (eg., Lockhart, *et al.*, 1996).

In many patients, different strains were isolated from recurrent infections. This suggests that the re-emergence of infection is most likely due to a decrease in immune response and that the infection is caused by one of many strains present in the patient's oral cavity at the time.

Different strains have been isolated from the same patient during asymptomatic carriage prior to infection and during post-infection carriage following therapy using fluconazole or amphotericin B. Additionally, during infection of a number of patients the strain obtained by swabbing an active lesion was rarely the same as that isolated in their concurrent oral rinse sample. The oral rinse technique has been assumed useful to obtain a sample of the

predominant strain present in a patient but the present study has demonstrated that oral rinse samples often do not contain the infecting strain.

Oral and systemic *C. albicans* isolates cluster, suggesting that both sites of infection are opportunistic. Since the isolates from oral and systemic sites are genetically indistinguishable, the oral cavity is a likely source for systemic infection. Considering this possibility, it may be wise to assess the oral carriage of *Candida* in a patient prior to oral surgery, particularly in immunocompromised individuals where there is an increased risk of ensuing systemic infection. The detection of oral *Candida* may warrant prophylactic antifungal treatment, or may alert the physician to the possibility of *Candida* colonisation at the first signs of systemic infection.

4.2.5 Antifungal treatment

Using 18S rRNA sequence comparisons, a close genetic proximity of *C. krusei*, which is innately resistant to fluconazole, and *C. lusitaniae*, which is more likely to become amphotericin B resistant than other *Candida* species, was identified. This observation suggests that these two species shared a common ancestor more recently than with other *Candida* species. Using the same data, their close genetic proximity to *C. tropicalis*, a species more commonly isolated from systemic infection, indicates the possible existence of a particularly pathogenic lineage of *Candida* species. Since this analysis was conducted using newly-purchased type and reference strains of these species, the relationships obtained using this gene cannot be attributed to contamination by other yeast species. It is, however, important to confirm these relationships using a broader range of isolates from each species, since relationships can alter with the addition of more sequences. It is also critical to assess this relationship using a range of characters of varying rates of evolution because, as highlighted in the introduction of this thesis, different characters can yield very different phylogenies.

Antifungal susceptibilities, both clinical and laboratory, were not determined for the majority of isolates assessed in this thesis. From the available data, fluconazole resistance does not appear to be restricted to a genetic subset of *Candida* strains, suggesting that it is randomly acquired by a strain. A rapid rate of fluconazole resistance acquisition has been reported in a single strain of *C. tropicalis* where it occurred during growth in a single broth containing the antifungal (Barchiesi *et al.* 2000). Additionally, the acquisition of fluconazole resistance by a *C. albicans* strain occurs more rapidly than changes in the DNA fingerprint or multilocus genotype of the strain (Cowen *et al.* 1999). Conversely, the rapid rate of strain turnover

currently observed suggests the selection of other more resistant *Candida* strains and species with antifungal treatment is far more relevant. *C. albicans* strains that are innately resistant to fluconazole have been isolated from the oral cavities of immunocompromised and healthy individuals (Xu *et al.* 2000).

Fluconazole treatment has been shown to increase the number of morphotypes, karyotypes and PCR types in a patient (Takasuka *et al.* 1998). The vast array of genetic types observed herein support this finding, suggesting that there is a huge number of strains present in the population analysed. The survival of a particular type is probably associated with its ability to withstand fluconazole, due to a chance mutation. There must be a large array of mutations that confer antifungal sensitivity since it occurs so readily in numerous genetically diverse strains. A number of authors (eg., Revankar *et al.* 1996, Tumbarello *et al.* 1996, Laguna *et al.* 1997) found that the immune status of an HIV-positive individual was an important risk factor for acquiring fluconazole resistant infections. If this is the case, the inability for an HIV-positive individual to combat *Candida* colonisation probably allows the survival of a larger number of *Candida* strains and, by chance alone, they are therefore more likely to acquire a fluconazole resistant strain. Additionally, since many immunocompromised individuals acquire *Candida* infection, they have probably been treated with the first antifungal drug of choice, fluconazole. These circumstances would lead to the appearance of a correlation between previous fluconazole exposure and the acquisition of a resistant strain. If the immune status of an individual is an important risk factor, as their HIV infection progresses, they will more often become infected with *Candida* and are therefore more likely to encounter an azole resistant strain. This would negate the importance of previous exposure to fluconazole as a risk factor and explain the disagreement between different authors on its importance (eg., Maenza *et al.* 1997, Boschman *et al.* 1998). Instead, it is more important to identify the reservoirs for the vast array of strains causing these recurrent infections and the origins of species other than *C. albicans* of paramount clinical importance.

Persistence of a fluconazole-resistant strain during treatment with fluconazole and the subsequent return of the same strain with repeat fluconazole treatment was detected in patient 9 (study 7, p., 155). The persistence of this strain is most likely due to reseeding of the patient's oral cavity from a consistent source of infection, such as their partner. This highlights the need to examine the transmission of *Candida* species between individuals and the possible requirement for concurrent antifungal therapy of the partners of infected individuals.

An amphotericin B lozenge trial was undertaken on some of the HIV-positive patients during collection of oral *Candida* isolates for this thesis. Of the sixteen patients in the trial who continued their participation and were sampled over time, nine acquired mixed infections possibly as a direct consequence of the lozenge. One of these patients started with what appeared to be an amphotericin B clinically-resistant *C. albicans* infection not resolved using the lozenges, which then led to a mixed infection that still included the original infecting strain (patient 9, study 7, p., 155). Another patient started with a mixed infection directly after commencing the lozenge trial and eventually appeared to have an amphotericin B clinically-resistant *C. albicans* infection, which persisted during the treatment (patient 54, studies 7 and 9, p., 155 and 173). One patient acquired an infection caused by another *Candida* species that was resistant to amphotericin B (patient 19, study 6, p., 145) and another patient appeared to oscillate between clinically-resistant *C. albicans* strains and strains from other *Candida* species (patient 63, study 9, p., 173). Selection of what appears to be a clinically resistant amphotericin B strain of *C. albicans* with treatment using this antifungal has been noted in 8 of 16 patients herein. These combined observations suggest that the use of amphotericin B in a lozenge form allows the survival of populations of *Candida* in the oral cavity. This eventuates in the predominance of a single strain, usually *C. albicans*, which is tolerant to the dosage of amphotericin B provided by the lozenge. These results become more clinically significant when considering the possibility of a recombinatorial life cycle stage within *Candida* species, providing the opportunity for the acquisition of antifungal resistance from other *Candida* strains concurrently present in the oral cavity. This could lead to an increased occurrence of amphotericin B and other antifungal resistant infections.

Despite the observed non-significant decrease in the incidence of oral candidiasis in HIV-positive individuals on HAART therapy (eg., Ives *et al.* 2001), it remains a challenging infection for immunosuppressed individuals. Additionally, there is still restricted access to HAART therapy and oropharyngeal candidiasis has not disappeared from the HIV-positive population. Results from this study have suggested that there might be a resurgence of this infection in these populations as *Candida* species become less susceptible to current antifungals. A similar epidemiological study in other immunocompromised populations and sites of infection outlined in Section 1.3.2a-e (p., 30) may begin to unravel the obvious complexities of *Candida* colonisation and infection.

4.3 *Taxonomy of Candida and related genera*

Genetic variation within and between *Candida* species and related genera were effectively assessed using allozyme electrophoresis at twenty metabolic enzyme loci. Following allozyme electrophoresis, isolates representing the range of genetic variation observed were selected for an investigation of the phylogenetic and epidemiological utility of the 18S and ITS 1 and 2 sequences. Sequence comparisons using the 18S rRNA gene provided an accurate phylogeny for *Candida* and related genera. ITS 1 and 2 sequences were conserved within *C. albicans* and so this sequence was not epidemiologically informative. Additionally, the ITS sequence was too variable to deduce phylogenetic information for the species within *Candida*. Consequently, the ITS sequence analysis results are not further discussed in this Chapter. Allozyme electrophoresis is discriminatory at the individual strain to species level and 18S rRNA sequence comparisons discriminate at the species to genus level (Figure 4.1 p., 267). Their combined results provide information concerning the systematics and epidemiology of medically and dentally relevant *Candida* species.

Before discussing the possibility that the current *Candida* genus is comprised of a number of genera, it is timely to revisit the definitions of genera and species. In sexually reproducing organisms, a species is comprised of "groups of interbreeding or potentially interbreeding natural populations that are reproductively isolated from other groups" (Prescott *et al.* 1999). In asexual organisms, a species cannot be defined using the same criteria, so they comprise "collections of strains that share many stable properties and differ significantly from other groups of strains" (Prescott *et al.* 1999). These other characters may be morphological, biochemical, and more recently genetic. There are obvious difficulties associated with the use of these definitions when dealing with fungal taxonomy due to the ability of some species to exist in both sexual and asexual forms that are genetically related but morphologically and biochemically distinct. The relative importance of each character forms the basis for the taxonomic confusion evident for *Candida* and related genera. Also, as stated in the Introduction (Section 1.2.1 p., 11), *Candida* comprises yeast species that do not belong to other genera rather than species that form a natural cluster.

Based on the results presented in this thesis, using allozyme electrophoresis, 18S rRNA gene sequence comparisons and traditional identification methods confirm that *Basidiomycota* (ie., *Cr. neoformans* and *T. beigelii*) constitutes a genetically distinct phylum from *Ascomycota* (ie., *Candida* and *S. cerevisiae*). Unfortunately, this appears to be where the limits of taxonomic

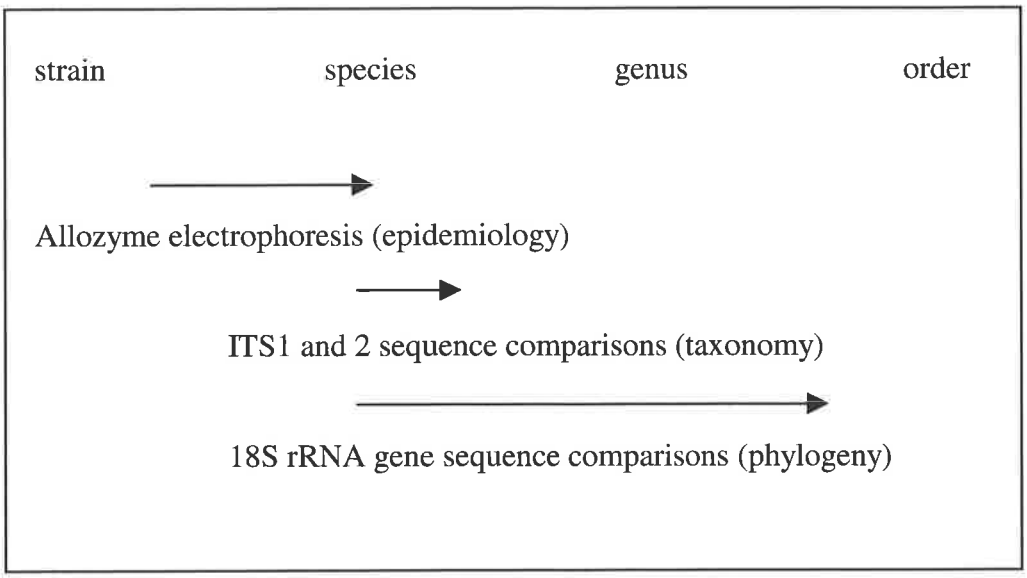


Figure 4.1; Diagrammatic representation of the discriminatory power of the techniques assessed in the present study, for medically relevant species of the genus *Candida* and related genera

The most appropriate resolution level for each technique is in brackets.

resolution for these phyla lie using these techniques ie., taxonomic divisions below the phylum level are not strongly supported.

Within the basidiomycete species *Cr. neoformans*, the two recognised serovars are genetically distinct suggesting their separation into distinct species. Since each of these serovars originates from different environmental sources, causes infections in different subpopulations, constitutes distinct mating types and has distinct biochemical properties that allow their diagnostic identification, the genetic distinction observed herein provides further evidence for taxonomic reclassification. The two serovars have also been discriminated using a number of other molecular techniques (eg., Meyer *et al.* 1993, Brandt *et al.* 1995, Sandhu *et al.* 1995). However, as has been observed for other fungal species, there is a single teleomorph associated with both serovars. Perhaps *Filobasidiella neoformans* is also comprised of two distinct "cryptic" or previously undescribed species that remain undiscovered at this time.

The results from the present study confirm the existence of discrete species within the genus *Candida* and the clusters of isolates associated with type and reference strains of currently described species. However, the *Candida* species were quite distinct from each other, having fixed differences at 50 to 80% of the loci assessed using allozyme electrophoresis and differing at one to one-hundred and forty-four bases of the 18S rRNA gene sequence compared. Using allozyme electrophoresis, prokaryotic species are usually defined by fixed differences exceeding 60% of loci (Andrews and Chilton 1999). Therefore, results in this thesis demonstrate that the genetic cohesiveness of the *Candida* genus is questionable, although the species within it appear well defined. As a consequence of this genetic diversity, *S. cerevisiae* appears closely related to and may in fact belong to the *Candida* genus. This is not the first time that the taxonomic discretion of *Saccharomyces* has been questioned (eg., Cai *et al.* 1996, James *et al.* 1997). *C. glabrata* is phenotypically distinct from other *Candida* species (Bikandi *et al.* 1998b). Using allozyme electrophoresis and 18S rRNA gene sequence analyses, results obtained in this thesis suggest that *C. glabrata* forms a distinct lineage, being most closely associated with *C. kefyr* and *S. cerevisiae*. In previous studies (eg., Barns *et al.* 1991, Fujita *et al.* 1995, Crist *et al.* 1996, Cohn *et al.* 1998), *S. cerevisiae* has been closely associated with *C. glabrata*. Using both allozyme electrophoresis and 18SrRNA gene sequence comparisons, the present results suggest that *C. kefyr* is more closely related to *S. cerevisiae*, with *C. glabrata* the next closest species.

The data presented for both allozyme electrophoresis and 18S sequence comparisons also confirm that *Candida* anamorphs and their sexual-state teleomorphs cluster closely. This observation supports the suggestion that they actually constitute two alternate life-cycle stages of a single genetically-defined species, confirming the need for taxonomic revision of the genus *Candida* and its various teleomorph genera. In order to construct a taxonomy that is consistent and reproducible using any identification method (traditional or contemporary), a reclassification of the holomorph (combined anamorph and teleomorph species) into a single species is required. Due to the genetic and phenotypic distinction between many of the identified teleomorphs, the genus *Candida* would be split between a number of distinct existing teleomorph genera. This observation has been published previously (eg., Barns *et al.* 1991), but has not led to taxonomic revision.

It may transpire that yeast taxonomic clustering is not valid above the species level using molecular data. This observation could arise due to the evolutionary age of these species, with the accumulation of mutations and diversion between species masking their true evolutionary origins. The 18S rRNA gene sequencing results already suggest this possibility; genetic differences that usually resolve relationships between genera of bacterial species, resolve at the species level in the genus *Candida*. Alternatively, another gene may provide a more accurate genetic structure for yeast genera. In order for taxonomic classification to be accurately reassessed, those *Candida* species for which a teleomorph has not been found yet would need to be reclassified according to their genetic relatedness to other yeast genera. This reclassification is obviously a massive task which will take many years to complete. Just taking the genus *Candida* as an example, there are still over 190 species to be investigated in this manner.

To add confusion to the issue, there are also controversies within some *Candida* species. *C. kefyr* has been associated with a number of teleomorphs from different *Kluyveromyces* species (Cai *et al.* 1996). The results from the partial 18S rRNA gene sequence comparisons in this thesis suggest that there may be more than one species within this genetic group. This observation needs further investigation, but may lead to the identification of alternative species associated with different *Kluyveromyces* species. Present comparisons of the 18S rRNA gene have confirmed the genetic distinction of *C. kefyr* from other *Candida* species, as was reported previously by Barns *et al.* (1991).

Using 18S rRNA gene sequence comparisons, Hendriks *et al.* (1991) determined that *C. lusitaniae* was the earliest species within the genus *Candida*. The results obtained herein go further to suggest that this species forms a distinct evolutionary lineage. Despite *C. lusitaniae* and *C. tropicalis* sharing biochemical characteristics, they are genetically quite distinct. This observation again indicates the importance of using a combination of techniques to provide an accurate systematic framework and the need for taxonomic revision. In the same 18S rRNA sequencing study, Hendriks *et al.* (1991), identified *C. krusei* as a distinct evolutionary lineage. The results of this thesis suggest that *C. krusei* is genetically distinct from other *Candida* species, being most closely associated with *C. haemulonii* according to partial 18S rRNA sequence comparisons. These results also confirm that *Isstachenkia orientalis* is a teleomorph for *C. krusei*.

Based on 18S rRNA sequence comparisons, Cai *et al.* (1996) and Nishikawa *et al.* (1997) identified a distinct lineage comprising *C. famata*, *C. guilliermondii* and *D. hansenii*. Using this same sequence, the current results refute the existence of this genetic group, probably as a consequence of the inclusion of more *Candida* species in this study. The inclusion of all species within the genus *Candida* provides the most accurate indication of the genetic relationships within the "genus".

It is obvious from the results of this thesis and those of many other authors that the taxonomy of yeasts requires urgent revision. The results demonstrate the danger in assigning a single "gold standard" method, such as 18S rRNA gene sequence comparisons, or phylogenetic characters, to resolve this issue. Molecular methods are universally applicable but there are problems associated with constructing phylogenies of organisms containing species with different rates of evolution and the selective pressures associated with the gene of choice that may bias the result. As an added difficulty, many of the genetic relationships currently identified between fungi cross traditionally-defined taxonomic groups. *Candida* species and their associated teleomorphs are a good example of these conflicts, where members of a single anamorph genus are genetically associated with a diverse range of teleomorph genera and phyla according to genetic characters. An indicator of the long-term taxonomic confusion that has persistently surrounded the genus is the one hundred and ten synonymous names associated just with *C. albicans*.

An additional consideration when conducting systematic investigations is whether the isolates used to obtain taxonomic data are accurate representatives of their species. The only

true representative of a species by definition is the type strain. In many investigations, conclusions have been made on other strains from the species.

In the present study, the results were based on a limited range of type, reference and clinical strains of species within the *Candida* genus and associated genera. An unequivocal taxonomic study would require the incorporation of a range of traditional and contemporary techniques. These should include supportive morphological, biochemical and biological characters such as ecological niche, pathogenicity, antifungal resistance, which are conserved within a defined taxonomic group. The current results support the use of 18S rRNA gene sequence comparisons for this type of systematic investigation of *Candida* and suggest that the 490bp fragment of this gene also provides sufficient data for this type of research.

Using multiple clinical, laboratory and environmental isolates from each of the species represented would confirm the validity of the current type and reference strains. To incorporate all recognised *Candida* species and a range of clinical and environmental isolates is extremely costly and time-consuming. Efficiency could be gained by first determining the most appropriate method or combination of methods for comparing isolates.

The investigation should also include a similar assessment of the remaining one hundred and eighty-three species within the genus *Candida* that are not investigated in this thesis. It would be expected that the observed levels of genetic variation within ecological niches would be lower than between niches. It could therefore be predicted that the other *Candida* species, not isolated from human infection, would be more diverse and branches would extend outside of the presently established framework. The results of such a study may begin to elucidate the mechanisms of yeast infection and aid in the prevention, correct diagnosis and treatment of infection.

The method of allozyme electrophoresis was able to accurately discriminate all of the species and genera examined herein but the relationships between them probably exceed the levels of resolution that allozyme electrophoresis can reliably detect. The framework obtained using allozyme electrophoresis was not identical to that obtained using partial 18S rRNA sequence analyses indicating that each method resolves at different taxonomic levels within the genus *Candida*.

Comparisons of the 490-bp fragment of the 18S rRNA gene sequence using additional taxa decreased some of the internal bootstrap values but the major lineages obtained using the complete 18S rRNA gene sequence remained well supported (Figure 3.19c p., 223). This indicates that although the partial 18S sequence provides an indication of the genetic relationships between *Candida* species, it is not as phylogenetically informative as the complete sequence. Also, as discussed previously (Section 3.5.3 p., 206), using the partial 18S sequence the branching order of *C. lusitaniae* and *C. krusei* swapped. This is probably another effect of analysing additional species, and suggests a need to analyse more genes to resolve this relationship unequivocally.

In conclusion, *Candida* infection is opportunistic, being more dependent on the host immune status than the infecting strain or species. Antifungal resistance is acquired by chance in a variety of different genetic types within *Candida* species. Clinically resistant infections result more often from superinfection by a resistant strain than genetic alteration of the same strain. There are no strain differences between those carried by HIV-positive and HIV-negative individuals or infection and carriage. Individuals are often colonised simultaneously by more than one strain in a mixed infection. An oral rinse does not give an accurate indication of the infecting strain or the number of strains present within the oral cavity. The genus *Candida* should be revised with a number of medically-important species being reassigned to different genera according to their associated teleomorphs taxonomic position. However, it should be borne in mind that, in this study, the genetic relationships between only thirteen medically relevant species of the two-hundred in the genus *Candida* were investigated.

