INVESTIGATION ON THE POPULATION VARIATION OF *Drosera indica* L. COMPLEX USING COMBINED MORPHOLOGICAL AND MOLECULAR TECHNIQUES

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ABSTRACT

Drosera indica L. is an annual, tropical species of carnivorous plant exhibiting a considerable amount of morphological variability, including plant size, flower colour, stamen form, seed size, and seed coat ornamentation pattern. Thus far there has been no study dealing with these morphological variability. The present study, therefore, is aiming at investigating the pattern of morphological and genetic variability in this species to determine whether there are morphologically distinguishable groups, and whether these groups are genetically distinct.

Materials used in this study consisted of air-dried herbarium specimens, water- and silica sand-preserved plant, and glasshouse- and tissue culture-grown plants germinated from seeds. The assessment of morphological variation was carried out on sixty two accessions of *D. indica* based on 62 accessions based on 14 vegetative and floral characters, as well as 12 micromorphological seed characters examined using scanning electron microscope. Multivariate numerical analysis on morphological data was performed using cluster analysis and two ordination techniques: the multidimensional scaling and principal component analysis. The pattern of genetic variation was evaluated on 15 accessions of *D. indica* using random amplified polymorphic DNA (RAPD). The DNA for RAPD analysis was obtained from fresh materials only, either from glasshouse- or tissue culture-grown plants germinated from seeds. The other types of materials failed to produce DNA of sufficient amounts and quality.

Results of morphological data analysis indicated that there are six morphotypes, each representing a distinctive combination of seed type and other morphological characters. Examination on the geographic distribution of accessions, coupled with the geology and the average annual rainfall data suggested that these morphotypes occurred sympatrically, and that they did not exhibit distinct geographical and ecological patterns. Based on this

evidence, therefore, these morphotypes might represent varieties within *D. indica*, or possibly even distinct species.

Cluster analysis and multidimensional scaling ordination on RAPD data revealed a high degree of genetic dissimilarity between accessions and between different morphotypes. The grouping of accessions based on RAPD data did not correspond to that resulted from morphological analysis. A comparison on the same set of samples (15 accessions) indicated that accessions from different morphotypes grouped together in the same cluster generated from RAPD data, and that there was no consistent pattern in the grouping of these morphotypes. This result indicated that there were differences in the pattern of within-species morphological and genetic variation. The discrepancy between results from morphological and molecular data was discussed. The two data sets, however, are in general agreement in detecting the degree of similarity between accessions.

The high degree of genetic dissimilarity revealed from RAPD analysis confirms the inbreeding nature of *D. indica*, and provides evidence on the reproductive isolation between sympatric morphotypes. This result, therefore, supports the recognition of the six defined morphotypes as distinct species. Considering the wide range of distribution of *D. indica* across different habitats and continents, however, further examination of specimens covering as much as possible its range of geographic distribution and morphological variation is required to justify the suggested taxonomic treatment.

DECLARATION

This work contains no material which has been accepted for the award of any degree or diploma in any university or other tertiary institutions and, to the best of my knowledge and belief, the thesis contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Ratna Susandarini 21/6/2001