4.4 Future directions

Following, are a number of future research projects proposed from the results of this study. Some have already received grant funding and are underway at Adelaide University. Some focus on new research areas introduced by other researchers since the commencement of this study.

The extreme genetic variability of *C. albicans* oral isolates observed in the present study may be used to contrast with the variability within extraoral isolates, particularly those from vaginal candidiasis, another particularly problematic recurrent infection. It would be clinically significant to determine whether the incidence of mixed infection and the rapid rate of turnover

of colonising strains is equivalent in other such ecological niches to again determine whether the infection is opportunistic or strain specific.

Multilocus sequence typing is a relatively new molecular biological technique based on multilocus enzyme electrophoresis. It was formulated for systematic investigations of *Neisseria meningitidis* and *Streptococcus pneumoniae* (Enright and Spratt 1998). Using this technique, sequences are obtained and compared for the enzyme loci that appear epidemiologically informative, providing increased resolution between closely related strains. This technique could be applied to *Candida* species using the enzyme loci that differentiate at the strain level herein (eg., pyruvate kinase) to further investigate the epidemiology of colonisation and infection (currently grant funded; Australian Dental Research Foundation). The advantage of this type of technique is that a universal database can be constructed and stored for long-term use allowing comparisons of data between laboratories.

It has been estimated that 40% of healthy individuals asymptomatically carry oral *Candida*. In order to determine possible sources of infection and routes of transmission, a prevalence survey of asymptomatic carriers is required. Studying this within families may give valuable insight into the genetic basis for carriage, transmission of genetic types between individuals, consistency of colonization and the species and strain level differences in colonization rates. Along these lines, a study of carriage patterns in monozygous and dizygous twins is currently grant funded (ADRF).

The framework obtained for the genus *Candida* and the species within it in this study, allows for the appropriate future selection of isolates for entire genome sequencing projects additional to type strains. It is unwise to select a readily available strain as the representative for a species before its relatedness to other strains is determined. By selecting a truly representative strain, comparative data generated from other species and strains can be used to make unequivocal conclusions regarding the systematics of yeast taxa, identification of putative virulence factors, the acquisition of antifungal resistance and epidemiologically informative sequence data.

The interaction between *Candida* species (and strains) with the human immunodeficiency virus is a research area of particular clinical importance. Previous studies have suggested a synergistic effect with co-colonization (eg., Pietrella *et al.* 1998, Teanpaisan and Nittayananta 1998). The ability of different genetic types of *C. albicans* identified herein to interact with

HIV would make an interesting research topic. The results of the current study suggest that oral candidiasis is opportunistic, but it is likely that the complex interactions between HIV, the host immune system, other oral micro-organisms and *Candida* strains may be critically important to clinical outcome. For example, it has been suggested previously that the level of salivary histatins has an effect on the transition from asymptomatic carriage of *C. albicans* to infection (Jainkittivong *et al.* 1998).

Whilst *Candida* infections associated with HIV-positive immunosuppression have been assessed in this thesis, there are a number of other well-known potential risk factors. These include smoking, dentures (eg., Kamma and Nakou 1997; Abu Elteen and Abu Alteen 1998), age of the individual and the geographic location from which they originate (Kleinegger *et al.* 1996). These risk groups could form the basis for further epidemiological analyses.

Transmission of *Candida* strains between partners is a potential source of sequential infections (eg., Schroppel *et al.* 1994, Lockhart *et al.* 1996). The recurrence of strains in some patients observed herein, before and after antifungal treatment or in successive recurrent infections, supports this observation in the HIV-positive population. Further investigation of this cross-transmission by obtaining *Candida* isolates from HIV-positive individuals and their partners and families may confirm this theory and suggest the requirement for concurrent antifungal treatment of potential sources of infection.

Using allozyme electrophoresis, two expressed copies of malate dehydrogenase and hexokinase in *Candida* species were identified. These enzymes are involved in the TCA cycle and glycolysis, respectively, making them essential for survival. However, it seems wasteful of the organism to maintain two functioning copies of these genes. The enzymes produced could be discriminated by their net charge. A future study could be to determine the biochemical effect of knocking out one copy of the genes. The presence of two functioning copies of these enzyme may have similarity to the isozymes of higher eukaryotes; the two forms of the enzymes could perform slightly different functions and the characterisation of each copy may identify these differences. Potentially there are differences between the enzymes at the structural level that could identify them as targets for antifungal therapy. One copy could have significant differences with human enzymes, negating associated side effects if it was targeted. This system would require that both copies of the enzyme are essential or that loss of one copy of the enzyme debilitates *Candida* enough so that the depleted host immune system may then be able to overcome an infection.

4.5 Summary

Allozyme electrophoresis has proved useful in distinguishing *Candida* strains and loosely grouping them into species. Using this technique a large degree of genetic diversity within and between *Candida* species has been identified. Clusters of isolates showed no association with clinically relevant characters such as infection, antifungal resistance or HIV-positive immunosuppression. From these results it can be concluded that oropharyngeal candidiasis is opportunistic. Using 18S rRNA gene sequence comparisons, particularly the variable 490-bp fragment of the gene, the taxonomic validity of the genus *Candida* has been questioned. The only true biologically and clinically-meaningful functional unit for asexual organisms is the species and levels below to the individual strain. These groups allow the identification of an organism, which brings with it information regarding its life cycle, nutritional requirements and in the case of organisms causing infection, treatment strategies, clinical outcomes and transmission routes. The genus, family and above is a hierarchy of convenience and not biologically relevant, except to allow the labelling and grouping of life. Despite this, there is still a great deal of importance placed on the accuracy of the taxonomy of organisms. Prior to the commencement of this research project, there existed a great deal of controversy surrounding the systematics of *Candida* and related genera. There continues to be disparity concerning the taxonomic status of this genus due to proposed divisions based on conflicting biochemical, morphological and genetic data. The results presented in this thesis do not resolve the complexities of fungal taxonomy, nor the epidemiology of infection, but provide a much more solidly defined framework depicting the systematics of the genus *Candida*. This systematic framework provides the foundation for further investigation.

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APPENDIX 1

Species	Cell morphology	Cell size	Pseudohyphae appearance	Clinical sites of isolation	Clinical Mycoses	Environmental sites of isolation
C. tropicalis	spherical to subspherical	3.0-5.5 x 4.0-9.0 um	Abundant long, wavy, branched with numerous ovoid blastoconidia	major cause of septicemia and disseminated candidiasis, especially in patients with lymphoma, leukemia and diabetes	Opportunistic	faeces, shrimp, kefir, and soil
C. albicans	spherical to subspherical	2.0-7.0 x 3.0-8.5 um	Pseudohyphae with blastoconidia and terminal vesicles (chlamydoconidia)	Represents up to 70% of Candida species isolated from infections	Cutaneous and opportunistic	usually from sources contaminated by human or animal excreta, such as polluted water, soil, air and plants.
C. dubliniensis	numerous ovoid	2.0-7.0 x 3.0-8.5 um	Pseudohyphae with blastoconidia and terminal vesicles (chlamydoconidia)	oral, most commonly isolated from recurrent infection after antifungal treatment	Cutaneous and opportunistic	
C. famata	spherical to ovoid	2.0-3.5 x 3.5-5.0 um	none	rare, usually from skin infections	Opportunistic	commonly environmental
C. glabrata	numerous ovoid	2.0-4.0 x 3.0-5.5 um	none	Common on the body surface often found in skin and urine. Opportunistic cause of septicemia, pyelonephritis, pulmonary infections, endocarditis and hyperalimentation.	Cutaneous and opportunistic	
C. guilliermondii	spherical to subspherical	2.0-4.0 x 3.0-6.5 um	Branched pseudohyphae with dense verticils of blastoconidia.	Numerous infections, mostly of cutaneous origin. Systemic infections are rare	Cutaneous	normal skin and in sea water, faeces of animals, fig wasps, buttermilk, leather, fish and beer.
C. haemulonii	numerous ovoid to globose	3.0-5.0 x 3.0-6.5 um	none	Rare, fungemia	Opportunistic	Fish, dolphins
C. kefyr	numerous short-ovoid to long-ovoid, sometimes elongated	3.0-6.5 x 5.5-16.0um	Abundant, long, wavy, branched, with ovoid blastoconidia, budding off singly, in pairs or chains, often in a verticillated position. Pseudohyphae may be scarce or almost absent.	Rare, superficial cutaneous manifestations rather than systemic disease. Isolated from nails and lung infections	Cutaneous and opportunistic	cheese and dairy products.

Table 1a; Morphological characters of 13 medically relevant *Candida* species.

Species	Cell morphology	Cell size	Pseudohyphae appearance	Clinical sites of isolation	Clinical Mycoses	Environmental sites of isolation
C. krusei	predominantly small, elongated to ovoid	2.0-5.5 x 4.0-15.0 um	Abundant long, wavy, branched pseudohyphae with elongated to ovoid blastoconidia, budding off in verticillate branches.	Infant diarrhoea and occasionally systemic disease. Colonizes the gastrointestinal, respiratory and urinary tracts of patients with granulocytopenia.	Opportunistic	beer, milk products, skin, faeces of animals and birds and pickle brine
C. lusitaniae	numerous subglobose, ovoid, or elliptical	1.5-6.0 x 2.5-10.0 um	Abundant pseudohyphae with short chains of blastoconidia.	disseminated candidiasis, including septicemia and pyelonephritis. Colonizes the human respiratory, gastrointestinal and urinary tracts	Opportunistic	cornmeal, citrus peel, fruit juices, and milk from cows with mastitis.
C. norvegensis	Spherical to ovoid	2.0-3.5 x 3.5-5.0 um	none	very rare, peritonitis and disseminated candidiasis in a patient on CAPD.	Opportunistic	
C. parapsilosis	predominantly small, globose to ovoid, sometimes elongated	2.0-3.5 x 3.0-4.5 um	Abundant, much-branched pseudohyphae in a delicate tree-like pattern with 2-3 blastoconidia in small clusters at intervals along the pseudohyphae.	superficial cutaneous infections (nail) and systemic disease (endocarditis). Also endophthalmitis and fungemia.	Opportunistic	intertidal and oceanic waters, pickle brine, cured meats, olives and normal skin, and faeces.
C. viswanathii	numerous globose, ovoid, or cylindrical	2.5-7.0 x 4.0-12.0 um	Abundant wavy pseudohyphae with verticillated branched chains of blastoconidia.	rarely isolated	Opportunistic	

Table 1b; Morphological characters of 13 medically relevant *Candida* species.

Species	Group	G+C content	Cell size	Capsule	Germ tube	Pseudo-hyphae	Urease	Growth on Cyclohexamide	Growth at 37°C
C. tropicalis	VII	35.9-36.1%		-	-	+	-	+	+
C. albicans	VII	34.3-35.6%		-	+	+	-	+	+
C. famata		36.9%		-	-	-	-	v	v
C. glabrata		39.6-40.2%		-	-	-	-	-	+
C. guilliermondii		44.1-44.4%		-	-	+	-	+	+
C. haemulonii		45.9-47.8%		-	-	-	-	+	+
C. kefyr		41.3%		-	-	most +	-	+	v
C. krusei		39.6%		-	-	+	v	-	+
C. lusitaniae		43.4-46.3%		-	-	+	-	-	+
C. norvegensis				-	-	-	-	-	+
C. parapsilosis		40.5%		-	-	+	-	-	+
C. dubliniensis				-	+	+	-	+	+
C. viswanathii		46.3%		-	-	+	-	+	+
C. neoformans var *gattii*				+	-	-	+	-	+ (weak)
C. neoformans var *neoformans*				+	-	-	+	-	+ (weak)
R. rubra				+ (small)	-	-	+	-	v
S. cerevisiae				-	-	v	-	-	v
T. beigelii				-	-	+	+	v	v

Table 2a; Biochemical characters of 13 medically relevant *Candida* species and 4 related genera (from Kreger van Rij, 1984).

Species	Fermentation[a]					
	Glu	**Mal**	**Gal**	**Tre**	**Suc**	**Lac**
C. tropicalis	+	+	+	+ (d)	v	-
C. albicans	+	+	v	v	- (some +)	-
C. famata	v	v	-	v	v	-
C. glabrata	+	-	-	v	-	-
C. guilliermondii	+	-	v	-	+	-
C. haemulonii	+	v	-	+	+	-
C. kefyr	v	v	+ (d)	-	v	v
C. krusei	+	-	-	-	-	-
C. lusitaniae	+	v	v	+ (d)	+ (d)	-
C. norvegensis	+ (d)	-	-	-	-	-
C. parapsilosis	+	- (some +)	v	- (some +)	- (some +)	-
C. dubliniensis						
C. viswanathii	+	+	+	+ (d)	v	-
C. neoformans var *gattii*	-	-	-	-	-	-
C. neoformans var *neoformans*	-	-	-	-	-	-
R. rubra	-	-	-	-	-	-
S. cerevisiae	+	v	v	v	v	v
T. beigelii	-	-	-	-	-	-

Table 2b; Biochemical characters of 13 medically relevant *Candida* species and 4 related genera (from Kreger van Rij, 1984).

a Fermentation is the production of gas independent of pH changes.

Species	Assimilation													
	Glu	**Gal**	**Mal**	**Tre**	**D-Xyl**	**Starch**	**Succ**	**D-Man**	**L-Ara**	**D-Gluci**	**Suc**	**Sal**	**Mel**	**Gly**
C. tropicalis	+	+	+	+	+	+	+	+	+ weak	+	v	v	v	v
C. albicans	+	+	+	+	+	+	v	+	v	+ (d)	+ (some -)	-	v	v
C. famata	+	+	+	+	+	v	+	+	+	+	+	+	+	+
C. glabrata	+	-	-	+	-	-	-	-	-	-	-	-	-	v
C. guilliermondii	+	+	+	+	+	-	+	+	+	+	+	+	+	+
C. haemulonii	+	+	+	+	+	-	+	+	+ (d)	+	+	-	+	+
C. kefyr	+	+	-	v	v	-	v	v	v	-	+	v	-	v
C. krusei	+	-	-	-	-	-	+	-	-	-	-	-	-	+ (d)
C. lusitaniae	+	v	+	+	+	-	+	+	v	+	+	+	v	+
C. norvegensis	+	-	-	-	-	v	+	-	-	-	-	-	-	-
C. parapsilosis	+	+	+	+	+	-	v	+	+	+	+	-	-	+
C. dubliniensis	+	+	+	v	-	?	?	+	-	?	+	?	-	-
C. viswanathii	+	+	+	+	+	+	+	+	+ (d)	-	+	+	+	+
C. neoformans var *gattii*	+	+	+	+	+	v	v	+	+ (d)	+	+	v	-	v
C. neoformans var *neoformans*	+	+	+	+	+	v	v	+	+ (d)	+	+	v	+	v
R. rubra	+	v	v	+	+	-	v	v	v	v	+	v	v	v
S. cerevisiae	+	v	+ (some -)	v	-	v	- (some +)	v	-	- (some +)	+ (some -)	-	v	v
T. beigelii	+	+	+	v	+	+ (d)	v	v	v	?	+	+ (d)	v	v

Table 2c; Biochemical characters of 13 medically relevant *Candida* species and 4 related genera (from Kreger van Rij, 1984).

Species	Assimilation														
	Cel	**D-Rib**	**Ribi**	**L-Sor**	**Cit**	**DL-Lac**	**Pot**	**L-Rham**	**Lac**	**Raf**	**Meli**	**Gala**	**Ery**	**Ino**	**D-Ara**
C. tropicalis	v	v	v	v	v	v	-	-	-	-	-	-	-	-	-
C. albicans	-	v (some +)	- (some +)	v	v	v	-	-	-	-	-	-	-	-	-
C. famata	+	v	+	v	v	+	-	v	v	+	v	v	v	-	v
C. glabrata	-	-	-	-	-	v	-	-	-	-	?	-	-	-	-
C. guilliermondii	+	v	+	+ (d)	+	v	-	+	-	+	+	+ (d)	-	-	+ (d)
C. haemulonii	-	-	+ (d)	-	+	-	-	+	-	+ (d)	-	+ (d)	-	-	-
C. kefyr	v	v	-	v	v	+	-	-	+	+	-	-	-	-	-
C. krusei	-	- (some +)	-	v	v	+	-	-	-	-	-	-	-	-	-
C. lusitaniae	+	+ (d)	+	v	v	+	-	+	-	-	-	-	-	-	v
C. norvegensis	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-
C. parapsilosis	-	v	+ (d)	v	v	v	-	-	-	-	-	-	-	-	-
C. dubliniensis	-	?	?	-	?	-	?	-	-	-	-	?	-	-	?
C. viswanathii	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-
C. neoformans var *gattii*	+ (w)	+ (w)	v	v	v	v	-	+	v	+ (w)	-		+ (d)	+	+
C. neoformans var *neoformans*	+ (w)	+ (w)	v	v	v	v	-	+	-	+ (w)	-	+ (d)	-	+	+
R. rubra	v	v	+ (some -)	v	v	v	-	v	-	+	-	v	-	-	+ (d)
S. cerevisiae	-	-	-	-	-	v	-	-	-	+	v	-	-	-	-
T. beigelii	+	+ (some -)	v	v	v	+ (d)	-	v	+	v	v	- (some +)	v	v	v

Table 2d; Biochemical characters of 13 medically relevant *Candida* species and 4 related genera (from Kreger van Rij, 1984).

Additional characters for *Candida dubliniensis* identification

Species	Sor	2-Keto	N-Ag	Gluc	Pal	D-Rib	Glucur	Glucon	Esculin
C. dubliniensis	+	+	+	+	v		-	-	-

Antifungal	Class	Mode of action
Amphotericin B	macrocyclic polyene	Binds to ergosterol, a major cell membrane component, compromising its strength. Can cause oxidative damage to fungal cells
Fluconazole	synthetic bis-triazole	Inhibits step in ergosterol synthesis, decreasing cellular ergosterol and increasing accumulation of methylated sterols.
5-fluorocytosine	synthetic fluorinated pyrimidine	Converted into 5-fluorouracil and incorporated into RNA, blocking protein synthesis. Blocks thymidylate synthetase and inhibits DNA synthesis
Itraconazole	synthetic dioxolane triazole	Same mode of action as fluconazole
Ketoconazole	synthetic dioxolane imidazole	Interferes with ergosterol synthesis
Miconazole	synthetic phenethyl imidazole	Interferes with ergosterol synthesis. At high concentration, interacts with membrane lipids causing direct membrane damage

Table 3; Classification of antifungals commonly used to treat *Candida* infections. Derived from Richardson and Warnock (1994) *Fungal infection; diagnosis and management* (London Blackwell Scientific Publications).