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

R. SUSANDARINI
ERRATA: page/paragraph/line

3/2/4-5 '*D. ramentacea* Burch, ex DC., *D. madagascariensis* DC., *D. burmanni* Vahl and *D. peltata* Thunb. is recognised as the'
7/2/1 'areas, and' = 'areas, that'
7/2/10 'Asia to' = 'Asia, Japan'
9/3/1 remove 'L.'
9/3/2 remove 'a set of characters such as'
10/2 remove all 'L.'
11/2/1 'resulted in a new'
12/1/1 replace 'amount' with 'number'
13/2/5 'of a specific'
13/2/6 'by a factor'
15/2/8 'researchers: Welsh'
18/1/5 'from the heterozygote
18/3/2 replace 'overcome' with 'reduced'
19/1/9 'in conjunction with'
23/2/5 'kinds'
31/2/11 'data may have'
35/1/8 remove 'each'
35/2/1 remove 'research'
35/2/2 'variability between specimens'
36/1/6 replace 'Provided' with 'Combined'; 'evident' with 'evidence'
38/2/4 'either help to define'
38/2/14 'At the infraspecific level'
39/1/10 'species on seed morphology'
40/1/5 delete 'genus'
40/1/9 delete 'was'
40/2/7 delete 'the family'
40/2/9 'as in the study'
49/1/3 'SEO meant that'
51/1/4 'excavations'
52/2/2 'Type II seeds'
52/3/4 'belonging to'
53/1/2-3 'which no additional character was found to support the'
55/1/2 replace 'quality' with 'concentration'
55/2/5 delete 'cell'
66/3/6 'sources'
69/2/11 delete 'because'
69/2/12 'hydration, so the'
69/3/1 delete 'using'
69/3/4 delete 'from'
76/3/title 'plants'
77/3/4 '1986;'
79/2/5 'thus does not necessarily'
80/2/8 'character overlap with'
81/2/4 'replace 'to use' with 'that'
81/2/5 'analysis to be used in'
84/1/3 delete 'in the application of numerical phenetic methods'
84/2/12 delete 'two'
85/2/3 'fifty-nine complete specimens'
88/2/1 'dendrogram'
91/1/2 'of the clusters (Fig. 5.4)'
91/4/1 replace 'Despite' with 'In addition to'
102/1/3 'with a distinctive'
102/1/4 delete 'and thus'
102/1/8 'population systems'
102/1/17 'in the case of *D. indica*'
103/2/1 'that of the six defined morphotypes, 3 (A, B and C)
104/1/2 delete 'six defined'
104/2/1 'comprised three morphotypes and a further three subtypes'
105/1/10 'inbreeding'
105/1/11 ' the three morphotypes'
106/2/7 'similarity and difference'
108/1/1 replace 'in which the' with 'so that a'
108/2/1 'replace' from with 'of'
108/2/6 'which is a commonly used'
108/2/9 'reproductive'
109/1/1 'fingerprinting technique using RAPD'
110/1/2 delete 'random amplified polymorphic DNA'
112/2/2 'B2 and C, corresponding to the main clusters identified in the earlier morphological analysis'
112/2/3 'Cluster A (seed Type II)
112/2/5 'Cluster B1 (seed Type I)
112/2/7 'Cluster B2 (seed Type I)
112/2/9 'Cluster C (seed Type III)
112/2/10 'and red-striped petiolate leaves'
117/1/3 delete 'result'
118/1/5 '(Abbott *et al.* 1985)'
119/2/2 replace 'inbreeding' with 'inbred'
119/2/3 'that is responsible'
119/2/5 replace 'explaining' with 'explains'
119/2/10 'populations from exchanging genes'
120/2/10 'Whitkus, 1997), whereas'
121/2/2 'populations'
122/1/2 'difference, and'
122/1/8 'as much as'
122/1/9 'conclusion could'
124/2/8 replace 'defining' with 'studying'
126/1/1 delete 'are'
127/3/2 'cases where a pair'
128/1/14 'is the nature of morphology'
128/1/21 'and that with a large'
128/1/22 'there is a possibility that'
129/3/2 replace ', thus confirms its inbreeding nature' with 'providing evidence of reproductive isolation between accessions, possibly through inbreeding'
130/2/3 'distinct species. The anecdotal pollination observations where there is clear pollinator preference between the different morphs also supports the idea of genetic isolation. However, considering'
130/2/5 'as possible of its range'

***Drosera indica* L.**

Chapter One

Introduction

The definition of population, within the framework of taxonomy, was stated by Briggs and Walters (1984) to be the total number of organisms belonging to a particular taxonomic group found in a particular place at a particular time. From studies of population biology (Snaydon, 1973; Briggs and Walter, 1984), it has been widely recognised that organisms in a population vary in almost all of the measurable characters that make up the overall phenotype.

Variation between individuals within a population may be found in morphological, anatomical, karyological, biochemical, and molecular characters (Kreitman, 1991), and can be either genetically or environmentally determined. This means that for some characters there is a genetic basis underlying phenotypic variation, and this genetic variation can be measured using various methods.

The knowledge of the amount and distribution of genetic variation in natural populations is critical for their conservation and management strategies (Schaal *et al.*, 1991; Dawson *et al.*, 1993). This important issue originates from the fact that genetic variation is a necessary prerequisite for long- and short-term survival of the species (Schaal *et al.*,1991; Black-Samuelson *et al.*, 1997). Therefore, populations with low genetic variability have a reduced

potential to adapt to environmental changes (Ellstrand and Elam, 1993) and have a greater risk of extinction (Schaal *et al.*, 1991). In addition, Ayres and Ryan (1999) point out that both the amount and distribution of genetic variation within and among populations are indicative of the maintenance of variability, inbreeding and gene flow.

In dealing with genetic variation in a particular population, Schaal *et al.* (1991) argue that the assessment of genetic variation is most easily obtained by measuring morphological or phenotypic variation. Their argument is based on the fact that this approach has an advantage of requiring neither breeding not laboratory studies, and, most importantly, this work can be done directly from the field collection. With the invention of new methods in molecular analysis, however, the assessment of genetic variation is become increasingly based on polymorphic molecular markers.

Methods that have been developed as a tool to estimate population genetic variation at the molecular level include electrophoresis of seed storage proteins, isozymes, and DNA variation using restriction enzyme digestion and PCR-based techniques. Among those methods, the direct analysis of DNA variation has become the method of choice because of its greater potential sensitivity, greater number of polymorphisms, and higher probability of variation being neutral (i.e. not correlated or linked to differences in phenotype or adaptation to habitat) (Slade, 1993; Heaton *et al.*, 1999). In particular, it has been claimed that the estimation of genetic variation using PCR-based molecular markers has a number of advantages such as relatively

low cost, and the requirement for small amounts of DNA (Ellstrand and Elam, 1993). One of the most widely used PCR-based molecular techniques is random amplified polymorphic DNA (RAPD) which has become popular for the estimation of genetic variation at the population, species, and cultivars level (Williams *et al.*, 1990).

Drosera indica L. is an annual tropical species of carnivorous plant with a wide range of distribution including South Africa, Asia, and Australia (van Steenis, 1953; Ohwi, 1965; Obermeyer, 1970, Marchant *et al.*, 1982). This species, together with *D. ramentacea, D. madagariensis, D. burmannii,* and *D. peltata,* is recognised as crude drug 'Herba Droserae' from the southern hemisphere (Länger *et al.*, 1993) which is used for the treatment of cough and ailments of the respiratory tract (Wawrosch *et al.*, 1993).