APPENDIX 2

Isolate Code	Sex	HIV status	Site of Isolation	Candidiasis Infection	Antifungal Resistance	Patient's Therapy	Other Notes
CBS 562			skin disorder				C. albicans Type strain
17484			human				C. albicans
CBS 834			kefir grains				Kluveromyces marxianus (C. kefyr) b Type strain
ATCC 46764							C. kefyr
15526			human				C. dubliniensis
CBS 7987							C. dubliniensis Type strain
CBS 4024			cerebrospinal fluid				C. viswanathii Type strain
ATCC 22981							C. viswanathii
ATCC 2601							Saccharomyces cerevisiae
CBS 566							Pichia guilliermondii (C. guilliermondii) Type strain
ATCC 6260							C. guilliermondii
CBS 604							C. parapsilosis Type strain
ATCC 22019							C. parapsilosis
CBS 6564			pregnant woman's vagina				Pichia norvogensis (C. norvogensis) Type strain
CBS 2466							Trichosporon cutaneum Type strain
CBS 6289			human				Cryptococcus neoformans var. gattii Type strain
CBS 132			fermenting fruit juice				C. neoformans var. neoformans Type strain
CBS 138			human faeces				C. glabrata (Torulopsis glabrata) Type strain
CBS 6936			citrus peel juice				Clavispora lusitaniae (C. lusitaniae) Type strain
ATCC 42720							C. lusitaniae
CBS 94			human				C. tropicalis Type strain
ATCC 13803							C. tropicalis
CBS 573							Issatchenkia orientalis (C. krusei) Type strain
ATCC 6258							C. krusei
CBS 5149			gut of Haemulon sciurus				C. haemulonii Type strain
CBS 940			air				C. famata Type strain
ATCC 90028			Blood	systemic			C. albicans
ATCC 90029			Blood	systemic			C. albicans
ATCC 90030			Blood	systemic			C. glabrata
ATCC 90118			Sputum	gastric			C. parapsilosis
ATCC 90112			Cerebrospinal fluid	nervous			C. neoformans var. neoformans
ATCC 90113			Cerebrospinal fluid	nervous			C. neoformans var. gattii
C1	M	-	Undiluted Saliva	-			
S1	M	-	Undiluted Saliva	-			Not C. albicans
S2	M	-	Undiluted Saliva	-			Taking antibiotics
S3	F	-	Undiluted Saliva	-			BHSc
S4	F	-	Undiluted Saliva	-			
S5	M	-	Undiluted Saliva	-			
S6	F	-	Undiluted Saliva	-			Taking antibiotics
S7	F	-	Undiluted Saliva	-			BHSc
S8	M	-	Undiluted Saliva	-			
S9	F	-	Undiluted Saliva	-			
S10	M	-	Undiluted Saliva	-			BHSc
S11	F	-	Undiluted Saliva	-			BHSc, taking antibiotics
S12	M	-	Undiluted Saliva	-			
S13	M	-	Undiluted Saliva	-			
S14	F	-	Undiluted Saliva	-			Taking antibiotics
S15	M	-	Undiluted Saliva	-			
S16	M	-	Undiluted Saliva	-			
S17	F	-	Undiluted Saliva	-			BHSc
S18	M	-	Undiluted Saliva	-			
S19	F	-	Undiluted Saliva	-			Not C. albicans
S20	F	-	Undiluted Saliva	-			
S21	F	-	Undiluted Saliva	-			Not C. albicans
S22	M	-	Undiluted Saliva	-			Betadine mouth rinse
S23	F	-	Undiluted Saliva	-			

Table 1 Details of the clinical Candida isolates used

Isolate Code	Sex	HIV status	Site of Isolation	Candidiasis Infection	Antifungal Resistance	Patient's Therapy	Other Notes
*12716	M	+	Oral Swab	+	Fluconazole, moderate 5-flurocytosine		Mixed infection with C. krusei; resistant to Fluconazole, Itraconazole and 5-flurocytosine
14130	M	+	Oral	+	-	Fluconazole	Outpatient. Reidentified WCH on 14/12/95 - Trichosporon cutaneum
15526	M	?	Oral	+	-	Ketoconazole	Germ-tube negative, recurrent infection
16272	M	?	Systemic; Blood	?	-		
16517	M	?	Systemic; Blood	?	-	Penicillin	
17130	F	+	Oesophageal Biopsy	+	Fluconazole, Itraconazole	Fluconazole	
17484	M	+	Throat Swab	+	Fluconazole, Itraconazole, 5 flurocytosine	Ketoconazole	
17640	M	+	Oral	+	-		
18502	M	+	Oral	+	-	Itraconazole trial	Low CD4 count
18527	M	?	Pancreas	?	-		Multiple abscess and on liver taken at laparotomy; from C. albicans, enterococci and multiple resistant P. aeruginosa
18715	M	+	Oral Buccal Mucosa Swab	+	Fluconazole	Fluconazole	
18735	F	?	Systemic; Blood	?	-		
1A	M	+	Oral Rinse	+			
1B	M	+	Oral Rinse	+			Genetically identical
2A	M	+	Oral Rinse	+			
2B	M	+	Oral Rinse	+			Genetically identical
5A	M	+	Oral Rinse	+			
5B	M	+	Oral Rinse	+			Genetically different. Two colonial morphologies.
P1							Different virulence
A2							in mouse model
19426; 1A.1 to 6	M	+	Swab OHL tongue	+			Previous microscopy +ve for fungal elements but not cultured - recultured
20719; 1B.1 to 6	M	+	Oral Rinse	+			
23603; 1C.1 to 6	M	+	Oral Rinse	+			
24852; 1D.1 to 6	M	+	Oral Rinse	?			
26403; 1E.1 to 6	M	+	Palate Swab	Oropharyngeal candidiasis		nil at present	Sensitivities requested especially ketoconazole
26404; 1F.1 to 6	M	+	Oral Rinse	Oropharyngeal candidiasis		nil at present	Sensitivities requested especially ketoconazole
19436; 2A.1 to 6	M	+	Buccal Mucosa swab	+		Was on Itraconazole trial (placebo?), 14 days Ketoconazole, now fungalin lozenges	Oropharyngeal and oesophageal candida
19437; 2B.1 to 6	M	+	Oral Rinse	+		Was on Itraconazole trial (placebo?), 14 days Ketoconazole, now fungalin lozenges	Oropharyngeal and oesophageal candida
19438; 3A.1 to 6	M	+	Swab floor of mouth	+			Fom. candidiasis
19453; 4A.1 to 6	M	?	Oral Rinse	?			Culture for C. albicans presence
19468; 5A.1 to 6	M	-	Tongue swab	?			Radiotherapy for ?. Xerostomia Glossitis and coated tongue
19483; 6A.1 to 6	M	?	Oral Rinse	+			
19502; 7A.1 to 6	M	+	Oral Rinse	+			
19503; 8A.1 to 6	M	+	Oral Rinse	+			
21561; 8B.1 to 6	M	+	Oral Rinse	+			
27108; 8C.1 to 6	M	+	Oral Rinse	?			
19596; 9A.1 to 6	M	+	?	+			
22487; 9B.1 to 6	M	+	Oral Rinse	?			
22488; 9C.1 to 6	M	+	Swab Palate/Throat	?	fluconazole	Fluconazole	Low CD4+ count
26072; 9D.1 to 6	M	+	Oral Rinse	?			
27262; 9E.1 to 6	M	+	Swab Pharynx	Oesophageal?		Fluconazole	Sensitivity to FCZ requested
27263; 9F.1 to 6	M	+	Oral Rinse	Oesophageal?		Fluconazole	Sensitivity to FCZ requested

Table 1 Details of the clinical Candida isolates used

Isolate Code	Sex	HIV status	Site of Isolation	Candidiasis Infection	Antifungal Resistance	Patient's Therapy	Other Notes
27379; 9G.1 to 6	M	+	Oral Swab	?			Sensitivity to FCZ requested
27380; 9H.1 to 6	M	+	Oral Rinse	?			Sensitivity to FCZ requested
19597; 10A.1 to 6	M?	+	?	+			
19636; 11A.1 to 6	F	+	Oral Rinse	+			
19717; 12A.1 to 6	M	+	Mouth Swab	?			
19718; 13A.1 to 6	M	+	Mouth Swab	?			
19719; 14A.1 to 6	M	+	Oral Swab	+			
19775; 15A.1 to 6	M	+	?	?			*C. glabrata*
19776; 16A.1 to 6	M	+	?	?			
19777; 17A.1 to 6	M	+	?	?			
20937; 17B.1 to 6	M	+	Oral Rinse	-			
22055; 17C.1 to 6	M	+	Oral Rinse	?			
22056; 17D.1 to 6	M	+	Swab of Palate	?			
22151; 17E.1 to 6	M	+	Oral Rinse	+			
22490; 17F.1 to 6	M	+	Oral Rinse	+			
22939; 17G.1 to 6	M	+	Oral Rinse	+			
22940; 17H.1 to 6	M	+	Palate Swab	+			Previous cultures taken - A Candida strain resistant to fluconazole
19889; 18A.1 to 6	M	+	Mouth Swab	?			
19890; 19A.1 to 6	M	+	Oral Rinse	?			
22836; 19B.1 to 6	M	+	Buccal mucosal swab	?			
23720; 19C.1 to 6	M	+	Oral Rinse	?			
25002; 19D.1 to 6	M	+	Oral Rinse	?			
19905; 20A.1 to 6	M	+	?	?			
19995; 21A.1 to 6	F	+	Oral Rinse	+			
24090; 21B.1 to 6	F	+	Oral Rinse	+			
19996; 22A.1 to 6	M	+	Oral Rinse	?			
26861; 22B.1 to 6	M	+	Oral Rinse	+	Fluconazole	100mg/day fluconazole (trial)	Fluconazole resistant candidiasis, Germ tube positive, FCZ resistance required
27104; 22C.1 to 6	M	+	Oral Swab Hard Palate	+			Resistance to fluconazole, ketaconazole and amphoteracin B requested
27105; 22D.1 to 6	M	+	Oral Swab Floor of Mouth	+			Resistance to fluconazole, ketaconazole and amphoteracin B requested
?	M	+	Retromolar ?	Pseudomembranous candidiasis			Resistance to amphoteracin B requested
?	M	+	Oral Rinse	+			Resistance to fluconazole, ketaconazole and amphoteracin B requested
27290; 22G.1 to 6	M	+	Hard Palate Swab	Pseudomembranous candidiasis on hard palate			Resistance to amphoteracin B requested
27291; 22H.1 to 6	M	+	Oral Rinse	Pseudomembranous candidiasis on hard palate			Resistance to amphoteracin B requested
27397; 22I.1 to 6	M	+	Oral Rinse	?			
19941; 23A.1 to 6	M	+	Oral Rinse	+			
20454; 23B.1 to 6	M	+	OHL Tounge Swab	OHL		Fungilin	
20473; 23C.1 to 6	M	+	Oral Rinse	?			
22390; 23D.1 to 6	M	+	Oral Rinse	?			
19942; 24A.1 to 6	M	+	Swab Fom	+		Nilstat oral drops	Resection for fom scc & rnd & rt to bilateral neck.

Table 1 Details of the clinical Candida isolates used

Isolate Code	Sex	HIV status	Site of Isolation	Candidiasis Infection	Antifungal Resistance	Patient's Therapy	Other Notes
20019; 25A.1 to 6	M	+	Swab	+			
20044; 26A.1 to 6	M	+	Oral Rinse	?			
20939; 26B.1 to 6	M	+	Tongue Swab	?		AZT	
20069; 27A.1 to 6	M	+	Oral Rinse	?			
20082; 27B.1 to 6	M	+	Tongue Swab	?			
23117; 27C.1 to 6	M	+	Oral Rinse	?			
20084; 28A.1 to 6	M	+	Throat Swab	Red Throat			Red Throat
20089; 29A.1 to 6	M	+	Oral Rinse	?			
21507; 29B.1 to 6	M	+	Oral Rinse	?			
20090; 30A.1 to 6	M	+	Oral Rinse	+	fluconazole?	On 50mg fluconazole a day	Previous isolate fluconazole sensitive
20168; 31A.1 to 6	M	+	Oral Rinse	?			
20986; 31B.1 to 6	M	+	Oral Rinse	OHL			OHL
20987; 31C.1 to 6	M	+	Tongue swab	OHL			OHL
21527; 31D.1 to 6	M	+	Oral Rinse	?			
24049; 31E.1 to 6	M	+	Oral Rinse	?			
20169; 32A.1 to 6	M	+	Swab OHL Tongue	?			
20170; 32B.1 to 6	M	+	Oral Rinse	?			
20171; 33A.1 to 6	M	+	Oral Rinse	?			
22789; 33B.1 to 6	M	+	Oral Rinse	?			
20187; 34A.1 to 6	M	+	Oral Rinse	?			Growth on agar slopes very adhesive and granular
22788; 34B.1 to 6	M	+	Oral Rinse	?			Growth on agar slopes very adhesive and granular
27107; 34C.1 to 6	M	+	Oral Rinse	?			Candida species
20196; 35A.1 to 6	M	+	Swab Buccal Mucosa	?			
20197; 35B.1 to 6	M	+	Swab	?			
20198; 35C.1 to 6	M	+	Oral Rinse	?			
20199; 36A.1 to 6	M	+	Oral Rinse	?			
20200; 37A.1 to 6	M	+	Oral Rinse	?			
20418; 37B.1 to 6	M	+	Tongue Swab	?			
20419; 37C.1 to 6	M	+	Oral Rinse	?			
20339; 38A.1 to 6	M	?	Oral Rinse	?			Only One Colony
20384; 39A.1 to 6	M	+	Oral Rinse	?			
21852; 39B.1 to 6	M	+	Oral Rinse	?			
24442; 39C.1 to 6	M	+	Oral Rinse	?			
26269; 39D.1 to 6	M	+	Oral Rinse	+		fluconazole	Chemotherapy for lymphoma, fluconazole and miconazole sensitivities requested
26270; 39E.1 to 6	M	+	Oral Swab	+		fluconazole	Chemotherapy for lymphoma, fluconazole and miconazole sensitivities requested
20386; 40B.1 to 6	M	+	Oral Rinse	?			
22389; 40C.1 to 6	M	+	Oral Rinse	?			
23931; 40D.1 to 6	M	+	Oral Rinse	?			
24132; 40E.1 to 6	M	+	Oral Rinse	?			
24270; 40F.1 to 6	M	+	Oral Rinse	?			Only two colonies
24408; 40G.1 to 6	M	+	Oral Rinse	?			Candida species (not Ca) yeast, germ tube +, looks mixed

Table 1 Details of the clinical Candida isolates used

Isolate Code	Sex	HIV status	Site of Isolation	Candidiasis Infection	Antifungal Resistance	Patient's Therapy	Other Notes
24853; 40H.1 to 6	M	+	Oral Rinse	?			
25361; 40I.1 to 6	M	+	Oral Rinse	?			Candida species
25811; 40J.1 to 6	M	+	Oral Rinse	?		Amphoteracin B?	Amp B sensitivity testing requested
2036?; 41A.1 to 6	M	+	Oral Rinse	?			
20400; 42A.1 to 6	M	+	Oral Rinse	?		Not previously treated	
20401; 42B.1 to 6	M	+	Tongue Swab	?		Not previously treated	
21724; 42C.1 to 6	M	+	Oral Rinse	?			
23721; 42D.1 to 6	M	+	Oral Rinse	?			
23721; 42E.1 to 6	M	+	Oral Rinse	?			
20402; 43A.1 to 6	M	+	Tongue Swab	?		Fluconazole, dosage recently increased	43A.3-6 Growth on agar slopes very adhesive and granular
21369; 43B.1 to 6	M	+	Oral Rinse	?			Growth on agar slopes very adhesive
20417; 44A.1 to 6	M	+	Oral Rinse	?			
26711; 44B.1 to 6	M	+	Oral Rinse	+			Candida palate and tongue
26712; 44C.1 to 6	M	+	Palate Swab	+			Candida palate and tongue
27106; 44D.1 to 6	M	+	Oral Rinse	?			
27214; 44E.1 to 6	M	+	Oral Rinse	?			Resistance to amphoteracin B requested
20445; 45A.1 to 6	M	+	Swab from OHL on Tongue	OHL			
20471; 46A.1 to 6	M	+	Oral Rinse	?			
21911; 46B.1 to 6	M	+	Oral Rinse	?			
23934; 46C.1 to 6	M	+	Oral Rinse	+			
24146; 46D.1 to 6	M	+	Oral Rinse	+			
25467; 46F.1 to 6	M	+	Oral Rinse	?			Amp-B sensitivity requested Trial completed
26071; 46G.1 to 6	M	+	Oral Swab	?			
20472; 47A.1 to 6	M	+	Oral Rinse	?			
20544; 48A.1 to 6	M	+	Tongue Swab, OHL	OHL			OHL lateral ? tongue
20545; 48B.1 to 6	M	+	Oral Rinse	OHL			OHL lateral ? tongue
20546; 49A.1 to 6	M	+	Oral Rinse	?			
20547; 50A.1 to 6	M	+	Oral Rinse	?			
22180; 50B.1 to 6	M	+	Oral Rinse	?			
23933; 50C.1 to 6	M	+	Oral Rinse	?			
20559; 51A.1 to 6	M	+	Oral Rinse	?			
21722; 51B.1 to 6	M	+	Oral Rinse	?			
20560; 52A.1 to 6	M	+	Oral Rinse	?			Colony 52A.6 Labelled 26A.6 on McCartney, 2 colony types - one very adhesive
20561; 52B.1 to 6	M	+	Tongue Swab	?			White ? tongue
23724; 52C.1 to 6	M	+	Oral Rinse	+			candidiasis - dorsum of tongue.
20652; 53A.1 to 6	M	+	Oral Rinse	?			53A.1-6 growth on agar slopes very adhesive
21122; 53B.1 to 6	M	+	Oral Rinse	?			
21562; 53C.1 to 6	M	+	Oral Rinse	?			
20651; 54A.1 to 6	M	+	Oral Rinse	?			54A.1 growth on agar slopes very adhesive
20720; 54B.1 to 6	M	+	Oral Rinse	?			
20938; 54C.1 to 6	M	+	Oral Rinse	?			

Table 1 Details of the clinical Candida isolates used

Isolate Code	Sex	HIV status	Site of Isolation	Candidiasis Infection	Antifungal Resistance	Patient's Therapy	Other Notes
21066; 54D.1 to 6	M	+	Oral Rinse	?			
21265; 54E.1 to 6	M	+	Oral Rinse	?			
21681; 54F.1 to 6	M	+	Oral Rinse	?			Amphotericin B trial
22323; 54G.1 to 6	M	+	Oral Rinse	?			
22326; 54H.1 to 6	M	+	Oral Rinse	?			Amphotericin B trial
22808; 54I.1 to 6	M	+	Oral Rinse	?			Amphotericin B trial
24394; 54J.1 to 6	M	+	Oral Rinse	Oral pseudomembranous candidiasis			Amphotericin B trial
24395; 54K.1 to 6	M	+	Oral Swab	Oral pseudomembranous candidiasis			Amphotericin B trial
20649; 55A.1 to 6	M	+	Oral Rinse	?			
22125; 55B.1 to 6	M	+	Oral Rinse	?			
20650; 56A.1 to 6	M	+	Oral Rinse	carrier			Post radiotherapy patient, not HIV+. 56A.2 and .4 growth on agar slopes very adhesive
21680; 56B.1 to 6	M	+	Oral Rinse	carrier			Post radiotherapy patient
20701; 57A.1 to 6	M	+	Tongue Scrape	?			
20702; 57B.1 to 6	M	+	Oral Rinse	?			
21618; 57C.1 to 6	M	+	Oral Rinse	?			
20698; 58A.1 to 6	M	+	Oral Rinse	OHL			CD4<200, OHL tongue and coated with white plaques
20699; 58B.1 to 6	M	+	Tongue Swab	OHL			CD4<200, OHL tongue and coated with white plaques. 58B.5-6 Growth on agar slopes very adhesive and granular
22305; 58C.1 to 6	M	+	Oral Rinse	?			
27264; 58D.1 to 6	M	+	Oral Rinse	?			
20700; 59A.1 to 6	M	+	Oral Rinse	?			59A.4 Growth on agar slopes very adhesive and granular
24877; 59B.1 to 6	M	+	Tongue Swab	Median Rhomboid ?			
24878; 59C.1 to 6	M	+	Palate Swab	Erythematous candidasis hard palate			
24879; 59D.1 to 6	M	+	Oral Rinse	+			
25003; 59E.1 to 6	M	+	Oral Rinse	?			
25004; 59F.1 to 6	M	+	Palate Swab	?			
25362; 59G.1 to 6	M	+	Oral Rinse	?			
25884; 59H.1 to 6	M	+	Oral Rinse	?			
26625; 59I.1 to 6	M	+	Oral Rinse	?			
20734; 60A.1 to 6	M	+	Mouth Swab	?			
25952; 60B.1 to 6	M	+	Oral Rinse	?			
25953; 60C.1 to 6	M	+	Tongue Swab	?			
25954; 60D.1 to 6	M	+	Palate Swab	?			
21006; 61A.1 to 6	M	+	Tongue swab	?			
21123; 62A.1 to 6	M	+	Mouth swab (penile swab not sent over)	?			HIVAb+, immunosuppressed, clinical HSV of penile shaft
25885; 62B.1 to 6	M	+	Oral Swab	?			
21128; 63A.1 to 6	M	+	Tongue? Swab	OHL and candidiasis			
21129; 63B.1 to 6	M	+	Oral Rinse	OHL and candidiasis			
21130; 63C.1 to 6	M	+	Oral swab	OHL and candidiasis			White plaques on retromolar mucosa
22057; 63'C.1 to 6	M	+	Oral swab	?			

Table 1 Details of the clinical Candida isolates used

Isolate Code	Sex	HIV status	Site of Isolation	Candidiasis Infection	Antifungal Resistance	Patient's Therapy	Other Notes
22058; 63D.1 to 6	M	+	Oral Rinse	?			
22236; 63E.1 to 6	M	+	Tongue Swab	?			
22237; 63F.1 to 6	M	+	Palate Swab	?			
22238; 63G.1 to 6	M	+	Oral Rinse	?			
22325; 63H.1 to 6	M	+	Oral Rinse	?			
22616; 63I.1 to 6	M	+	Oral Rinse	?			
22619; 63J.1 to 6	M	+	Oral Swab	?			
22620; 63K.1 to 6	M	+	Oral Swab Tongue	?			
23116; 63L.1 to 6	M	+	Oral Swab	pseudomembranous candidiasis			
23118; 63M.1 to 6	M	+	Oral Rinse	pseudomembranous candidiasis			
23317; 63N.1 to 6	M	+	Oral Swab, lateral border of tongue	pseudomembranous candidiasis, OHL likelihood			
23318; 63O.1 to 6	M	+	Oral Swab	Candidiasis (buccal mucosa)			Amphoteracin B topical
23319; 63P.1 to 6	M	+	Oral Rinse	?			
23722; 63Q.1 to 6	M	+	Oral Rinse	?			
23723; 63R.1 to 6	M	+	Swab	Angular chelita?			
24131; 63S.1 to 6	M	+	Oral Rinse				
21264; 64A.1 to 6	M	+	Oral Rinse	?			
21369; 64B.1 to 6	M	+	Oral Rinse	?			
25043; 64C.1 to 6	M	+	Oral Rinse	?			
21333; 65A.1 to 6	M	+	Oral Rinse	OHL			
21334; 65B.1 to 6	M	+	Oral Swab	OHL			
21367; 66A.1 to 6	M	+	Oral Rinse	?			
21563; 66B.1 to 6	M	+	Candida culture	?			
2223?; 66D.1 to 6	M	+	Oral Rinse	?			
22618; 66E.1 to 6	M	+	Swab	OHL?			
22623; 66F.1 to 6	M	+	Oral Rinse	?			
23119; 66G.1 to 6	M	+	Oral Rinse	?			
21368; 67A.1 to 6	M	+	Oral Rinse	?			
21560; 68A.1 to 6	M	+	Oral Rinse	?			not Candida albicans, unidentified Candida species
23932; 68B.1 to 6	M	+	Oral Rinse	?			not Candida albicans, unidentified Candida species
25316; 68C.1 to 6	M	+	Oral Rinse	?			
25317; 68D.1 to 6	M	+	Pharynx Swab	?			Amp-B sensitivity requested
25810; 68E.1 to 6	M	+	Oral Rinse	?		Amphoteracin B topical therapy	Both cultures labelled 68E.1
26283; 68F.1 to 6	M	+	Oral Rinse	?			
26557; 68G.1 to 6	M	+	Oral Rinse	?			
27197; 68H.1 to 6	M	+	?	?			
27198; 68I.1 to 6	M	+	Oral Rinse	?			
21682; 69A.1 to 6	M	+	Oral Rinse	?			
21683; 70A.1 to 6	M	+	Oral Rinse	?			Candida glabrata
21723; 71A.1 to 6	M	+	Oral Rinse	?			
21853; 71B.1 to 6	M	+	Oral Rinse	?			
21912; 72A.1 to 6	M	+	Oral Rinse	?			