In its natural habitat, *D. indica* exhibits polymorphic populations varying in colour, size, seeds and anthers. The present project, therefore, is aiming to investigate the morphological and genetic variability in this species. Multivariate approaches will be used on morphological data to determine if there are discrete morphotypes, while genetic analysis (RAPD) will be employed to ascertain whether these are genetically isolated.

Chapter Two

Literature Review

2.1 The Genus *Drosera*

Drosera (sundew) is the second largest genus of carnivorous plants (Seine and Barthlott, 1994), and, together with *Dionea*, *Drosophyllum* and *Aldrovanda*, forms the family Droseraceae. The glistening mucilage on the leaves of this group of plants attracts small insects which are then captured and digested (Lloyd, 1942). The natural habitat of these plants is nutrient-poor, and the carnivorous nature of these plants is reported to play an important role in their survival (Adamec, 1997; Zamora *et al.*, 1997). Having such characteristic nature, carnivorous plants have been the subjects of many studies.

The ecophysiological aspects of carnivorous plants, especially in relation to nutrient uptake and the role of carnivory, have been reported by many authors (Wilson, 1985; Haslin and Karlsson, 1996; Zamora *et al.*, 1997). The effects of competition on carnivorous plants with other plant taxa have been observed by Wilson (1985) and Brewer (1998), while the effects of habitat disturbances on the growth and survival of carnivorous plants, particularly the genus *Drosera*, have been reported by Brewer (1999). Those studies, however, can be considered as fragmentary, in that only one or two particular aspects were investigated. The recent review by Adamec (1997) reconsidered

much of the original data and conclusions from previous studies, including both greenhouse and natural habitat growing conditions. The phylogenetic and structural evolution of carnivorous plants has been described by Albert *et al.* (1992) in which the carnivore diversity was assessed from a historical perspective. This study, unlike some previously reported evolutionary accounts of carnivorous plants which were based on trap mechanisms and diversity, was based on a cladistic analysis of nucleotide sequence variation. Therefore, it could be considered as an example of a molecular study of carnivorous plants.

Drosera consists of both perennial and annual plants. This genus is divided into four subgenera: *Drosera, Rorella,* and *Ergaleium*, each with five, four, and three sections, respectively (Marchant *et al.*, 1982), and the South African endemic *Ptycnostigma* with three polymorphic species (Obermeyer, 1970), although other authors favour fewer subgeneric divisions (Seine and Barthlott, 1993, 1994; Culham and Gornall, 1994). Covering more than 200 species worldwide, *Drosera* has a main distribution in the Southern hemisphere, and the greatest speciation occurs in Australia and New Zealand (Lloyd, 1942; van Steenis, 1953; Marchant *et al.*, 1982; Boesenwinkel, 1989). About two thirds of its species are Australian, and more than half of those species grow in Western Australia (Marchant *et al.*, 1982; Lowrie, 1998). This group of plants has a distinct preference for sandy or otherwise acid, mostly poor, oligotrophic soils (Lloyd, 1942; van Steenis, 1953).

Drosera, as the most diverse genus in the family Droseraceae, has been the subject of various studies. One study on the relationships of Droseraceae, based on the sequence of the gene encoding the large subunit of ribulose-1,5-biphosphate carboxylase (*rbc*L) and morphological data (Williams *et al.*, 1994), revealed that *Drosera* is monophyletic with three major lineages: the *regia, capensis*, and *peltata* clades. A phytotaxonomic study showed that the presence of naphthoquinones within the genus *Drosera* (Culham and Gornall, 1994) supports the classifications of de Candolle (1824) and Diels (1906) to some extent, as well as the family's position in the ordinal classification of flowering plants (APG, 1998). To date, there is a growing interest in the phytochemistry of *Drosera*, with some of the species such as *D. rotundifolia, D. intermedia, D. spatulata,* and *D. capensis* under extensive study for their pharmacological properties (e.g. Budzianowski *et al.*, 1993; Wawrosch *et al.*, 1993; Budzianowski, 1997). These phytochemical studies emphasise the role of specific naphthoquinones in the genus, particularly plumbagin and 7-methyljuglone. These two chemicals have been recognised as antispasmatic and antibiotic and have been used in the treatment of respiratory tract infections (Perica and Berljak, 1996).

Generally, phytochemical studies of *Drosera* have been done in conjunction with tissue culture techniques. Tissue culture, in this particular case, is increasingly important not only as an alternative way to mass-produce medically important secondary metabolites but also for the purpose of conservation. There is a noted relationship between the two, in which the increasing collection of the plants from their natural habitat to be used as

medicine has resulted in reducing the population. The concern about conservation has lead some countries, such as United States and Europe, to consider protecting some species of *Drosera* that are under threat (Wawrosch *et al.*, 1993; Länger *et al.*, 1995).

2.1.1 *Drosera indica* L.