Table 1 Details of the clinical Candida isolates used

Isolate Code	Sex	HIV status	Site of Isolation	Candidiasis Infection	Antifungal Resistance	Patient's Therapy	Other Notes
22126; 73A.1 to 6	M	+	Oral Rinse	?			
22322; 74A.1 to 6	M	+	Oral Swab	?			
22324; 75A.1 to 6	M	+	Oral Rinse	?			
22364; 76A.1 to 6	M	+	Oral Rinse	?			Not *C. albicans*
22365; 76B.1 to 6	M	+	Oral Rinse	?			Not *C. albicans*
22366; 77A.1 to 6	M	+	Oral Swab	?			
22367; 77B.1 to 6	M	+	Oral Rinse	?			
22422; 78A.1 to 6	M	+	Oral Rinse	?			
22423; 79A.1 to 6	M	+	Swab - Floor of Mouth	?			CD4 160
22489; 80A.1 to 6	M	+	Oral Rinse	?			
22617; 81A.1 to 6	M	+	Oral Rinse	?			two 81A.2 and 81A.3, no 81A.1 or 81A.4 - relabelled
22621; 82A.1 to 6	M	+	Oral Rinse	?			
22622; 83A.1 to 6	M	+	Oral Rinse	?			
22790; 84A.1 to 6	M	+	Oral Rinse	+			
22791; 84B.1 to 6	M	+	Palate Swab	+			
26410; 84C.1 to 6	M	+	Tongue Swab	+		Fluconazole	Candida species
27196; 84D.1 to 6	M	+	?	?		Fluconazole	
27230; 84E.1 to 6	M	+	Oral Rinse	?			
27311; 84F.1 to 6	M	+	Oral Rinse	?			Sensitivity to fluconazole, itraconazole, ketoconazole and amphoteracin B requested
27312; 84G.1 to 6	M	+	Oral Swab	?			Sensitivity to fluconazole, itraconazole, ketoconazole and amphoteracin B requested
23316; 85A.1 to 6	M	+	Oral Swab	?			
23501; 86A.1 to 6	M	+	Oral Rinse	?			Candida species, not C.albicans
23559; 87A.1 to 6	M	+	Tongue Swab	?		none	
24050; 88A.1 to 6	M	+	Oral Rinse	?			
26170; 88B.1 to 6	M	+	Oral Rinse	+			Oral pseudomembranous candidiasis, sensitivity to amphotericin B requested
26171; 88C.1 to 6	M	+	Oral Swab	+			Oral pseudomembranous candidiasis, sensitivity to amphotericin B requested
26256; 88D.1 to 6	M	+	Oral Rinse	+			candidiasis, oral hairy leukoplakia
26257; 88E.1 to 6	M	+	Left buccal mucosal swab	+			candidiasis, oral hairy leukoplakia, sensitivity to amphoteracin B requested
26258; 88F.1 to 6	M	+	Right buccal mucosal swab	+			candidiasis, oral hairy leukoplakia, sensitivity to amphoteracin B requested
26367; 88G.1 to 6	M	+	Left Buccal Mucosal Swab	Pseudomembranous candidiasis over OHL			pseudomembranous candidiasis, oral hairy leukoplakia, sensitivity to amphoteracin B requested
26368; 88H.1 to 6	M	+	Oral Rinse	Pseudomembranous candidiasis over OHL			pseudomembranous candidiasis, oral hairy leukoplakia, sensitivity to amphoteracin B requested
26860; 88I.1 to 6	M	+	Oral Rinse	?			Germ tube positive
27212; 88J.1 to 4	M	+	Swab Buccal Mucosa Left	?			Resistance to amphoteracin B requested
27213; 88K.1 to 4	M	+	Oral Rinse	?			
24130; 89A.1 to 6	M	+	Oral Rinse	?			
24213; 90A.1 to 6	M	+	Oral Rinse	?			
24256; 91A.1 to 6	M	+	Oral Rinse	?			Candida species, germ tube + but ?, mixed with other yeasts

Table 1 Details of the clinical Candida isolates used

Isolate Code	Sex	HIV status	Site of Isolation	Candidiasis Infection	Antifungal Resistance	Patient's Therapy	Other Notes
25436; 92A.1 to 6	M	+	Oral Rinse	?			
26172; 93A.1 to 6	M	+	Oral Swab Tongue	+			Pseudomembranous candidiasis, left tongue, lateral border, sensitivity to amphotericin B requested
26369; 93B.1 to 6	M	+	Oral Rinse	?			sensitivity to amphotericin B requested
26459; 93C.1 to 6	M	+	Oral Rinse	?			
26460; 93D.1 to 6	M	+	Left Swab Floor of Mouth	Pseudomembranous candidiasis?			sensitivity to amphotericin B requested
26461; 93E.1 to 6	M	+	Right FOM Swab	Pseudomembranous candidiasis?			sensitivity to amphotericin B requested
26458; 94A.1 to 6	M	+	Oral Rinse	?			
26603; 95A.1 to 6	M	+	Oral Rinse	?			
26859; 96A.1 to 6	M	+	Cheek Swab	?			Sensitivity to fluconazole requested
27072; 97A.1 to 6	M	+	Oral Rinse	?			
27110; 97B.1 to 6	M	+	Oral Swab corners of mouth	angular chelitis			
27229; 97D.1 to 6	M	+	Oral Swab angle of mouth				
27073; 98A.1 to 6	M	+	Oral Rinse	?			Candida species, Sensitivity to fluconazole requested
27199; 99A.1 to 6	M	+	Oral Rinse	?			
27265; 100A.1 to 6	F	+	Oral Rinse	?			
27289; 101A.1 to 6	M	+	Oral Rinse	?			Sensitivity to amphoteracin B requested

Table 1 Details of the clinical Candida isolates used

Enzyme locus	Loading position	Running time	Running buffer	Special conditions
ACON	cathode	1½ hr	Phos	
ACP	cathode	1½ hr	TM	
ADA	cathode	1½ hr	Phos	
ADH	cathode	1 hr	CP	1.2 ml 70% EtOH
ADH	cathode	1½ hr	Phos	1.6 ml 70% EtOH
ADH[a]	**cathode**	**1¼ hr**	**TM**	
AK	cathode	1½ hr	TM	
ALD	cathode	1½ hr	TM	
ALD	**cathode**	**1¼ hr**	**TM**	
AP	cathode	1½ hr	TEB	
AP	cathode	1¼ hr	TM	Post couple 1½ hr Freeze thawed + sonicated samples
AP	cathode	1¼ hr	TM	Post couple 2½ hr Freeze thawed + sonicated samples
DIA	cathode	1 hr	Phos	1.2ml DCIP
ENOL	cathode	1 hr	CP	
ENOL	**cathode**	**1¼ hr**	**Phos**	
EST	cathode	1 hr	Phos	
EST	**cathode**	**1¼ hr**	**TM**	
FDP	cathode	1½ hr	TM	
FDP	**cathode**	**1¼ hr**	**Phos**	
FUM	cathode	1 hr	TM	
FUM	**cathode**	**1¼ hr**	**TM**	
GDA	cathode	1 hr	Phos	
GDH	cathode	1 hr	TM	
GDH	cathode	1½ hr	TM	sample preparation testing
GDH	**cathode**	**1¼ hr**	**Phos**	
GLDH	cathode	1½ hr	TEB	
GOT	cathode	1 hr	Phos	negative stain
G6PD	cathode	1 hr	TM	
G6PD	cathode	2 hr	CP	Freeze thawed + sonicated samples
G6PD	middle	2 hr	CP	Freeze thawed + sonicated samples
G6PD	anode	2 hr	CP	Freeze thawed + sonicated samples
G6PD	cathode	2 hr	Phos	Freeze thawed + sonicated samples
G6PD	middle	2 hr	Phos	Freeze thawed + sonicated samples

[a] Enzymes in bold are those used in the study and the final conditions optimised for them (contained within Table 2 of the Materials and Methods Chapter).

Table 2; Details of the enzyme loci screened for the allozyme electrophoresis component of this thesis.

Enzyme locus	Loading position	Running time	Running buffer	Special conditions
G6PD	anode	2 hr	Phos	Freeze thawed + sonicated samples
G6PD	**cathode**	**1¼ hr**	**Phos**	
GPI	cathode	1 hr	TM	
GPI	cathode	1½ hr	TM	sample preparation testing
GPI	**cathode**	**1¼ hr**	**Phos**	
GPT	cathode	1 hr	Phos	
HK	cathode	1 hr	TM	
HK	cathode	1½ hr	CP	
HK	middle	1½ hr	CP	
HK	anode	1½ hr	CP	
HK	cathode	1½ hr	Phos	
HK	middle	1½ hr	Phos	
HK	anode	1½ hr	Phos	
HK	cathode	1½ hr	TEB	
HK	middle	1½ hr	TEB	
HK	anode	1½ hr	TEB	
HK	cathode	1½ hr	TM	
HK	middle	1½ hr	TM	
HK	anode	1½ hr	TM	
HK	cathode	2 hr	CP	Freeze thawed + sonicated samples
HK	middle	2 hr	CP	Freeze thawed + sonicated samples
HK	anode	2 hr	CP	Freeze thawed + sonicated samples
HK	cathode	2 hr	Phos	Freeze thawed + sonicated samples
HK	middle	2 hr	Phos	Freeze thawed + sonicated samples
HK	anode	2 hr	Phos	Freeze thawed + sonicated samples
HK	**cathode**	**1¼ hr**	**TM**	
IDH	cathode	1 hr	Phos	
IDH	cathode	1½ hr	Phos	
IDH	**cathode**	**1¼ hr**	**Phos**	
LDH	cathode	1 hr	Phos	
LDH	middle	1½ hr	TM	
LDH	anode	1½ hr	TM	
MDH	cathode	1 hr	TM	
MDH	cathode	1½ hr	Phos	
MDH	cathode	1½ hr	CP	
MDH	middle	1½ hr	CP	

[a] Enzymes in bold are those used in the study and the final conditions optimised for them (contained within Table 2 of the Materials and Methods Chapter).

Table 2; Details of the enzyme loci screened for the allozyme electrophoresis component of this thesis.

Enzyme locus	Loading position	Running time	Running buffer	Special conditions
MDH	anode	1½ hr	CP	
MDH	cathode	1½ hr	Phos	
MDH	middle	1½ hr	Phos	
MDH	anode	1½ hr	Phos	
MDH	cathode	1½ hr	TEB	
MDH	middle	1½ hr	TEB	
MDH	anode	1½ hr	TEB	
MDH	cathode	1½ hr	TM	
MDH	middle	1½ hr	TM	
MDH	anode	1½ hr	TM	
MDH	cathode	1¼ hr	CP	Freeze thawed + sonicated samples
MDH	cathode	1¼ hr	TM	Freeze thawed + sonicated samples
ME	cathode	1½ hr	Phos	
ME	cathode	1¼ hr	CP	Freeze thawed + sonicated samples
ME	cathode	1¼ hr	TM	Freeze thawed + sonicated samples
MPI	cathode	1½ hr	TM	
NDPK	cathode	1½ hr	TEB	
NDPK	**cathode**	**1¼ hr**	**Phos**	
NP	cathode	1½ hr	Phos	
NP	**cathode**	**1¼ hr**	**Phos**	
OGR	cathode	1½ hr	Phos	
PepA	cathode	1 hr	CP	
PepA	cathode	1 hr	CP	
PepA	**cathode**	**1¼ hr**	**CP**	
PepB	cathode	1 hr	CP	
PepB	**cathode**	**1¼ hr**	**CP**	
PepC	cathode	1 hr	CP	
PepD	cathode	1 hr	CP	
PGAM	cathode	1 hr	CP	
PGAM	**cathode**	**1¼ hr**	**Phos**	
6PGD	cathode	1 hr	TM	
6PGD	cathode	1½ hr	TM	
6PGD	**cathode**	**1¼ hr**	**Phos**	
PGK	cathode	1 hr	CP	
PGM	cathode	1 hr	TM	
PGM	**cathode**	**1¼ hr**	**Phos**	
PK	cathode	1 hr	CP	
PK	**cathode**	**1¼ hr**	**Phos**	
UMPK	cathode	1 hr	Phos	

[a] Enzymes in bold are those used in the study and the final conditions optimised for them (contained within Table 2 of the Materials and Methods Chapter).

Table 2; Details of the enzyme loci screened for the allozyme electrophoresis component of this thesis.

100µl of 5% Chelex beads

5µl cell pellet [a]

Place the beads and cells into a reaction tube.

Incubate the mixture at 56°C for 20 minutes

Vortex

Incubate for 8 minutes at 100°C

Centrifuge for 3 minutes to pellet the Chelex beads

Remove the supernatant and place into a fresh reaction tube.

Re-centrifuge, then retain all but the last 10-20µl of supernatant for PCR amplification [b].

[a] Use the cell pellets harvested for allozyme electrophoresis, after sonication and lysate collection.

[b] One Chelex bead is enough to inhibit PCR amplification.

Table 3; Protocol for the extraction of DNA from *Candida* using Chelex beads

PCR mix	PCR protocol	
5µl of one of the initial PCR primers [a]	94°C for 30 seconds	
8µl dye terminator sequencing kit mix	25 cycles of	94°C for 30 seconds
5µl PCR product		50°C for 15 seconds
2µl H_2O		60°C for 4 minutes
	hold at 4°C	

[a] One reaction per primer

Table 4; Protocol for the sequencing PCR used.

Add 80µl H_2O to the 20µl sequencing PCR product
Transfer to a reaction tube containing 10% v/v Na-acetate and 2.5 volumes 100% EtOH
Incubate overnight at -20°C
Centrifuge for 30 minutes at 15,000rpm at 4°C
Discard the supernatant
Add 500µl of 70% EtOH to the pellet
Centrifuge for 20 minutes at 15,000rpm at 4°C
Discard the supernatant
Dry the pellet at approximately 60°C for no more than 5 minutes

Table 5; Ethanol precipitation protocol used for purifying the sequencing PCR product

APPENDIX 3

Isolate	16272	17640	18735	12716	18502	17484	16517	18527	14130	17130	18715
17640	43										
18735	43	54									
12716	57	38	75								
18502	40	23	46	46							
17484	92	75	85	50	75						
16517	46	58	25	75	58	92					
18527	27	43	46	57	36	83	50				
14130	77	58	67	82	67	82	73	75			
17130	50	31	58	23	38	55	67	50	73		
18715	40	21	62	29	29	58	67	47	75	29	
15526	80	85	86	85	100	92	83	86	75	92	86

Table 1; Matrix of the percentages of loci at which fixed differences occurred in the Pilot Screen.

Isolate	18527	17B.1	18502	17B.5	ATCC 90029	1A	18735	17A.5	17A.1	17A.2	1B	17484	17640	15526	ATCC 90113	ATCC 90112	ATCC 90028	17B.4	18715	ATCC 90030	14B.3	2A	17A.3	16517	2B	17B.6	A2	17B.2	12716	ATCC 90118	5A	P1	14130	16272	17A.6	5B	17130
17B.1	58																																		
18502	52	35																																	
17B.5	53	15	40																																
ATCC 90029	60	32	48	42																															
1A	50	6	35	19	35																														
18735	40	37	43	37	50	38																													
17A.5	50	22	40	11	35	13	32																												
17A.1	62	15	36	20	38	12	43	15																											
17A.2	52	20	36	15	43	12	48	15	9																										
1B	50	13	25	20	44	6	40	19	13	13																									
17484	50	47	26	35	58	47	53	33	42	42	36																								
17640	59	13	17	19	47	25	53	31	17	17	13	38																							
15526	94	88	89	88	83	80	72	88	89	89	86	94	94																						
ATCC 90113	86	69	71	77	64	83	71	79	64	71	83	79	75	85																					
ATCC 90112	71	73	71	67	76	79	76	71	71	59	79	75	79	93	38																				
ATCC 90028	48	10	32	25	33	6	33	20	23	23	13	47	22	89	71	71																			
17B.4	57	5	45	10	43	12	48	20	23	23	19	42	28	89	79	71	23																		
18715	52	45	14	40	52	41	43	40	45	45	31	26	28	89	79	76	41	55																	
ATCC 90030	53	6	26	18	32	13	33	26	21	21	20	44	13	82	71	75	5	21	37																
17B.3	50	5	43	10	40	6	40	16	19	14	13	44	24	89	79	69	14	5	52	17															
2A	52	26	24	37	45	19	40	30	29	38	13	33	24	89	79	82	24	33	29	26	30														
17A.3	57	15	45	20	38	6	48	10	9	9	13	42	28	89	71	65	23	14	55	26	10	33													
16517	43	40	41	40	38	29	29	30	41	36	31	53	50	79	79	71	36	41	50	37	33	38	36												
2B	50	33	24	33	53	44	35	31	35	47	33	25	38	93	75	80	41	41	18	40	44	19	41	53											
17B.6	52	10	41	5	48	12	43	20	18	14	13	42	22	89	79	71	23	14	50	21	5	33	18	36	47										
A2	39	47	58	47	37	41	39	44	53	53	44	59	59	71	85	81	47	53	58	41	50	50	53	32	53	53									
17B.2	55	5	40	11	37	13	32	21	15	20	19	47	19	88	64	69	15	15	45	17	10	30	20	40	33	10	47								
12716	43	40	45	40	52	29	52	35	50	41	31	42	39	89	86	71	36	41	41	37	38	43	41	50	47	45	47	45							
ATCC 90118	92	69	85	77	62	77	77	77	69	77	77	92	69	92	45	58	69	77	85	69	77	77	77	85	85	77	85	69	77						
5A	60	50	43	55	60	50	50	47	52	52	40	44	41	89	77	69	43	57	48	39	55	50	57	62	38	57	61	47	48	77					
P1	50	16	38	21	35	18	45	21	29	24	19	39	28	84	79	71	24	19	48	17	15	35	19	38	47	24	47	26	29	77	50				
14130	94	89	95	89	89	93	94	94	89	89	92	88	88	88	92	93	95	89	95	94	89	94	89	95	93	84	94	82	95	100	89	89			
16272	38	40	45	35	38	29	38	25	41	32	31	42	44	84	79	65	32	41	50	32	33	43	36	23	47	36	42	35	41	85	38	38	95		
17A.6	50	21	33	16	38	12	40	5	10	10	13	32	24	89	79	71	19	19	43	21	15	25	10	29	41	14	47	21	38	77	50	20	94	29	
5B	81	60	59	60	67	71	76	70	59	64	69	58	61	84	71	88	64	68	64	53	67	62	68	82	65	64	84	60	73	92	62	62	89	73	67
17130	60	21	52	26	40	19	48	32	38	38	27	47	41	83	79	76	29	29	57	28	25	45	33	48	53	33	39	26	33	77	40	30	89	43	35
17A.4	52	20	45	25	38	6	48	10	14	14	13	42	33	89	71	65	23	18	55	26	14	33	5	36	41	23	53	25	41	77	52	24	95	32	10

Table 2; Matrix of the percentages of loci at which fixed differences occurred in Pilot Study 1.