Drosera indica L. is an annual, erect sundew growing in tropical areas, and only reproduces sexually. *D. indica* is a herb with a fibrous root, 2 - 50 cm high; leaves cauline, scattered, glandular, shortly petiolate or with petiole up to 10 mm long, lamina narrowly linear, stipule absent; inflorescences cauline, terminal, racemose, up to 50 cm long, glandular, 2 – 25-flowered, bract linear, pedicel 5 - 20 mm long; sepals lanceolate to narrowly oblong, 3 – 5 mm long, entire or serrulate, glandular; petals cunnetae-obovate, 5 – 13 mm long, white, pink, orange, red or mauve; style 3, bipartite at base; stigmas papillose (Marchant *et al.*, 1982; Wheeler, 1992). *D. indica* is widely distributed from the tropical Old World extending to South East Asia to Japan and India. In Australia *D. indica* mainly occurs in the northern and eastern areas, with some isolated occurrences in New South Wales, southern Western Australia, and the inner regions of Queensland (Figure 2.1).

D. indica is distinguished from other members of the genus by a set of characters including its annual habit, elongated stem, cauline leaves with a narrowly linear lamina, and absence of stipules. *D. indica* has a wide range of variation in sizes and flower colour (Marchant *et al.*, 1982; Wheeler, 1992; Lian-li and Kondo, 1997), and different morphotypes can be found growing

together in mixed populations. Taking into account the considerable variation observed in some populations, Lowrie (1998) argued that some of these morphotypes may need to be recognised as distinct and given taxonomic status.

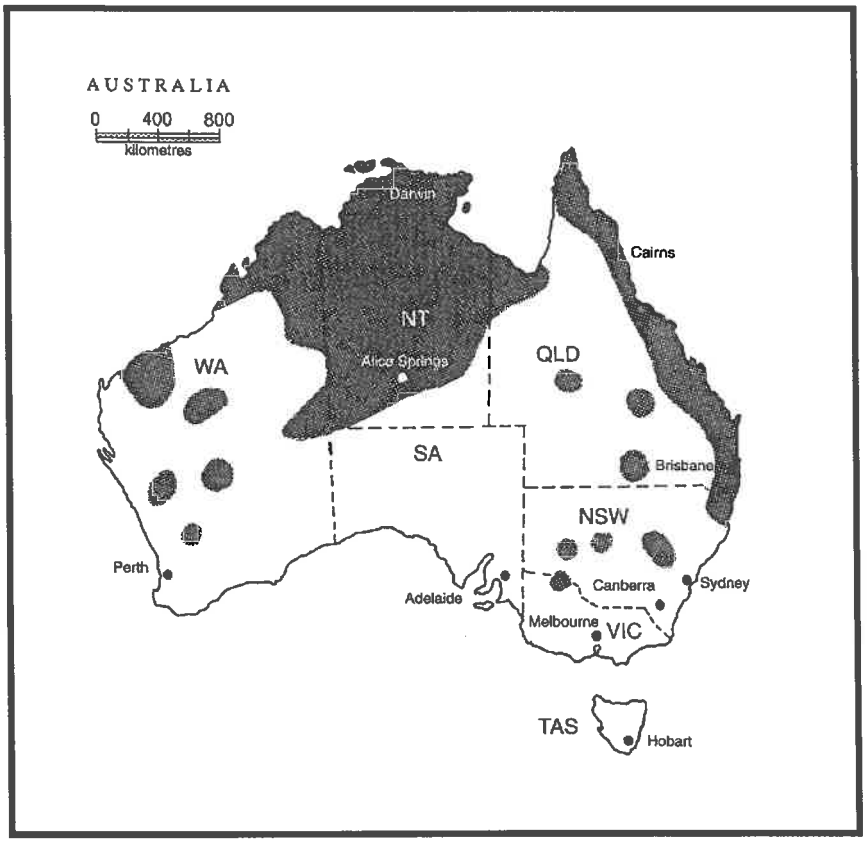

Figure 2.1 Distribution map of *Drosera indica* L. in Australia (Lowrie, 1998).

Unlike some other species in the genus *Drosera* that have been extensively explored (mainly for their chemical properties), only a limited amount of research is available on *D. indica*. A number of studies that included *D. indica* have been undertaken from various different aspects: anatomy

(Länger *et al.*, 1995), seed morphology (Boesenwinkel, 1989), seedling morphology (Conran *et al.*, 1997), cytotaxonomy (Kondo and Lavarack, 1984), phytochemistry (Zenk *et al.*, 1969), and a combined study of morphological and molecular data (Williams *et al.*, 1994). All of those studies, however, were carried out as to family or generic level, and were not specifically addressed to *D. indica* and/or the variation within this widespread and polymorphic species.

The lack of specific research on *D. indica* can be seen from the fact that until recently only few studies have been published, such as the study on the growth and reproductive status of this species by Maeyama and Ino (1996) and the vegetation control in a community complex of *D. indica* (Nakanishi, 1998). Moreover, no particular studies have been published on the species complex of *D. indica* referring to the morphological variability observed in this species.

Within the genus *Drosera*, species *Drosera indica* L. is classified under subgenus *Drosera* based on a set of characters such as the absence of a tuber, and roots arising from the stem base. In sectional level, this species belongs to the section *Arachnopus*, which is distinguished from its closest relative, section *Phycopsis*, on the basis of leaf characters (Marchant *et al.*, 1982).

Drosera indica L. was described by Linnaeus (1753). The species name refers to the location of its type specimen which was thought to be collected from India, although it was later found to have been collected from Ceylon

(Obermeyer, 1970). Diels (1906) monograph of the genus pointed out the variation in plant size within *D. indica,* especially in the specimens from Australia.