Isolate	ATCC 90028	ATCC 90030	70A	54A	56A	C1	ATCC 90029	S21	S8	S23	34B	18715	17484	34A	54K	62A	30A
ATCC 90030	65																
70A	59	10															
54A	41	80	75														
56A	53	30	30	70													
C1	53	56	44	67	39												
ATCC 90029	59	10	0	75	30	44											
S21	59	70	65	35	80	83	65										
S8	38	74	68	32	53	65	68	47									
S23	53	30	20	65	30	28	20	75	63								
34B	65	0	10	80	30	56	10	70	74	30							
18715	53	35	35	75	20	50	35	75	58	35	35						
17484	59	75	70	15	70	78	70	35	37	70	75	70					
34A	65	0	10	80	30	56	10	70	74	30	0	35	75				
54K	76	85	85	85	85	83	85	90	84	85	85	75	90	85			
62A	59	80	80	45	80	78	80	60	58	75	80	80	45	80	85		
30A	47	80	75	5	70	72	75	35	37	60	80	75	15	80	90	40	
15526	65	0	10	80	30	56	10	70	74	30	0	35	75	0	85	80	80

Table 3; Matrix of the percentages of loci at which fixed differences occurred in Pilot Study 2

Isolate	CBS 2466	ATCC 46764	ATCC 6258	3630	ATCC 13803	CBS 573	ATCC 90113	CBS 6936	CBS 834	ATCC 22019	ATCC 6260	RA4C	CBS 604	ATCC 42720	CBS 5149	ATCC 2601	CBS 562	CBS 132	ATCC 90029	CBS 94	CBS 940	RA1B	CBS 7987	CBS 4024	RA3C	ATCC 90113	ATCC 90118	CBS 566	ATCC 90030	CBS 6564	ATCC 90028	ATCC 90112	ATCC 22981	CBS 138
ATCC 46764	93																																
ATCC 6258	100	90																															
3630	73	60	95																														
ATCC 13803	100	79	84	95																													
CBS 573	100	95	0	95	89																												
ATCC 90113	67	76	82	65	100	88																											
CBS 6936	92	83	72	72	76	76	100																										
CBS 834	87	5	85	65	74	89	71	78																									
ATCC 22019	80	84	100	79	95	100	75	94	84																								
ATCC 6260	91	86	93	79	100	93	73	69	86	79																							
RA4C	73	60	95	0	95	95	65	72	65	79	79																						
CBS 604	80	85	100	75	95	100	76	94	85	0	79	75																					
ATCC 42720	92	83	78	83	71	82	100	18	78	94	75	83	94																				
CBS 5149	93	70	80	85	79	84	100	72	70	95	86	85	95	72																			
ATCC 2601	86	61	83	67	94	82	93	94	61	88	92	67	83	94	78																		
CBS 562	80	60	90	5	95	89	65	67	65	84	79	5	80	83	85	72																	
CBS 132	77	82	82	88	94	88	60	87	82	81	100	88	76	93	94	93	82																
ATCC 90029	73	60	95	0	95	95	65	72	65	79	79	0	75	83	85	67	5	88															
CBS 94	100	75	85	95	11	89	94	78	70	95	100	95	95	72	80	94	95	88	95														
CBS 940	86	78	89	83	76	94	88	69	83	100	92	83	100	75	72	100	78	88	83	78													
RA1B	73	60	95	0	95	95	65	72	65	79	79	0	75	83	85	67	5	88	0	95	83												
CBS 7987	93	80	90	80	84	89	94	83	80	79	79	80	75	89	85	72	80	88	80	85	94	80											
CBS 4024	73	70	75	75	68	84	82	72	75	89	93	75	90	67	75	78	80	82	75	65	67	75	85										
RA3C	73	60	95	0	95	95	65	72	65	79	79	0	75	83	85	67	5	88	0	95	83	0	80	75									
ATCC 90113	38	71	100	59	100	100	40	100	65	81	83	59	82	100	94	69	65	60	59	94	94	59	71	71	59								
ATCC 90118	73	84	100	79	95	100	75	94	84	5	86	79	5	94	95	88	84	81	79	95	100	79	84	89	79	88							
CBS 566	69	94	89	67	100	88	80	75	94	71	8	67	61	82	89	76	67	94	67	100	100	67	72	100	67	93	65						
ATCC 90030	75	75	94	81	93	93	79	93	81	73	100	81	75	87	94	75	88	71	81	88	94	81	88	69	81	73	73	93					
CBS 6564	93	80	75	85	89	79	82	72	80	79	71	85	80	72	80	89	85	82	85	90	89	85	80	80	85	94	79	72	88				
ATCC 90028	73	65	90	10	100	89	65	78	70	79	86	10	75	78	80	61	15	88	10	100	89	10	80	70	10	59	79	72	81	90			
ATCC 90112	67	94	88	82	100	94	67	88	94	69	92	82	71	87	94	93	88	50	82	100	94	82	88	76	82	60	75	100	73	76	82		
ATCC 22981	73	68	74	79	68	83	81	76	74	89	93	79	89	71	79	76	84	81	79	63	71	79	84	0	79	69	89	100	67	79	74	75	
CBS 138	92	71	94	82	94	94	87	87	76	81	100	82	82	80	82	88	82	73	82	88	88	82	88	71	82	88	81	100	13	94	82	80	75
RA2B	73	60	95	0	95	95	65	72	65	79	79	0	75	83	85	67	5	88	0	95	83	0	80	75	0	59	79	67	81	85	10	82	79	82

Table 4; Matrix of the percentages of loci at which fixed differences occurred between clinically-important species within the genus *Candida* and related genera according to allozyme electrophoresis results in the Taxonomy study.

Isolate	5B	14130	17130	CBS 94	ATCC 2601	17484	56A	CBS 604	12716	CBS 132	34A	62A	18527	S21	68A	ATCC 90028	CBS 834	CBS 562	S1	ATCC 90113	40I	CBS 6289	CBS 4024	CBS 6936	18502	C1	18735	15526	CBS 138	S8	CBS 2466	CBS 6564	CBS 566	58A	CBS 7987
14130	80																																	
17130	11	67																																
CBS 94	94	93	94																															
ATCC 2601	75	86	75	73																														
17484	28	79	28	100	75																													
56A	11	73	11	94	69	17																												
CBS 604	82	86	88	88	87	94	88																											
12716	26	73	26	89	75	28	16	94																										
CBS 132	81	79	75	80	86	80	75	100	69																									
34A	63	79	69	100	85	67	56	93	56	93																								
62A	37	87	37	94	81	28	32	88	32	81	63																							
18527	26	73	26	94	69	22	16	82	26	81	56	37																						
S21	84	100	84	83	94	89	89	71	95	94	88	95	89																					
68A	88	92	81	60	77	87	81	86	88	85	86	94	81	81																				
ATCC 90028	17	79	17	100	75	17	6	88	22	80	60	33	17	83	87																			
CBS 834	83	87	83	82	73	82	83	100	83	94	81	72	83	89	87	82																		
CBS 562	11	80	22	88	67	24	11	88	22	81	56	33	17	89	80	18	83																	
S1	100	80	95	89	88	89	89	82	89	94	88	95	89	68	88	83	94	94																
ATCC 90113	73	67	73	73	85	86	73	77	67	85	92	80	73	100	83	79	93	71	87															
40I	89	93	89	89	94	78	84	94	79	81	56	68	84	79	81	83	89	83	79	87														
CBS 6289	71	67	79	71	83	77	71	100	64	69	83	86	79	100	92	77	77	69	93	46	86													
CBS 4024	81	93	88	93	64	80	88	93	88	100	93	81	81	94	92	80	75	75	81	79	100	83												
CBS 6936	88	100	88	88	100	81	88	93	94	93	93	88	82	65	87	88	94	88	82	100	88	92	100											
18502	22	79	22	100	81	11	11	94	22	80	60	28	22	83	87	6	82	18	83	79	78	77	80	88										
C1	81	85	81	93	85	80	81	100	81	86	73	69	81	94	92	80	13	81	100	92	81	73	79	93	80									
18735	22	73	22	100	80	18	11	94	11	81	56	33	17	83	87	6	83	22	83	79	83	77	81	88	6	81								
15526	69	80	56	93	79	53	63	93	69	93	73	63	69	88	93	53	50	69	88	92	88	85	64	93	53	36	63							
CBS 138	83	73	83	76	50	82	83	88	83	80	87	83	89	94	80	76	71	88	83	80	94	77	75	100	76	67	76	73						
S8	63	87	68	83	88	83	68	71	68	56	81	79	68	95	81	72	89	67	95	60	84	79	88	100	78	88	78	94	94					
CBS 2466	68	73	74	83	75	83	79	88	79	75	88	84	84	89	94	83	78	72	95	67	95	57	69	100	83	69	83	75	67	79				
CBS 6564	82	77	82	82	71	81	82	93	82	86	100	82	82	88	86	81	88	81	94	71	100	69	86	100	81	93	81	93	81	88	65			
CBS 566	68	67	68	72	75	67	68	82	79	88	81	68	68	95	100	67	67	72	100	87	95	86	81	82	72	69	78	63	78	95	68	82		
58A	11	73	11	94	69	17	0	88	16	75	56	32	16	89	81	6	83	11	89	73	84	71	88	88	11	81	11	63	83	68	79	82	68	
CBS 7987	79	93	84	94	88	78	79	94	84	81	63	84	79	79	69	72	83	72	89	87	68	86	81	82	72	69	72	69	78	89	74	88	79	79
18715	16	80	21	100	75	22	11	88	26	88	56	32	21	84	94	6	83	17	89	80	84	79	81	88	11	81	11	63	78	74	84	82	68	11	74

Table 5; Matrix of the percentages of loci at which fixed differences occurred between clinical isolates and type and reference strains from various *Candida* species, particularly those other than *C. albicans*.

Isolate	CBS 6564	58A	S1	CBS 138	CBS 566	15526	CBS 4024	CBS 562	CBS 132	C1	ATCC 6258	14130	CBS 6289	CBS 834	56A	CBS 940	18715	40I	18502	18527	17130	ATCC 90028	ATCC 90113	CBS 604	76A	CBS 6936	CBS 94	CBS 7987	18735	CBS 5149	17484	5B	ATCC 2601	ATCC 28592	12716	CBS 2466	82I	70A
58A	64																																				
S1	64	80																																			
CBS 138	93	100	93																																		
CBS 566	69	71	71	77																																	
15526	92	86	93	85	85																																
CBS 4024	92	79	86	85	85	85																															
CBS 562	57	29	71	100	85	92	77																														
CBS 132	89	80	80	100	80	80	89	78																													
C1	50	27	67	100	86	86	79	7	80																												
ATCC 6258	86	100	93	93	93	86	93	100	70	100																											
14130	100	93	86	100	100	77	93	92	100	93	93																										
CBS 6289	83	85	85	75	100	83	75	75	78	69	92	92																									
CBS 834	91	91	100	91	90	73	73	91	75	91	82	82	89																								
56A	64	27	73	93	86	86	79	21	80	13	100	93	77	82																							
CBS 940	93	86	86	93	92	92	77	100	100	93	79	92	100	100	93																						
18715	57	27	73	100	79	86	79	21	80	13	100	93	69	91	20	93																					
15A	85	93	86	85	85	23	85	92	80	86	86	69	83	80	86	100	86																				
18502	57	27	73	100	79	79	79	21	80	13	100	93	77	91	20	93	13	79																			
18527	71	40	73	100	71	86	71	21	80	20	100	93	77	91	27	100	33	86	33																		
17130	71	27	80	100	71	86	71	29	80	20	100	93	77	91	20	93	20	86	20	13																	
ATCC 90028	64	33	67	100	86	86	79	29	80	20	93	93	69	91	27	86	20	86	20	40	27																
ATCC 90113	91	83	83	100	83	91	82	73	33	75	83	100	64	67	83	91	83	91	92	67	83	83															
CBS 604	75	100	83	83	82	91	91	83	100	83	100	100	64	78	100	92	92	83	92	92	100	92	89														
76A	77	86	100	69	100	85	92	92	78	86	79	100	77	60	79	85	86	85	93	93	86	86	67	73													
CBS 6936	69	71	86	85	62	86	54	92	100	93	86	92	92	73	93	92	86	85	86	100	93	79	91	91	77											
CBS 94	86	87	87	100	93	86	79	86	90	87	87	93	92	82	87	79	87	86	93	80	80	80	75	83	79	93										
CBS 7987	75	85	100	83	83	42	77	83	78	77	85	85	91	73	77	83	77	50	77	85	77	77	91	90	83	100	92									
18735	71	27	80	100	71	86	71	29	80	20	100	93	77	91	20	93	20	86	20	13	0	27	83	100	86	93	80	77								
CBS 5149	85	85	92	85	92	83	83	85	100	77	69	92	91	100	77	69	77	92	77	77	69	69	100	91	75	92	92	73	69							
17484	62	29	71	100	85	86	85	23	80	14	100	92	67	91	21	92	14	85	14	36	21	7	82	91	85	79	86	75	21	83						
5B	69	31	85	92	75	100	67	31	75	23	100	100	82	90	15	92	31	100	31	15	8	38	80	100	25	100	77	82	8	67	33					
ATCC 2601	90	82	91	100	90	55	91	90	75	82	73	100	78	67	82	100	73	64	91	82	82	82	75	89	70	82	73	80	82	89	82	89				
ATCC 28592	85	93	86	92	85	62	100	92	80	86	79	100	83	90	86	85	86	71	79	86	86	86	82	92	69	85	86	92	86	83	92	92	73			
12716	57	27	73	93	86	79	79	14	80	7	100	86	69	82	7	93	13	79	13	20	13	20	83	92	79	93	80	77	13	69	14	8	82	86			
CBS 2466	91	100	73	82	50	90	90	100	67	100	100	80	89	88	100	100	100	70	100	100	100	100	75	89	100	80	91	100	100	100	100	100	80	91		
82I	91	100	91	64	80	60	90	91	83	91	91	90	60	88	91	91	91	70	91	100	100	91	89	33	73	100	91	67	100	80	90	90	100	80	73	88	
70A	77	69	77	92	67	75	83	85	89	77	100	92	91	91	77	92	77	75	85	77	69	77	82	73	75	83	77	75	69	83	75	67	67	92	69	70	80
34A	57	67	60	100	93	79	93	57	80	53	100	86	85	82	60	100	60	79	60	60	67	60	83	75	86	86	80	92	67	92	64	77	91	86	53	82	82	85

Table 6; Matrix of the percentages of loci at which fixed differences occurred in Study 1

Isolate	2A	ATCC 22981	CBS 940	CBS 2466	CBS 6936	12716	5A	ATCC 2601	ATCC 6260	CBS 138	40I	16517	86A	CBS 834	CBS 573	CBS 604	CBS 6564	17484	15526	CBS 7987	76A	S21	A2	14130	ATCC 6258	56A	P1	68B	88	CBS 132	91A	CBS 5149
ATCC 22981	81																															
CBS 940	81	86																														
CBS 2466	74	88	88																													
CBS 6936	76	93	80	100																												
12716	17	80	80	78	75																											
5A	13	71	93	69	88	31																										
ATCC 2601	81	85	100	81	100	81	80																									
ATCC 6260	81	92	71	75	67	81	100	94																								
CBS 138	93	93	75	79	92	71	100	92	100																							
40I	83	87	93	83	94	76	80	73	87	100																						
16517	16	75	81	79	82	22	19	69	88	93	78																					
86A	82	87	93	94	94	75	88	93	100	62	88	82																				
CBS 834	82	86	86	82	80	81	79	80	93	85	81	82	93																			
CBS 573	87	92	93	100	86	87	79	93	100	100	93	87	100	85																		
CBS 604	83	93	80	83	81	82	87	93	80	85	82	78	94	76	100																	
CBS 6564	79	100	83	86	62	79	77	100	85	100	86	71	92	85	75	62																
17484	0	81	81	74	76	17	13	81	81	93	83	16	82	82	87	83	79															
15526	79	83	92	79	93	64	79	85	92	91	8	71	86	83	92	92	91	79														
CBS 7987	78	87	93	83	94	67	75	75	94	86	18	72	88	81	93	88	93	78	21													
76A	79	75	92	50	100	86	79	86	93	58	92	93	64	100	100	92	92	79	83	86												
S21	79	92	85	79	92	79	85	85	92	91	77	71	69	100	92	64	82	79	64	79	75											
A2	19	80	92	69	73	33	21	92	92	92	80	25	80	79	92	87	75	19	75	80	77	83										
14130	61	60	80	89	88	65	60	87	93	92	76	61	88	88	93	88	85	61	77	71	85	85	60									
ATCC 6258	94	86	85	100	87	94	87	100	100	92	87	94	93	86	21	87	69	94	92	88	100	83	93	88								
56A	13	69	86	87	80	21	29	62	85	100	73	7	80	77	83	93	83	13	69	64	92	82	38	57	92							
P1	6	81	87	83	76	18	19	73	87	93	76	11	82	81	86	88	79	6	71	71	86	77	25	59	94	7						
68B	50	79	86	94	73	60	71	85	62	92	75	50	80	86	100	100	92	50	69	73	83	73	57	67	100	47	44					
88	79	85	92	79	93	64	79	77	92	92	8	79	86	85	83	100	82	79	17	21	83	83	77	62	85	75	79	75				
CBS 132	64	92	83	57	86	79	64	71	93	83	85	79	71	77	100	92	83	64	85	93	62	73	67	92	92	75	71	92	83			
91A	35	86	73	76	75	50	53	87	73	92	88	53	94	93	100	81	77	35	85	81	77	85	50	63	86	53	44	53	85	69		
CBS 5149	83	93	80	94	100	82	87	80	100	85	100	83	100	81	79	88	100	83	100	88	92	85	93	88	93	86	82	80	100	100	94	
34B	93	100	100	93	100	87	93	79	93	100	14	87	86	92	85	93	91	93	29	33	92	83	92	93	92	85	86	77	33	92	86	100

Table 7; Matrix of the percentages of loci at which fixed differences occurred in Study 2

Isolate	71A	44A	71B	17130	CBS 7987	CBS 562	40B	46A	ATCC 90028	CBS 604	84B	80A	46B	40H	12716	40J	68D
44A	33																
71B	0	37															
17130	33	21	37														
CBS 7987	83	74	84	74													
CBS 562	11	37	11	42	84												
40B	28	28	28	22	72	33											
46A	17	21	21	21	74	26	11										
ATCC 90028	17	16	21	16	79	26	17	5									
CBS 604	78	68	79	79	74	79	78	68	74								
84B	17	21	21	21	79	26	22	11	5	74							
80A	17	26	21	37	79	21	28	21	21	74	21						
46B	28	17	28	22	72	28	22	11	17	67	22	17					
40H	61	61	61	56	44	67	50	50	56	61	56	61	56				
12716	50	42	47	26	74	53	39	42	37	79	42	53	39	56			
40J	39	39	39	39	72	44	44	33	28	72	28	39	39	50	50		
68D	17	32	21	37	84	16	28	21	21	79	16	11	22	61	53	39	
ATCC 90029	17	32	21	21	79	26	22	26	26	79	26	26	22	50	37	39	26

Table 8; Matrix of the percentages of loci at which fixed differences occurred in Study 3

Isolate	34A	23A	88D	CBS 604	31A	19A	40D	22B	17D	40F	17130	80A	84A	46B	CBS 7987	23C	17E	30A	19D	ATCC 90028	17A	68C	31E	22A	71A	23B	CBS 562	17C	12716	ATCC 90029
23A	65																													
88D	75	35																												
CBS 604	65	79	76																											
31A	81	47	31	71																										
19A	59	37	18	74	35																									
40D	71	47	36	73	31	33																								
22B	71	39	35	72	47	33	36																							
17D	71	33	29	78	35	28	29	11																						
40F	76	78	65	72	71	72	71	83	83																					
17130	65	21	24	79	41	26	40	44	33	78																				
80A	65	21	24	79	29	16	27	33	22	78	11																			
84A	65	22	35	78	35	22	29	28	22	78	22	11																		
46B	71	26	41	74	29	37	27	33	28	72	32	21	11																	
CBS 7987	81	78	81	72	76	83	64	82	88	41	72	72	76	67																
23C	56	17	24	72	44	33	47	41	41	65	33	33	35	39	82															
17E	69	29	36	79	43	36	33	21	21	86	43	29	14	14	79	36														
30A	81	72	76	61	75	78	60	76	82	41	72	72	76	72	24	72	86													
19D	65	53	35	79	53	26	47	44	39	78	37	32	44	42	83	50	50	83												
ATCC 90028	65	26	29	79	35	21	33	28	17	83	16	5	17	26	78	39	29	78	26											
17A	71	22	18	78	35	17	29	28	22	78	22	11	22	28	76	29	14	76	39	17										
68C	77	47	43	73	23	40	17	29	14	79	40	27	29	27	71	53	42	67	47	27	36									
31E	64	60	33	80	47	33	42	33	27	80	33	33	33	40	87	60	31	93	33	27	33	42								
22A	71	33	29	78	35	33	36	11	11	78	39	28	22	28	88	35	21	82	44	22	28	29	33							
71A	57	43	50	79	29	36	42	50	43	86	43	29	21	29	71	50	33	71	57	36	43	50	46	43						
23B	75	22	35	78	38	39	27	29	24	76	39	28	18	22	82	28	21	72	56	33	29	33	47	24	36					
CBS 562	75	29	35	76	38	35	36	12	12	82	35	24	18	24	81	41	14	82	47	18	24	29	33	6	43	24				
17C	71	42	18	79	41	32	33	33	28	72	42	32	44	47	83	28	36	78	37	26	28	40	40	28	50	39	29			
12716	71	42	29	74	41	37	40	56	44	83	21	21	33	37	67	50	36	72	42	26	33	47	33	50	29	44	41	47		
ATCC 90029	71	21	29	74	41	37	40	39	33	78	37	26	28	32	83	22	29	72	53	32	28	40	47	33	29	11	29	37	37	
71B	59	28	35	78	29	22	36	33	28	78	28	17	11	22	82	35	29	76	44	22	28	36	33	28	7	24	29	44	39	22

Table 9; Matrix of the percentages of loci at which fixed differences occurred in Study 4

Isolate	27A	24A	CBS 7987	12716	28B	21B	27B	ATCC 90029	34A	CBS 604	34B	87A	25A	ATCC 90028	29A	28A
24A	44															
CBS 7987	50	75														
12716	69	69	59													
28B	85	57	79	57												
21B	64	27	73	69	50											
27B	14	36	43	86	83	58										
ATCC 90029	88	69	76	35	29	53	93									
34A	93	81	88	69	67	73	92	69								
CBS 604	56	59	65	76	80	69	64	82	88							
34B	47	71	53	69	92	79	50	88	79	67						
87A	75	77	92	54	45	75	82	38	69	79	83					
25A	83	31	93	77	45	42	73	62	85	71	75	58				
ATCC 90028	86	47	80	50	29	56	75	27	73	69	86	50	33			
29A	86	54	86	47	27	62	73	33	54	86	87	42	55	15		
28A	80	40	73	53	29	60	75	44	67	75	86	50	50	27	31	
CBS 562	75	65	88	59	47	63	79	41	71	72	87	36	71	50	43	56

Table 10; Matrix of the percentages of loci at which fixed differences occurred in Study 5

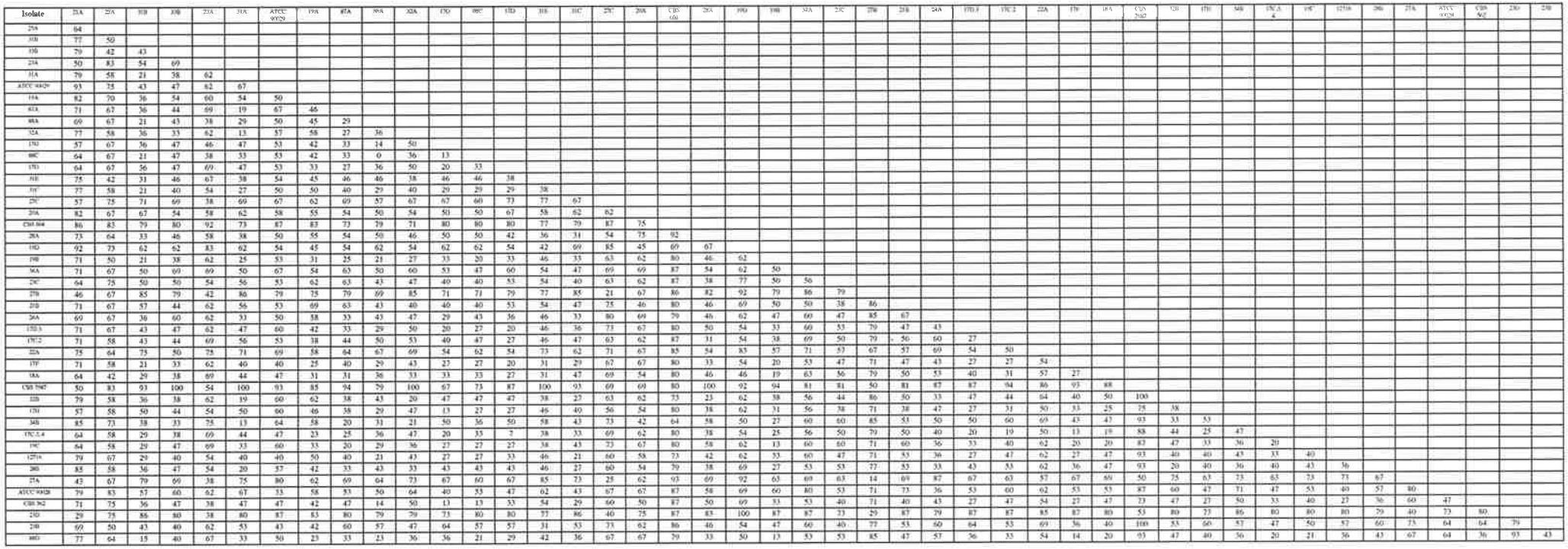


Table 11; Matrix of the percentages of loci at which fixed differences occurred in Study 6

Isolate	29B	42D	38A	42C	12716	ATCC 90028	42A	42B	9I	41A	9C	9F	9E	42E	39C	11A	ATCC 90029	CBS 7987	CBS 562	22B	9G	12A	16A	22A	22D	18A	15A	CBS 604	9D	29A
42D	62																													
38A	31	50																												
42C	77	79	69																											
12716	62	57	44	69																										
ATCC 90028	36	55	23	62	23																									
42A	38	50	25	63	44	31																								
42B	46	43	38	75	44	31	19																							
9I	46	54	43	79	57	42	36	36																						
41A	38	50	25	69	44	15	25	19	29																					
9C	33	54	36	71	57	27	36	36	8	14																				
9F	36	55	42	75	58	27	42	42	8	25	0																			
9E	33	50	38	77	54	27	38	38	8	23	0	0																		
42E	92	85	85	62	77	80	85	77	75	77	67	70	73																	
39C	46	43	31	81	56	38	44	38	50	25	43	50	46	77																
11A	42	62	43	71	64	33	50	50	23	29	8	8	8	58	50															
ATCC 90029	38	62	46	77	38	27	54	46	46	38	42	36	33	92	46	50														
CBS 7987	91	73	83	58	58	73	75	58	83	75	83	91	91	60	83	92	73													
CBS 562	31	50	25	69	44	15	31	25	36	13	21	33	31	77	38	36	31	67												
22B	46	54	29	71	36	25	29	29	29	29	31	25	23	75	43	38	31	67	29											
9G	30	50	45	82	64	30	45	45	18	27	0	9	9	78	45	9	40	90	36	36										
12A	54	46	50	93	64	50	57	50	36	50	31	25	31	75	57	31	62	92	50	50	27									
16A	36	55	31	69	38	17	38	38	42	23	27	27	27	73	46	33	36	82	23	33	30	42								
22A	46	50	27	73	40	25	33	33	29	27	29	25	23	69	40	36	31	67	27	7	36	43	25							
22D	46	43	25	69	25	15	31	31	29	25	29	25	23	69	38	36	23	58	25	7	36	43	23	0						
18A	50	77	40	67	47	17	47	53	46	40	46	27	33	92	60	46	33	82	40	31	40	62	33	36	27					
15A	64	75	64	79	64	67	71	71	50	57	50	50	50	82	57	54	55	82	64	58	55	67	58	54	43	62				
CBS 604	83	62	67	73	60	75	67	73	69	67	62	73	67	69	67	69	75	55	73	62	70	85	85	57	53	86	77			
9D	36	55	42	75	58	30	42	42	17	25	8	9	8	60	50	9	45	82	33	33	10	33	30	33	33	36	55	73		
29A	15	64	27	80	53	42	33	40	43	40	36	42	38	69	53	43	54	83	27	36	45	50	42	33	33	57	69	71	42	
42D	31	57	25	69	38	23	19	31	43	38	43	42	38	77	50	50	46	83	38	29	45	57	23	33	25	40	71	73	42	27

Table 12; Matrix of the percentages of loci at which fixed differences occurred in Study 7

Isolate	ATCC 90028	44G	50A	53C	50B	52B	46G	47A	23D	44H	44D	32B	CBS 562	ATCC 90029	54G	57A	45A	46D	52C	46B	50C	46F	46C	52A	17130	12716	CBS 7987
44G	77																										
50A	50	71																									
53C	77	86	79																								
50B	64	92	33	46																							
52B	50	79	21	71	25																						
46G	62	85	58	64	36	42																					
47A	54	93	50	69	31	43	29																				
23D	85	47	57	71	67	71	92	86																			
44H	64	40	67	81	79	67	79	81	67																		
44D	50	87	53	75	36	40	50	44	73	76																	
32B	62	69	69	100	64	62	58	69	85	86	64																
CBS 562	31	79	36	73	38	27	38	33	79	63	38	62															
ATCC 90029	10	90	45	82	40	36	33	27	90	75	17	40	17														
55B	64	73	73	50	71	67	79	69	73	71	76	86	50	67													
57A	64	73	60	63	57	67	79	56	73	71	71	86	50	58	29												
45A	38	86	43	87	50	43	54	40	86	69	44	38	47	25	81	63											
46D	43	87	47	81	36	40	36	31	73	76	24	50	31	8	76	65	25										
52C	67	91	50	73	36	36	44	36	91	67	50	60	36	38	67	67	36	33									
46B	42	75	17	85	33	23	50	38	75	64	50	42	36	36	79	57	14	36	22								
50C	38	87	40	80	46	13	54	40	80	69	31	64	27	18	63	63	27	31	42	31							
46F	50	93	50	86	33	43	42	36	71	87	20	54	36	10	73	67	29	0	36	42	33						
46C	46	80	67	93	62	60	54	53	93	75	44	29	47	18	81	81	27	25	42	38	50	27					
52A	46	80	40	73	38	13	54	47	80	63	44	64	40	36	69	69	40	44	50	38	13	47	56				
17130	18	77	42	75	55	42	55	58	85	77	62	55	25	22	54	62	62	54	56	36	54	58	54	62			
12716	23	92	58	69	55	42	54	46	92	71	43	58	31	0	64	64	38	36	44	50	31	42	46	38	27		
CBS 7987	33	75	58	92	80	58	73	67	83	62	62	77	50	33	77	77	50	54	67	55	46	58	62	54	45	27	
23A	67	58	45	91	67	64	64	64	42	67	67	67	55	63	67	67	73	58	67	60	67	64	75	67	60	73	64

Table 13; Matrix of the percentages of loci at which fixed differences occurred in Study 8

Isolate	54I	63G	59B	56B	63B	63P	2A	37C	37B	63R	63F	63M	CBS 604	59G	63E	27C	59I	59D	63J	7A	60B	54J	6A	1C	59A	1E	63H	CBS 7987	1A	58B	60A	60D	63N	63S	54H	5A	1B	63Q	63I	63C	59H	61A	2B	60C	CBS 562	58C
63G	67																																												
59B	20	38																																											
56B	25	40	17																																										
63B	64	38	44	45																																									
63P	63	67	75	56	63																																								
2A	25	57	20	30	57	86																																							
37C	22	80	38	50	78	50	57																																						
37B	50	50	33	40	43	63	25	50																																					
63R	70	50	60	55	56	56	50	40	63																																				
63F	50	60	44	60	71	63	38	56	38	40																																			
63M	56	44	38	33	50	63	43	44	57	20	44																																			
CBS 604	88	70	88	80	88	67	71	60	75	33	70	50																																		
59G	42	55	55	31	45	50	44	73	60	75	70	60	70																																	
63E	70	36	56	45	56	67	57	60	63	27	60	22	60	67																																
27C	40	45	22	55	56	78	14	40	50	55	50	33	60	67	50																															
59I	30	56	27	33	56	86	0	63	38	60	40	50	67	45	60	30																														
59D	20	63	30	27	50	63	13	44	57	60	50	38	75	27	67	33	20																													
63J	25	22	22	20	43	67	25	44	44	22	33	13	67	50	22	22	25	29																												
7A	22	78	44	50	75	44	50	22	63	56	50	63	75	50	67	44	50	38	44																											
60B	25	40	27	8	45	56	22	60	56	55	67	33	67	23	45	45	27	18	22	50																										
54J	18	50	10	8	50	63	25	44	38	70	56	50	89	42	55	45	27	20	38	44	17																									
6A	44	30	40	36	38	50	44	60	40	60	60	44	60	36	50	40	44	50	36	70	30	56																								
1C	50	27	44	27	33	60	38	73	44	55	70	40	60	33	45	45	33	33	30	70	18	40	27																							
59A	17	70	25	21	55	56	40	30	40	73	50	56	90	46	73	64	42	27	50	50	31	17	45	55																						
1E	56	45	50	30	38	67	43	70	63	40	50	22	60	45	45	45	33	25	33	78	20	40	40	18	50																				
63H	83	55	75	71	82	89	80	73	73	50	45	30	55	79	50	67	75	82	45	90	77	83	58	67	79	55																			
CBS 7987	90	44	73	75	67	63	80	89	56	82	60	67	78	75	80	70	82	90	56	88	82	90	50	60	83	67	69																		
1A	67	36	63	40	50	78	43	90	38	70	80	44	70	45	45	55	44	50	44	89	30	50	40	18	70	36	64	56																	
58B	40	75	22	27	67	57	38	25	43	56	71	43	86	64	56	56	56	33	57	50	36	20	75	67	18	63	91	100	75																
60A	27	56	27	25	60	56	22	44	56	60	63	50	75	42	40	40	30	20	44	40	17	18	50	40	33	44	92	100	56	20															
60D	36	56	40	17	50	50	38	56	75	40	63	25	63	33	50	60	40	20	38	56	8	27	44	30	33	11	75	90	44	36	18														
63N	63	40	44	30	43	56	38	67	44	22	30	13	60	60	40	50	33	43	30	78	33	44	45	40	50	20	45	44	50	57	56	25													
63S	73	45	55	54	50	40	56	64	50	50	45	30	55	46	58	50	55	60	45	70	50	73	17	42	62	36	50	50	55	70	64	45	36											
54H	40	60	20	25	33	67	33	50	33	50	40	33	90	55	60	50	30	22	40	60	36	20	55	36	25	20	67	80	60	33	40	30	30	50										
5A	50	33	38	11	57	63	29	63	38	50	56	38	78	44	33	44	38	33	22	63	13	25	40	33	44	33	60	50	33	33	38	29	30	50	44									
1B	44	70	44	60	63	60	38	30	67	30	44	22	56	73	50	30	38	25	40	40	50	56	64	55	60	40	64	89	60	50	40	33	50	55	40	67								
63Q	56	60	60	45	63	33	67	50	56	45	45	30	60	45	60	60	70	56	40	44	50	67	36	55	55	50	42	55	70	63	67	44	40	17	55	44	60							
63I	56	18	38	30	50	67	43	70	50	40	50	22	70	55	18	45	44	50	11	67	30	40	40	36	60	36	45	67	36	63	44	44	30	55	50	22	50	60						
63C	50	44	38	30	78	57	33	50	43	33	50	38	38	55	30	50	44	63	29	57	30	40	38	44	50	44	55	78	56	44	33	40	25	50	63	38	63	50	33					
59H	33	50	27	31	45	56	22	60	44	73	67	56	78	31	64	45	36	27	44	50	23	25	40	36	38	50	85	82	50	36	17	33	56	58	45	38	50	60	50	50				
61A	27	56	30	25	45	56	13	50	50	60	50	38	78	33	60	40	10	9	38	44	17	18	44	30	25	22	83	90	44	30	18	18	38	45	20	29	33	56	44	56	25			
2B	56	40	25	50	50	75	14	67	29	70	56	50	78	64	55	27	33	38	38	63	40	40	33	40	60	40	73	70	40	56	33	56	44	55	44	25	44	78	40	56	40	33		
60C	17	50	18	15	45	56	11	50	44	64	56	44	78	31	55	36	18	9	33	40	8	8	40	27	23	30	85	91	40	27	8	17	44	58	27	25	40	60	40	40	15	8	30	
CBS 562	60	50	44	40	67	86	29	63	50	50	75	22	71	60	33	44	44	56	14	63	40	56	50	44	70	50	60	78	38	50	56	56	43	50	56	29	50	56	25	50	60	44	50	50
58C	44	20	11	20	44	57	29	71	57	57	43	33	88	56	50	38	33	38	29	71	22	22	38	43	30	29	70	63	57	29	33	25	25	44	25	38	57	57	29	38	33	33	29	22
59C	20	63	20	9	50	63	38	44	57	60	63	38	88	45	67	56	40	27	29	50	18	10	63	44	18	38	82	90	63	22	30	20	43	70	22	33	50	56	50	50	36	27	63	18

Table 14; Matrix of the percentages of loci at which fixed differences occurred in Study 9

Isolate	93C	CBS 604	96A	88L	82A	88D	93E	71A	90A	97E	88J	93A	66C	97D	84F	74A	76B	84G	93B	73A	CBS 7987	75A	65B	65A	92A	89A	77A	88K	CBS 562	67A	84E	95A
CBS 604	67																															
96A	9	63																														
88L	31	89	17																													
82A	30	75	30	45																												
88D	25	75	30	33	44																											
93E	0	67	8	29	27	25																										
71A	13	80	11	22	13	14	11																									
90A	58	67	50	46	55	64	54	44																								
97E	9	50	0	25	22	40	8	13	36																							
88J	18	44	18	42	30	30	25	25	50	18																						
93A	15	56	17	36	36	33	21	22	46	17	17																					
66C	25	43	13	44	38	71	22	33	56	11	33	33																				
97D	18	50	10	33	33	45	17	29	55	9	27	25	13																			
84F	44	67	63	78	50	25	44	40	67	50	44	56	57	63																		
74A	20	71	27	45	44	33	18	22	64	30	40	36	25	44	43																	
76B	38	67	22	33	50	43	33	43	56	29	38	33	50	29	67	50																
84G	8	50	18	38	30	18	8	11	50	17	18	23	33	27	25	18	38															
93B	0	67	8	29	27	25	0	11	54	8	25	21	22	17	44	18	33	8														
73A	0	50	0	33	33	38	0	14	56	0	25	22	14	13	33	25	17	11	0													
CBS 7987	89	88	78	70	70	63	80	57	90	78	80	90	75	67	75	75	86	67	80	75												
75A	38	80	25	22	33	25	33	29	25	22	38	33	50	33	40	50	33	33	33	33	67											
65B	10	57	0	18	25	33	9	13	40	0	20	18	13	10	57	30	29	18	9	0	75	22										
65A	10	63	0	18	22	33	9	14	50	0	20	18	13	10	63	33	25	20	9	0	78	25	0									
92A	36	57	45	58	70	60	42	56	64	40	60	42	43	60	71	50	63	45	42	38	100	75	40	40								
89A	45	88	33	18	63	50	45	43	70	40	56	45	43	56	75	56	50	55	45	43	71	43	33	25	33							
77A	25	100	25	22	29	14	22	25	25	22	38	33	50	38	40	38	50	22	22	33	71	0	22	25	67	43						
88K	8	56	0	21	27	33	7	11	46	0	17	14	11	8	56	27	22	15	7	0	80	22	0	0	42	36	22					
CBS 562	56	86	60	50	38	50	50	57	70	63	56	70	57	63	57	56	71	44	50	67	57	67	63	50	89	75	50	60				
67A	11	63	22	50	38	38	10	33	70	22	40	40	25	11	50	25	50	22	10	0	75	50	25	25	63	75	50	20	56			
84E	33	67	33	38	45	36	38	33	54	36	17	31	56	45	56	55	33	33	38	44	80	38	40	40	73	50	38	31	60	60		
95A	0	67	8	29	27	25	0	11	54	8	25	21	22	17	44	18	33	8	0	0	80	33	9	9	42	45	22	7	50	10	38	
93D	0	67	8	29	27	25	0	11	54	8	25	21	22	17	44	18	33	8	0	0	80	33	9	9	42	45	22	7	50	10	38	0

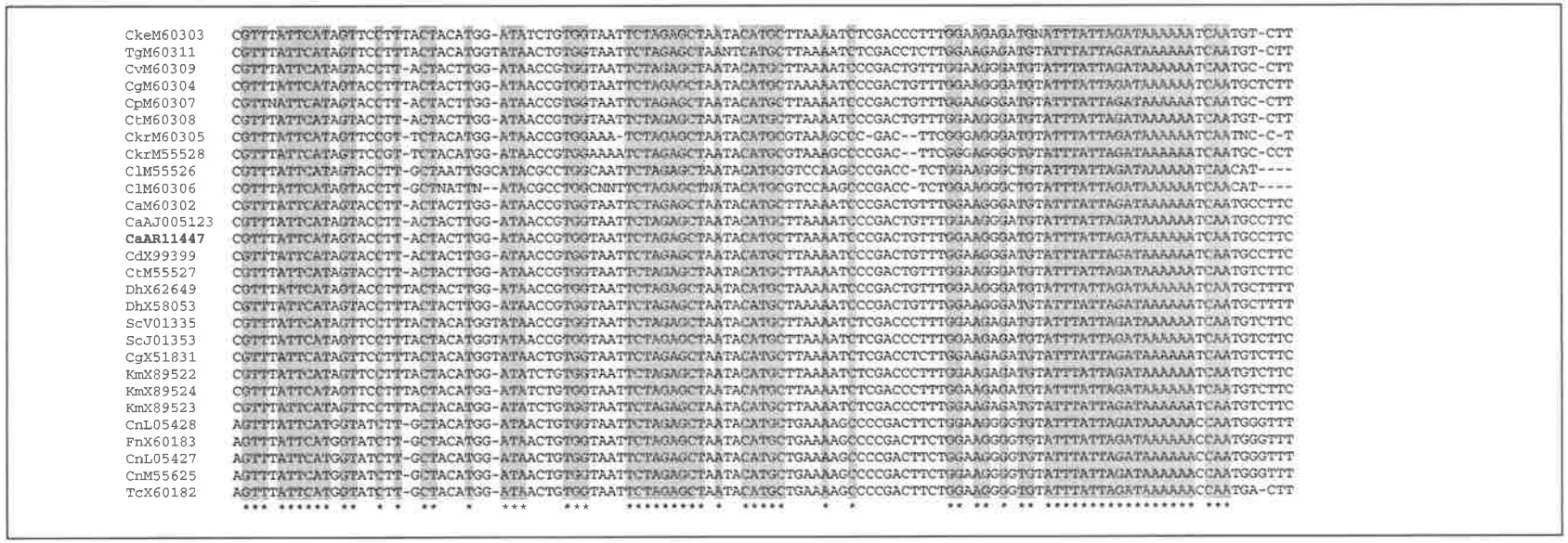
Table 15; Matrix of the percentages of loci at which fixed differences occurred in Study 10