Walker (1994) reported 18 synonyms for *D. indica*: ten at the species level while the rest are at the infraspecific level. Some of these synonyms clearly indicate the variability in plant size and flower colour: *Drosera indica* L. forma *robusta* F.M. Bail. (Bailey, 1913) describes a bigger and more robust form of the plant. Synonyms that reflect the variation in flower colour include: *D. indica* L. forma *albiflora* Makino, *D. indica* L. forma *rosea* Makino, and *D. indica* L. var. *albiflora* (Makino) Makino.

2.2 DNA-based Molecular Markers in Plant Systematics

Molecular systematics is developed from the pursuit by systematists for the most fundamental characters that can be applied to the classification of living organism. This goal is to obtain characters of a more fundamental nature than morphological characters, free of non-heritable perturbations that may obscure the true genetic relationships (Doyle, 1993). Traditionally, the identification of plant taxa, the estimation of genetic diversity, and of relationships between taxa was inferred mainly from the comparative analysis of phenological morphology-based characters. Despite their continuing role in plant systematics, it has been recognised, however, that these characters have several disadvantages. They may not be significantly distinct among accessions (Mailer *et al.*, 1994), they lack definition and objectivity (Wrigley *et al.*, 1987), they usually require growing plants to full

maturity (Mailer *et al.*, 1994; Ratnaparkhe *et al.,* 1995; Wachira *et al.*, 1995), and are often strongly influenced by the environment (Yang and Quiros, 1993; Wachira *et al.*, 1995; Weising *et al.*, 1995). Moreover, it has been claimed that they do not necessarily provide an accurate estimate of taxonomic or evolutionary relationships (Russell *et al.*, 1993).

The development of technology in molecular biology has resulted a new field in plant systematic study, molecular systematics, which allows systematists to study taxonomic characters from two classes of macromolecules: proteins and nucleic acids. The rationale of using macromolecules in plant systematics has been described by Crawford (1990). It starts with the fact that the taxonomic characters chosen in systematic studies are presumed to have a genetic basis, and the assumption that characters that directly reflect genetic differences are the strong basis for constructing classification and phylogeny. The following rationale is that the genetic material itself provides the most basic or fundamental characters that could be employed in classification and phylogeny.

DNA-based molecular markers have been considered as having more advantages than protein-based markers (Beckmann and Soller, 1983; Weising *et al.*, 1995; Crawford, 2000). Being the product of gene expression, protein-based markers have been known to vary depending on tissue, developmental stage of the plant, and environmental conditions (Beckman and Soller, 1983; Fabbri *et al.*, 1995; Link *et al.*, 1995). In addition, it has been recognised that protein-based markers such as allozymes and isozymes

generally produce a limited amount of polymorphisms (Wilde *et al.*, 1992; Hu and Quiros, 1991; Link *et al*, 1995; Gallego and Martinez, 1996). DNA-based markers, on the other hand, have been recognised as having several inherent advantages over morphological and other biochemical characters. These advantages include: the opportunity to examine the genotype directly, the possibility for choosing markers with different evolutionary rates, and the high number of polymorphisms produced (Doyle, 1993; Weising *et al.*, 1995). The DNA marker techniques commonly used in systematic studies are: restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and microsatellites.

Compared to protein-based markers, DNA-based marker have advantageous properties of not being influenced by the environment (Smith and Williams, 1994; Karp *et al.*, 1996; Rath *et al.*, 1998), and producing higher level of polymorphism (Karp *et al.*, 1996; Lazaro and Aguinalde, 1998). In addition, because DNA is more stable than protein, it can be obtained from preserved materials, which allows a longer time span between collection and processing (Weising *et al.*, 1995). From a systematic point of view, DNA markers provide an almost unlimited number of characters, and they are believed to be selectively neutral, i.e. the characters do not converge when the individuals are exposed to similar selection pressures (Rieseberg and Brunsfeld, 1992; Weising *et al.*, 1995). Moreover, because different genes and sequences have their own evolutionary tempo and mode, these markers are numerous enough to address questions at most taxonomic levels (Doyle, 1993).

Beginning in the early 1980s, the use of DNA data in plant systematics revitalised this field of study (Crawford, 2000). In particular Graham and McNicol (1995) point out that DNA-based markers should lead to a greater understanding of relationships between species, and an accurate taxonomic classification. An example of this positive impact is the study of Heun *et al.* (1994) in *Avena sterilis*, in which RAPD data results in a definitive grouping of accessions. Another significant contribution of DNA markers to taxonomy is in distinguishing plant taxa when other markers fail to do so, such as in the cases of cocoa (Russell *et al.*, 1993) and tea (Wachira *et al.*, 1995).

2.3 Polymerase Chain Reaction (PCR)

The invention of the polymerase chain reaction (PCR) represented major progress in molecular biology studies (Saiki *et al.*, 1985; Mullis *et al.*, 1986; Mullis and Faloona, 1987). PCR is an *in vitro* method of nucleic acid synthesis by which a particular segment of DNA can be specifically replicated (Saiki, 1990). This technique facilitates the manipulation of specific DNA sequence by producing a selective enrichment of the sequence of interest by factor of 10^6 (Saiki *et al.*, 1988), thus overcoming the problem in situations where only a very small amount of material is available for analysis.