Isolate	CBS 604	S17	100A	S25	S20	S13	C6	99A	S11	CBS 562	CBS 7987	S2	S15	C15	C3	C16	C1B	S5	S9
S17	75																		
100A	77	50																	
S25	92	77	77																
S20	83	33	42	62															
S13	73	31	82	67	38														
C6	73	0	60	82	45	27													
99A	85	23	50	71	46	25	27												
S11	83	7	50	79	29	31	9	23											
CBS 562	83	62	77	92	69	54	60	50	62										
CBS 7987	92	67	31	75	58	73	70	54	67	50									
S2	50	38	50	85	62	38	33	38	38	67	67								
S15	77	8	58	85	38	33	17	38	15	54	64	42							
C15	75	17	58	77	42	25	18	23	17	67	64	31	33						
C3	64	7	64	67	36	31	0	33	15	69	82	42	17	18					
C16	92	80	73	64	70	70	80	73	80	100	80	91	73	92	78				
C1B	83	33	67	75	58	64	36	46	42	69	73	64	50	64	36	80			
S5	70	18	44	80	36	25	18	30	18	55	67	27	25	10	18	89	55		
S9	67	23	46	54	31	33	18	29	23	77	62	31	33	17	17	80	58	20	
C12	77	14	50	71	36	31	9	27	14	60	62	31	21	23	23	82	43	9	21

Table 16; Matrix of the percentages of loci at which fixed differences occurred in Study 11

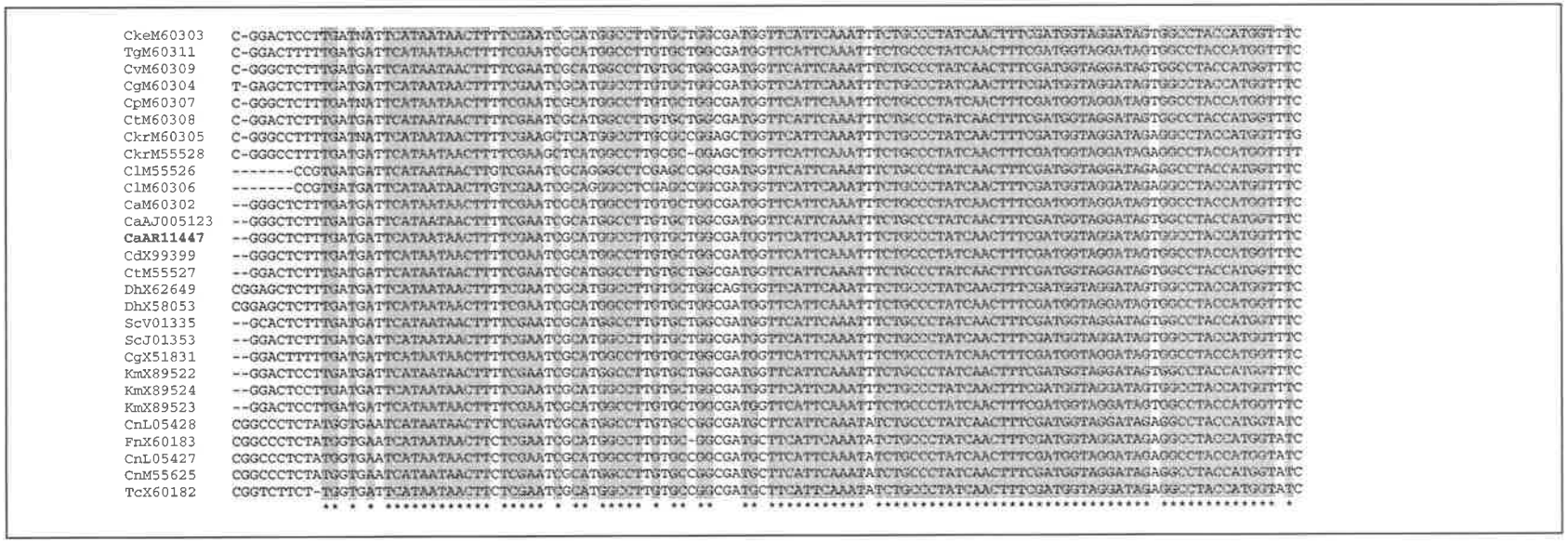
a; Areas of homology between all aligned sequences are highlighted
b; Emboldened sequence was obtained during the course of this study.

Table 17a; Alignment of Genebank sequences and the *C. albicans* type strain CBS 562 sequence of the full 18S rRNA gene obtained using ClustalX.



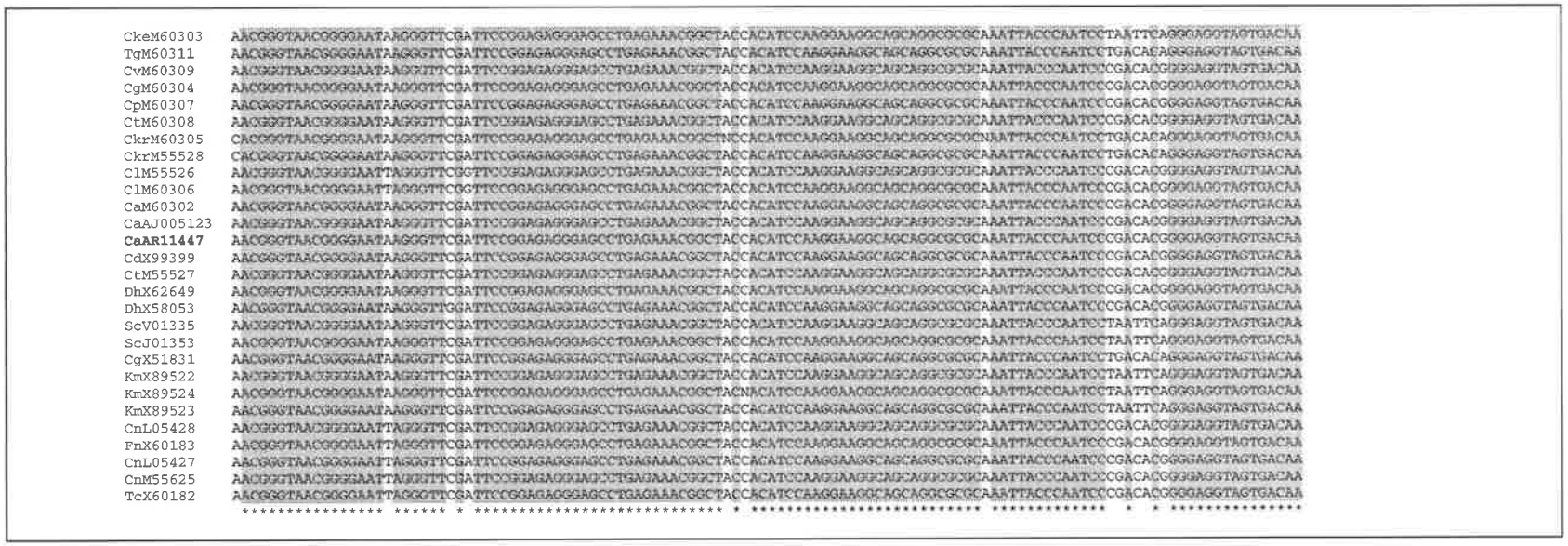
a; Areas of homology between all aligned sequences are highlighted
b; Emboldened sequence was obtained during the course of this study.

Table 17b; Alignment of Genebank sequences and the *C. albicans* type strain CBS 562 sequence of the full 18S rRNA gene obtained using ClustalX.



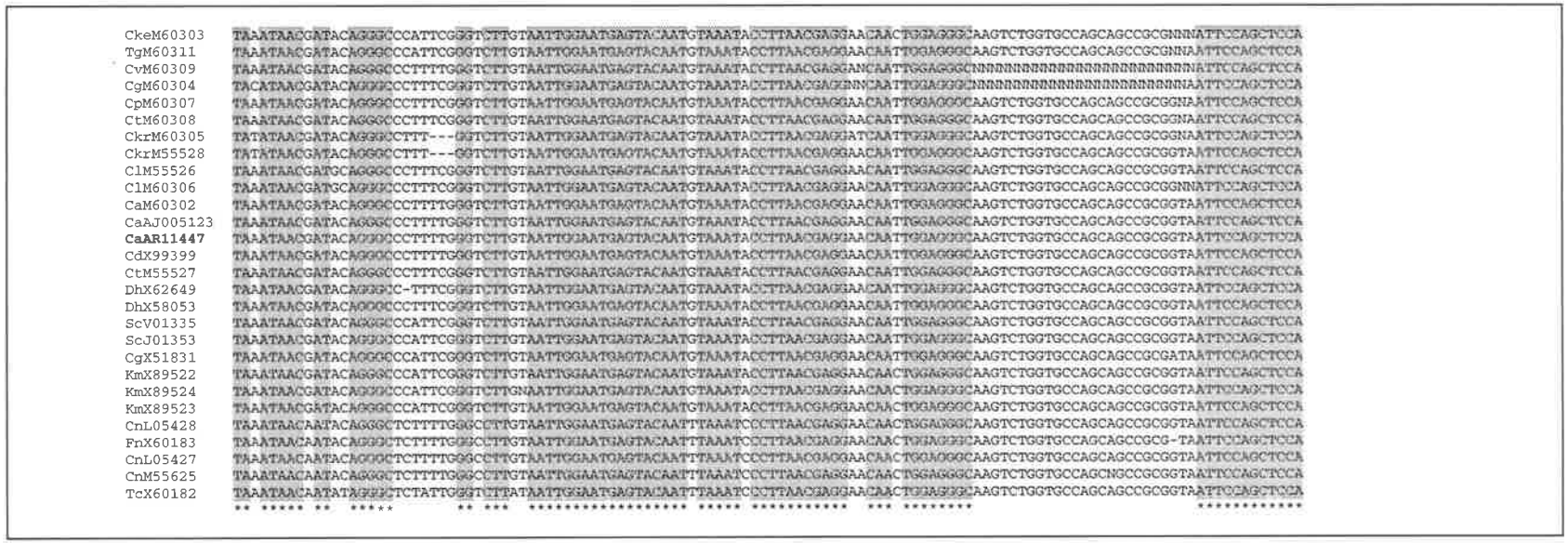
a; Areas of homology between all aligned sequences are highlighted
b; Emboldened sequence was obtained during the course of this study.

Table 17c; Alignment of Genebank sequences and the *C. albicans* type strain CBS 562 sequence of the full 18S rRNA gene obtained using ClustalX.



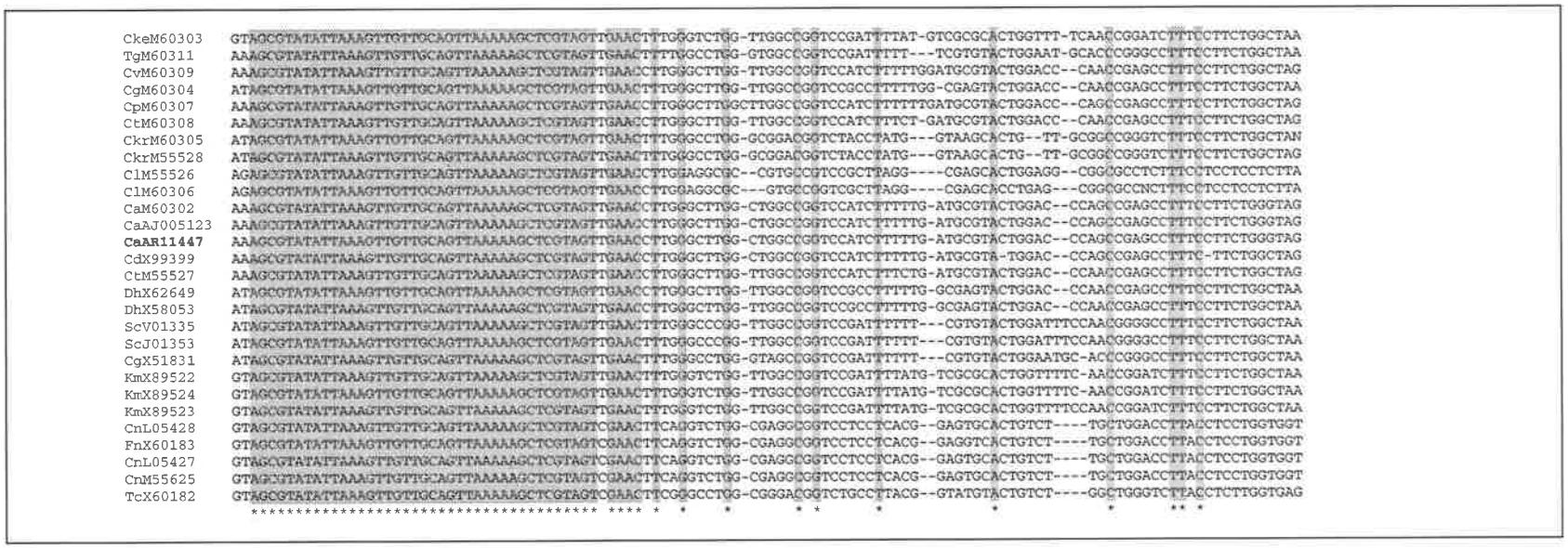
a; Areas of homology between all aligned sequences are highlighted
b; Emboldened sequence was obtained during the course of this study.

Table 17d; Alignment of Genebank sequences and the *C. albicans* type strain CBS 562 sequence of the full 18S rRNA gene obtained using ClustalX.



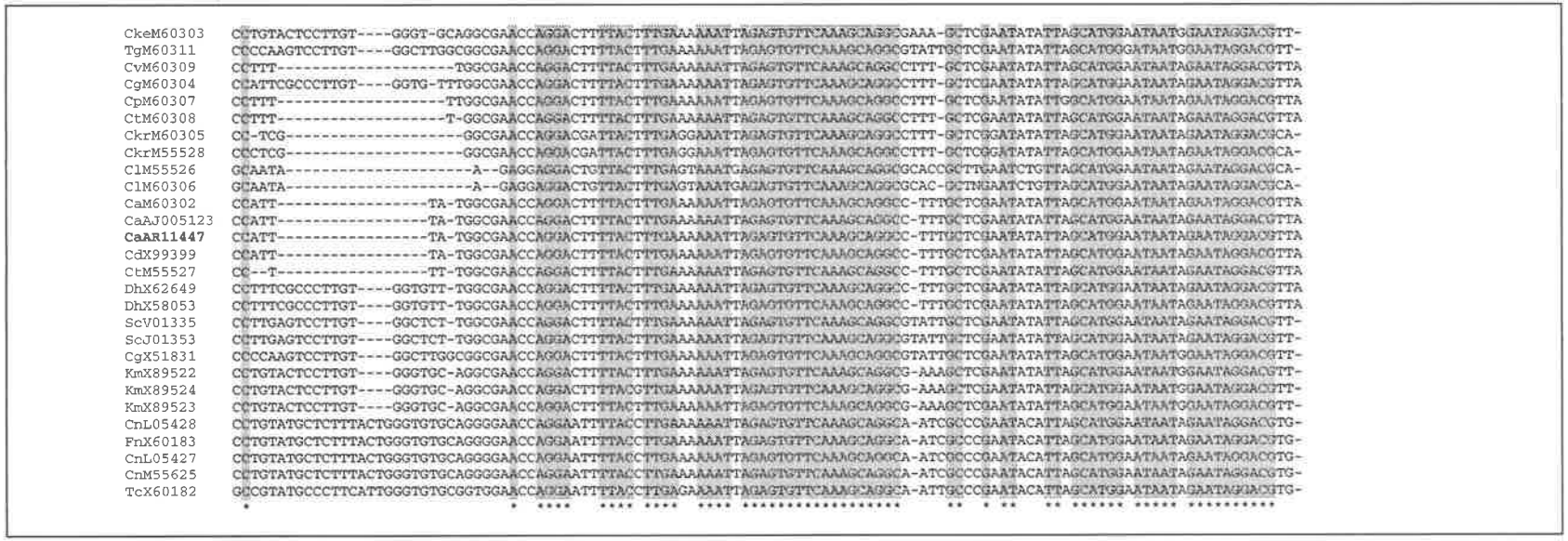
a; Areas of homology between all aligned sequences are highlighted
b; Emboldened sequence was obtained during the course of this study.

Table 17e; Alignment of Genebank sequences and the *C. albicans* type strain CBS 562 sequence of the full 18S rRNA gene obtained using ClustalX.



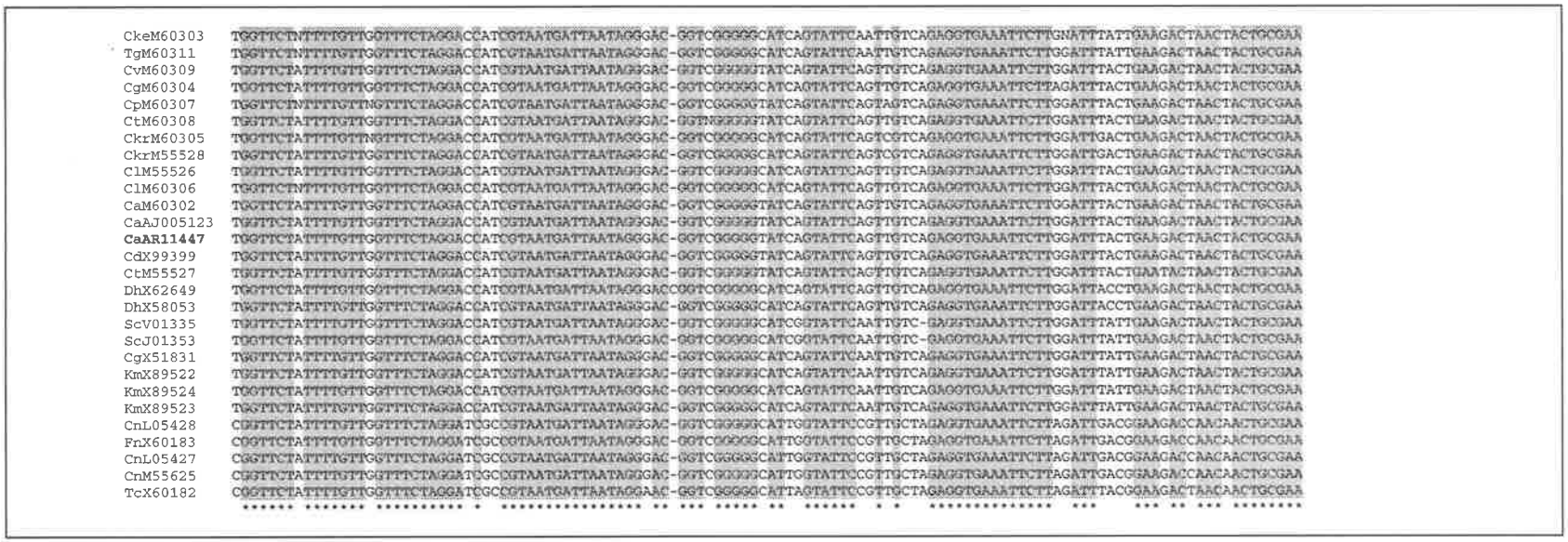
a; Areas of homology between all aligned sequences are highlighted
b; Emboldened sequence was obtained during the course of this study.

Table 17f; Alignment of Genebank sequences and the *C. albicans* type strain CBS 562 sequence of the full 18S rRNA gene obtained using ClustalX.