The materials involved in PCR are: template DNA, one or more primers complementary to the opposite strand on the template, buffer, the four deoxyribonucleotides in equal proportions, and a thermostable DNA polymerase enzyme. PCR is performed by incubating the samples at three temperatures corresponding to the three steps in a cycle of amplification. The

first step is the denaturation of the double stranded DNA, followed by annealing of the primers to their complementary sequences, and the last step is extension of the annealed primers catalysed by DNA polymerase. The power of PCR is essentially brought about by thermostable DNA polymerase, a DNA-copying enzyme that works optimally at 72°C (Newbury and Ford-Lloyd, 1993). When this technique was introduced for the first time (Saiki *et al.*, 1985; Mullis *et al.*, 1986), the Klenow fragment of the bacterium *Escherichia coli* DNA polymerase-I was used for the synthesis of specific DNA fragments. The thermolabile nature of this polymerase enzyme did not allow the automation of the amplification process, since it was necessary to add new enzyme for each amplification cycle. It was not until Saiki *et al.* (1988) demonstrated the use of the thermostable DNA polymerase purified from the thermophilic bacterium *Thermus aquaticus* (*Taq* polymerase) that PCR became an automated process.

This thermostable enzyme can maintain its catalytic function in a high temperature of denaturation during the amplification cycles (95°C). The heat-stable nature of this enzyme makes the PCR reaction more efficient by eliminating the need for adding new enzyme after the completion of each amplification cycle. This enzyme is now available in two forms: the native polymerase purified from *T. aquaticus*, and a genetically engineered polymerase from *E. coli* such as Ampli-Taq (Sambrook *et al.*, 1989). Another advantage of this thermostable polymerase is that it greatly reduces "mispriming" which can affect the heterogeneity in the length of the amplified DNA fragments.

The main advantage of PCR is its property of a chain reaction, in which the products from one amplification cycle become the reactant for the next cycle. Successive cycles of amplification essentially double the amount of the target DNA synthesised in the previous cycle. The result is an exponential accumulation of specific target fragments, approximately 2^n, where 'n' is the number of cycles of the amplification performed (Mullis, 1989; Saiki, 1990).

The success of PCR technology in molecular biology studies, with its application in a wide range of organisms, stimulated the development of PCR-based methods in DNA assays. Of particular interest is the development of DNA fingerprinting techniques. DNA fingerprinting was defined as any multilocus approach of visualising DNA polymorphisms, either generated from hybridisation or PCR-based techniques (Weising *et al.*, 1995). Two techniques of DNA fingerprinting analysis based on PCR technology were developed simultaneously in 1990 by different groups of researchers. Welsh and McClelland (1990) with their arbitrarily-primed PCR (AP-PCR) and Williams *et al.* (1990) with random amplified polymorphic DNA (RAPD). The similarity in these two techniques is that a single primer of arbitrary sequence is used to amplify random DNA segments. The main difference between the two techniques is that AP-PCR employs longer primers (20 base pairs or longer) than RAPD which uses 10 base pair primers. A similar technique called DNA amplification fingerprinting (DAF) was introduced by Caetano-Anolles *et al.* (1991). This technique uses shorter primers, usually of eight to ten or even five base pairs, and the resultant amplification product is resolved by polyacrylamide gel electrophoresis and visualised by silver

staining. Apart from the specificity in each of these three techniques, they all provide a genetic fingerprint. Since these three techniques cannot be named interchangeably, one general term was then proposed for three of them, that is multiple arbitrary amplicon profiling (MAAP) (Caetano-Anolles *et al.*, 1992). Since this project uses RAPD for generating molecular fingerprinting, this technique will be discussed in more detail.

2.4 Random Amplified Polymorphic DNA (RAPD)

RAPD is a PCR-based technique, in which random DNA sequences are amplified using single primers of arbitrary nucleotide sequence. RAPD markers are inherited in a Mendelian fashion (Williams *et al.*, 1990). In RAPD, a discrete band is produced when the primer binds to sites on opposite strands of the genomic DNA that are within an amplifiable distance, generally less than 3,000 base pairs (Wolfe and Liston, 1998). The amplification products form characteristic banding patterns showing a certain degree of polymorphism. The polymorphisms, represented by the presence or absence of the amplification products (amplicons), function as genetic markers (Williams *et al.*, 1990; Newbury and Ford-Lloyd, 1993), and provide highly informative characters for the evaluation of genetic similarity (Weising *et al.*, 1995). The polymorphisms in the amplification products may result from all classes of mutation: insertions, deletions, substitutions, and inversions in the primer binding sites (Williams *et al.*, 1990, Waugh and Powell, 1992; Gillies and Abbott, 1998). The high level of polymorphisms generated by RAPD has raised speculation about the molecular basis underlying the amplification process. In this regard, Venugopal *et al.* (1993)

claim that multiple mismatch-annealing between the DNA template and primer is the most probable mechanism responsible for generating highly polymorphic amplification products. In this case, the requirement for a DNA segment to be amplified is not always a perfect match, rather the occurrence of at least one perfect match annealing site was mandatory for a given target site to be amplified in RAPD.

The advantages of RAPD compared to the other PCR-based methods include: the use of only one short arbitrary sequence primer, there is no need for prior knowledge of target DNA sequence (Williams *et al.*, 1990; Bowditch *et al.*, 1993; Gillies and Abbott, 1998), its ease of methodology, low cost, and the ability to produce numerous and distinguishable polymorphisms (Williams *et al.*, 1990; Hadrys *et al.*, 1992; Stewart and Excoffier, 1996). Moreover, being based on the amplification process, RAPD has the ability to generate molecular markers from only a small amount of template DNA. This is especially significant in studies dealing with a limited source of materials such as those of rare and endangered species.

Despite these advantages, however, RAPD has some limitations, including the dominant nature of RAPD markers, the assessment of band homology, and the reproducibility of the markers (Williams *et al.*, 1990; Hadrys *et al.*, 1992; Lynch and Milligan, 1994; Jones *et al.*, 1998; Wolfe and Liston, 1998). The dominant nature of RAPD means that it is not possible to distinguish between homozygote and heterozygote. This is particularly problematic when the technique is to be used in the estimation of population genetic

parameters, such as for the estimation of gene and genotype frequencies, gene diversity within populations, and population subdivision (Lynch and Milligan, 1994). The same problem is found when RAPD is to be used in mapping studies using segregating F_2, in which the homozygote AA can not be discerned from heterozygote Aa (Halward *et al.*, 1992; Weising *et al.*, 1995).

The second problem, the assessment of band homology, is based on the assumption that co-migrating bands represent homologous loci. This is a particularly serious concern in studies dealing with higher taxonomic levels. This limitation, however, can be tested in two different ways: Southern hybridisation of the amplified bands, and restriction enzyme digestion of DNA fragments isolated from the gel (Wolfe and Liston, 1998). There are some studies using RAPD that include tests of homology either using Southern hybridisation (Wilkie *et al.*, 1993; Hilu and Stalker, 1995; Stammers *et al.*, 1995; Loo *et al.*, 1999; Song *et al.*, 2000), or restriction enzyme digestion (Gillies and Abbott, 1998). Those studies revealed that most of the co-migrating RAPD bands are indeed homologous, especially in within-species comparisons. However, there are also studies which show that a single RAPD band may consist of several similar-sized DNA fragments of different sequence (Hausner *et al.*, 1999; Genet Mekuria, pers. comm.).

The third limitation claimed for RAPD is the reproducibility of the markers, although it has been recognised that this problem could be overcome by optimising the procedure and by maintaining consistent reaction conditions

(Bowditch *et al.*, 1993; Hoey *et al.*, 1996). A study conducted by Mailer *et al.* (1994) using four different thermocyclers for RAPD revealed that each thermocycler produced the same banding pattern. This study provides evidence that, given consistent reaction conditions, RAPD can be a reproducible assay. However, from a test of reproducibility involving nine European laboratories, it was concluded that due to insufficient reproducibility, RAPD was not suitable for diversity studies in which several laboratories were involved to exchange and collate data sets Jones *et al.* (1998). In conjunction to the reproducibility problem, another issue raised for RAPD is the degree of artefacts observed between experiments (Ellsworth *et al.*, 1993; Pillay and Kenny, 1995). These artifacts may lead to the overestimation of the level of genetic variation. In addressing the problem of reproducibility in RAPD data in relation to its suitability in subsequent analysis, such as the estimation of genetic similarity, Skroch and Nienhuis (1995) demonstrated that there is no differences in the genetic distance value computed from replicated and unreplicated data. This result provides a counter argument that RAPD bands that cannot be consistently scored across repeated RAPD runs should not be used in studies of genetic similarity.

Having many technical advantages over other DNA-based assays, RAPD has been widely used in various studies, including systematics, evolutionary biology, plant ecology, conservation genetics, horticulture, and germplasm management. In addition, due to its simplicity, speed, and small amount of sample required in this technique, Waugh and Powell (1992) point out that RAPD is well suited for use in large sample-throughput systems such as

plant breeding, population genetics and biodiversity studies. The most commonly cited application of RAPD are in the:

1. generation of the genetic fingerprinting for species and cultivar identification;
2. assessment of genetic diversity within and between populations;
3. identification of chromosome-specific marker;
4. investigation of putative hybrid origin;
5. establishment of genetic maps;
6. generation of markers linked to disease resistance and agronomic traits;
7. examination of genetic similarity of species.

(Chalmers *et al.*, 1992; Black-Samuelson *et al.*, 1997; Gillies and Abbott, 1998).

2.4.1 RAPD in infaspecific study of plants

The application of RAPD in systematic studies has been reported at various taxonomic levels, ranging from familial (Chalmers *et al.*, 1994; Rath *et al.*, 1998), generic (Wilkie *et al.*, 1993; Gillies and Abbott, 1998), sectional or subgeneric (Adams and Demeke, 1993), to species, cultivars, individuals and populations levels (Chalmers *et al.*, 1992; Demeke *et al.*, 1992; Hadrys *et al.*, 1992). The most widely used applications of RAPD, however, have been reported at lower taxonomic levels, that is, at or below species (infraspecific). This is due to the ability of RAPD to detect polymorphisms in closely related organisms, such as in a species complex, in different populations of a single species, or individuals within a population (Kazan *et al.*, 1993a; Bowditch *et*

al., 1993). Moreover, some studies have indicated that RAPD is reliable for lower taxonomic levels such as infraspecific comparisons and among closely related species, but less reliable for higher taxonomic ranks (Bowditch *et al.*, 1993; Hallden *et al.*, 1994; Thormann *et al.*, 1994; Wolfe and Liston, 1998).