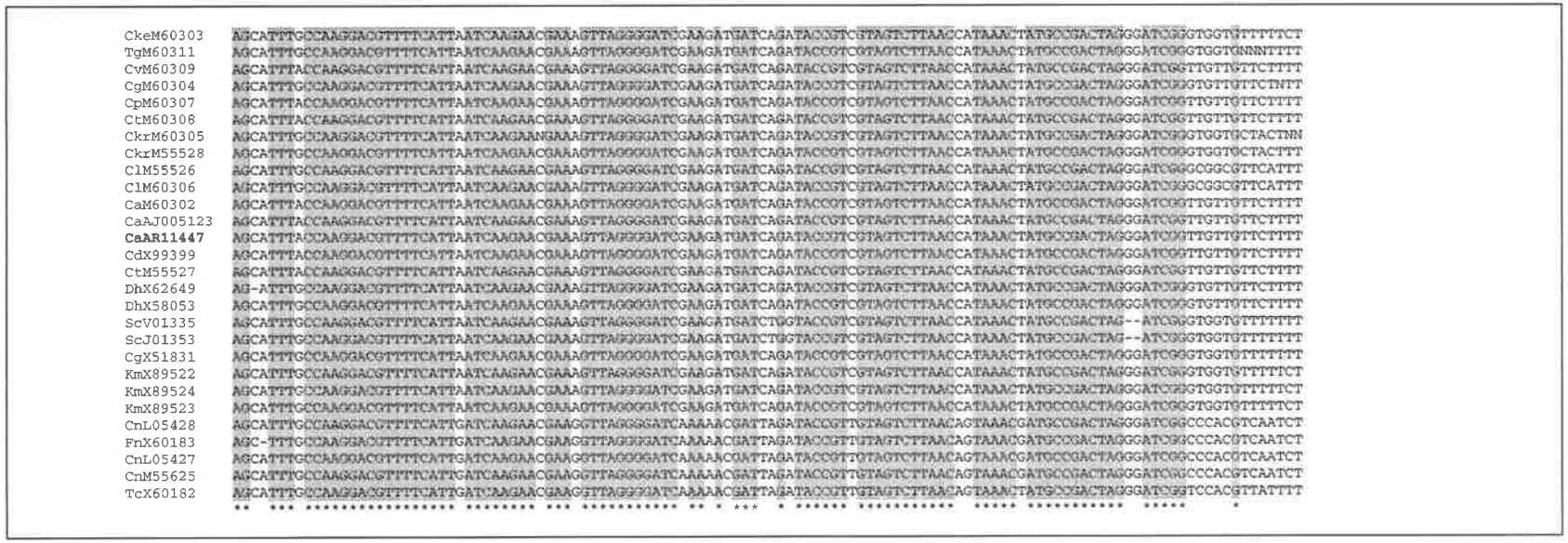
a; Areas of homology between all aligned sequences are highlighted
b; Emboldened sequence was obtained during the course of this study.

Table 17g; Alignment of Genebank sequences and the *C. albicans* type strain CBS 562 sequence of the full 18S rRNA gene obtained using ClustalX.



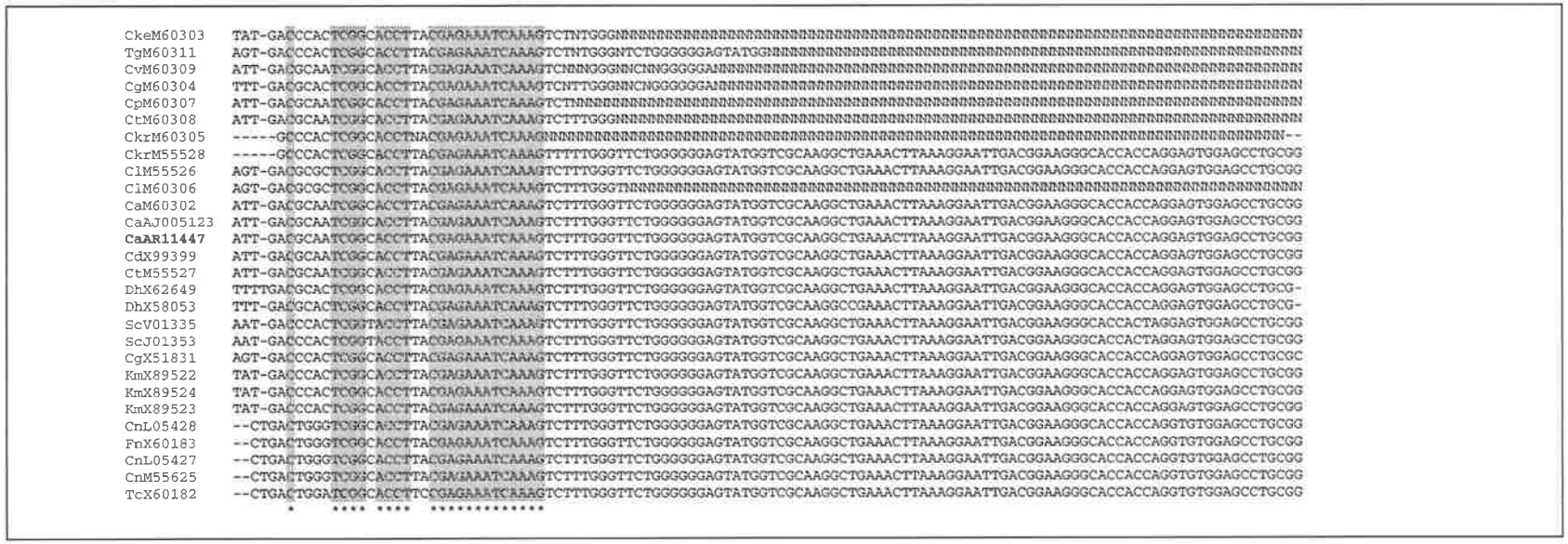
a; Areas of homology between all aligned sequences are highlighted
b; Emboldened sequence was obtained during the course of this study.

Table 17h; Alignment of Genebank sequences and the *C. albicans* type strain CBS 562 sequence of the full 18S rRNA gene obtained using ClustalX.



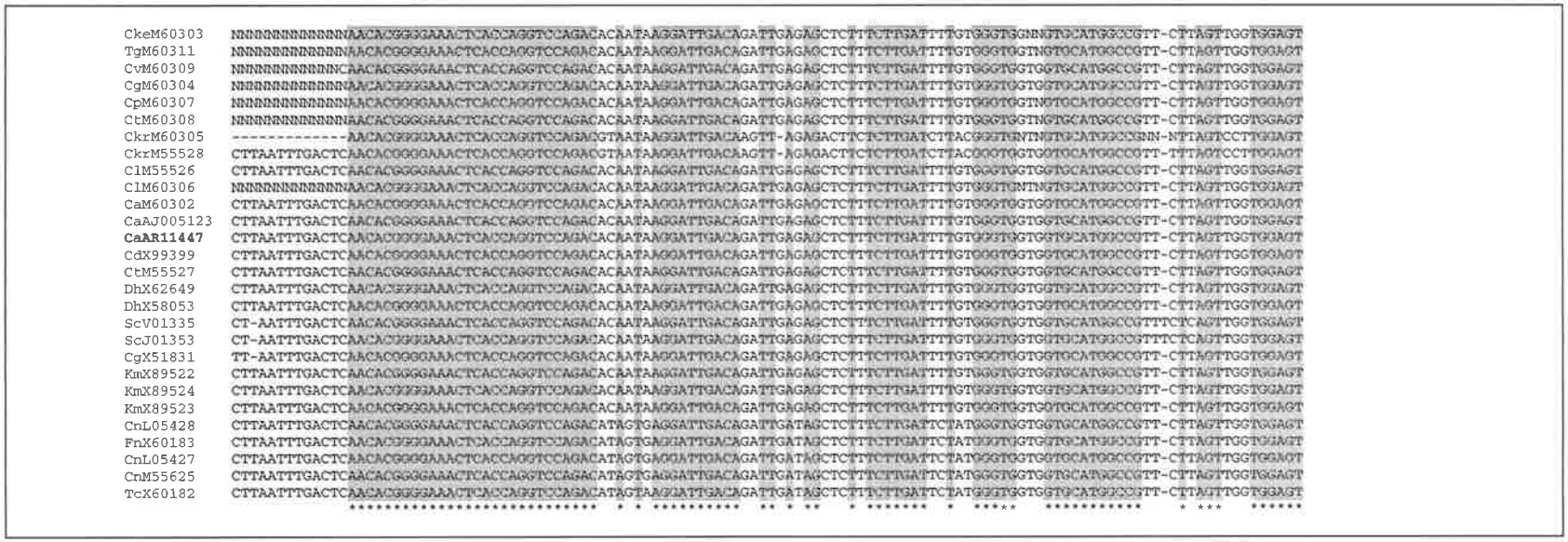
a; Areas of homology between all aligned sequences are highlighted
b; Emboldened sequence was obtained during the course of this study.

Table 17i; Alignment of Genebank sequences and the *C. albicans* type strain CBS 562 sequence of the full 18S rRNA gene obtained using ClustalX.



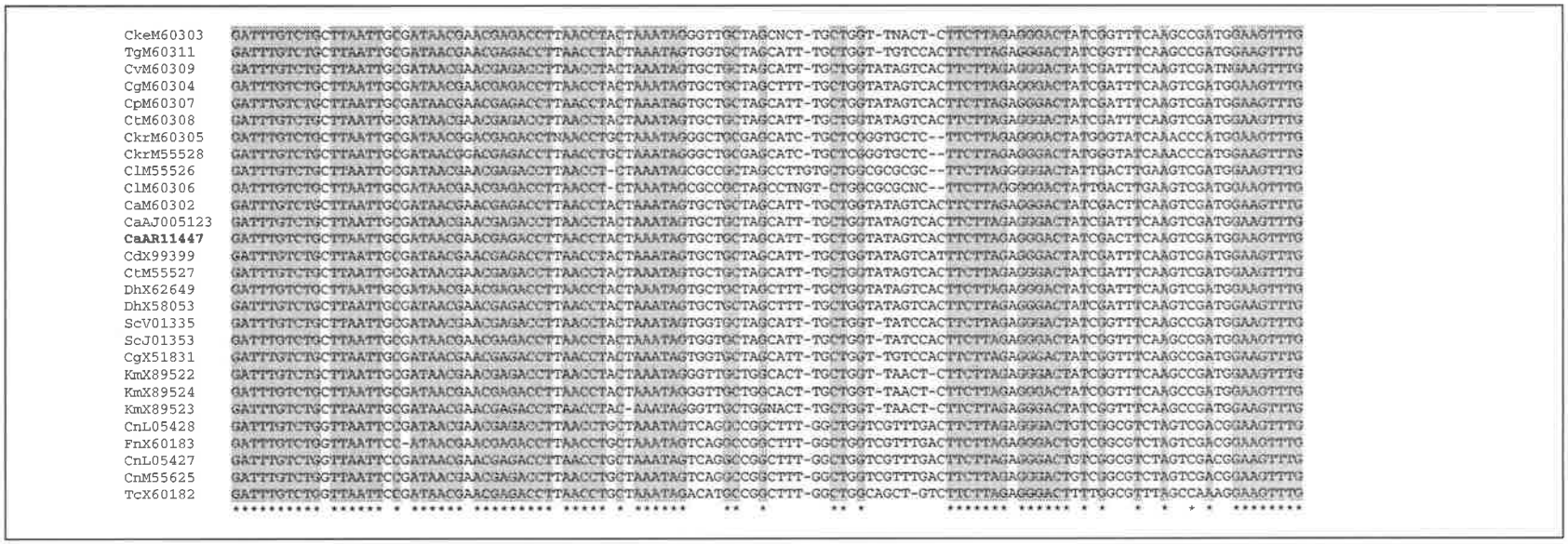
a; Areas of homology between all aligned sequences are highlighted
b; Emboldened sequence was obtained during the course of this study.

Table 17j; Alignment of Genebank sequences and the *C. albicans* type strain CBS 562 sequence of the full 18S rRNA gene obtained using ClustalX.



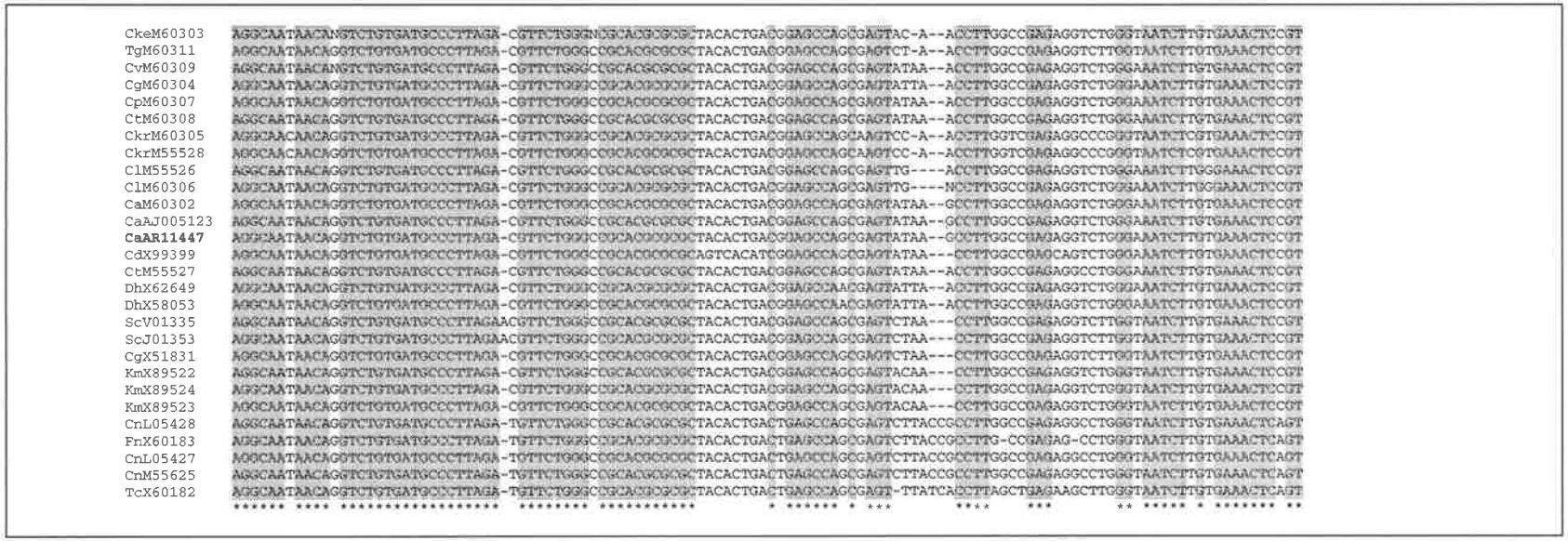
a; Areas of homology between all aligned sequences are highlighted
b; Emboldened sequence was obtained during the course of this study.

Table 17k; Alignment of Genebank sequences and the *C. albicans* type strain CBS 562 sequence of the full 18S rRNA gene obtained using ClustalX.



a; Areas of homology between all aligned sequences are highlighted
b; Emboldened sequence was obtained during the course of this study.

Table 17l; Alignment of Genebank sequences and the *C. albicans* type strain CBS 562 sequence of the full 18S rRNA gene obtained using ClustalX.

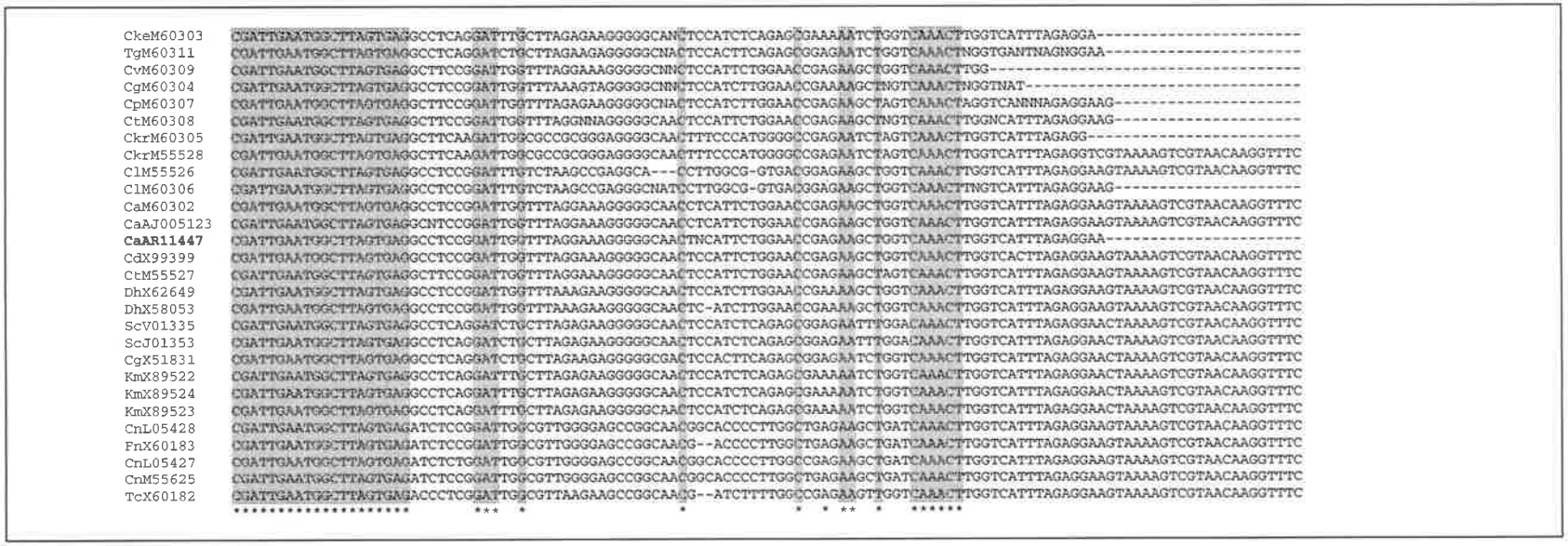


a; Areas of homology between all aligned sequences are highlighted
b; Emboldened sequence was obtained during the course of this study.

Table 17m; Alignment of Genebank sequences and the *C. albicans* type strain CBS 562 sequence of the full 18S rRNA gene obtained using ClustalX.

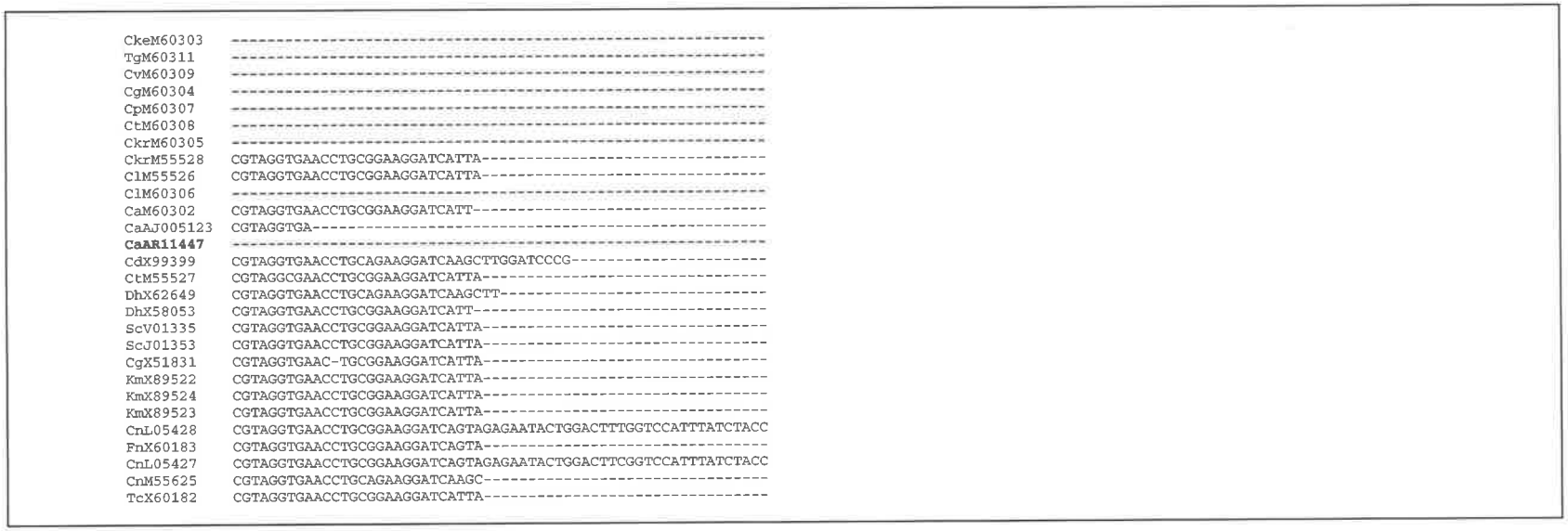
a; Areas of homology between all aligned sequences are highlighted
b; Emboldened sequence was obtained during the course of this study.

Table 17n; Alignment of Genebank sequences and the *C. albicans* type strain CBS 562 sequence of the full 18S rRNA gene obtained using ClustalX.



a; Areas of homology between all aligned sequences are highlighted
b; Emboldened sequence was obtained during the course of this study.

Table 17o; Alignment of Genebank sequences and the *C. albicans* type strain CBS 562 sequence of the full 18S rRNA gene obtained using ClustalX.



a; Areas of homology between all aligned sequences are highlighted
b; Emboldened sequence was obtained during the course of this study.

Table 17p; Alignment of Genebank sequences and the *C. albicans* type strain CBS 562 sequence of the full 18S rRNA gene obtained using ClustalX.