The published studies on the use of RAPD for infraspecific taxa have been concerned with two major aims, either for the estimation of genetic diversity or for cultivar identification. The combination of those two aims has also been reported (Lanham *et al.*, 1995; Schnell *et al.*, 1995). A number of studies will be reviewed in the following sections corresponding to those two major aims.

2.4.1.1 *The use of RAPD in the estimation of genetic diversity*

Traditionally, measures of genetic diversity were conducted using a combination of morphological and agronomic traits, but studies indicated that this approach does not necessarily provide an accurate estimation of taxonomic or evolutionary relationships (Dawson *et al.*, 1993; Russell *et al.*, 1993). This drawback can now be overcome using molecular markers, for example RAPD, which provide additional information, and function as a complement to the traditional approach. As far as the literature is concerned, the use of the RAPD technique for the estimation of genetic diversity has dominated studies at the infraspecific level. The focus of these studies is on the determination of the extent and the distribution of genetic diversity. Such studies have covered both cultivated and wild plant species. The sample used in those studies varies from:

1. cultivars or clones (Yu and Nguyen, 1994; Wachira *et al.*, 1995; Lanham *et al.*, 1995; Schnell *et al.*, 1995; Abo-elwafa *et al.*, 1995);
2. genotypes (Vierling and Nguyen, 1992; Tao *et al.*, 1993; N'goran *et al.*, 1994; Samec and Nasinec, 1996);
3. populations (Huff *et al.*, 1993; Smith and Pham, 1996; Dawson *et al.*, 1993; Yang *et al.*, 1998; Brunell and Whitkus, 1997; Wen and Hsiao, 1999, Loo *et al.*, 1999, Vazquez *et al.*, 1999); and
4. some accessions representing cultivars or populations under study (van Heusden and Bachmann, 1992a; van Heusden and Bachmann, 1992b; Russell *et al.*, 1993; Kazan *et al.*, 1993a; Heun *et al.*, 1994; Karihaloo *et al.*, 1995; Williams and St. Clair, 1993).

There are some general results observed from the studies on the estimation of genetic diversity in infraspecific taxa. The most significant one is that RAPD markers provide an accurate measure of the extent and the distribution of genetic diversity or variation. Moreover, there is a positive correlation between genetic variation and geographical distribution of the populations (Brunell and Whitkus, 1997; Irwin *et al.*, 1998; Lazaro and Aguinalde, 1998; Yang *et al.*, 1998; Wen and Hsiao, 1999). This is particularly important because the congruence of RAPD evidence with geographical distribution could be taken as an indication of the reliability of RAPD data in infraspecific studies (Purps and Kadereit, 1998). Another important point from the study of genetic diversity is the detection of gene flow and recombination in natural population. An example of this was the work of van Heusden and Bachmann (1992b) in *Microseris pygmaea*, in which the coastal and inland populations of

the species were proven to be two distinct groups but with weak differentiation, indicating the occurrence of the gene flow and recombinations among neighbouring populations.

In addition to its widely used application in estimating genetic diversity, RAPD has been used to assess genetic similarities of plant taxa. In most cases, RAPD markers are not only capable of producing a more definitive separation of accessions under study, but also show an agreement with other kind of data sets such as isozymes (van Heusden and Bachmann, 1992a; Yang and Quiros, 1993; Heun *et al.*, 1994; Karihaloo *et al.*, 1995; Irwin *et al.*, 1998), and in some cases, with morphology (Wen and Hsiao, 1992; Loo *et al.*, 1999). Some studies that compared different kinds of DNA-based molecular markers also indicate consistent results between RAPD and RFLP (N'goran *et al.*, 1994; Thormann *et al.*, 1994; Spooner *et al.*, 1996), AFLP (Mackill *et al.*, 1996; Paran *et al.*, 1998) and microsatellite data (Staub *et al.*, 2000).

2.4.1.2 *The use of RAPD in the identification of cultivars and species*

The use of RAPD markers as an identification tool for infraspecific taxa through DNA fingerprinting has been reported both for cultivated and wild plant species. The power of RAPD has been proven by its ability to fingerprint individual genotypes or cultivars (Russell *et al.*, 1993; Lanham *et al.*, 1995; Schnell *et al.*, 1995), even when only a low percentage of polymorphism is produced (Yang and Quiros, 1993). This kind of application is useful in the management of germplasm collections, by which the presence of duplicate accessions can be detected effectively and efficiently, and also for the

establishment of core collections. In this regard, Schnell *et al.* (1995) point out that RAPD provides an additional cultivar verification method for germplasm collections such as in cocoa (Wilde *et al.*, 1992), tomato (Williams and St. Clair, 1993), banana and plantain (Howell *et al.*, 1994), mango (Schnell *et al.*, 1995), rice (Virk *et al.*, 1995), and sweet potato (Zhang *et al.*, 2000).

Another significant contribution from RAPD fingerprinting is in the assessment of cultivar identity for economically important crops. The uncertainty of cultivar identity has been a major problem in some important crop species. This problem usually starts from the fact that many successfully established important crops were historically introduced without adequate information on their origin. Subsequently, many homonyms or synonyms to a single cultivar are present, such as in olive (Mekuria *et al.*, 1999). The need for an accurate and effective method for cultivar identification and characterisation is also important for the protection of Plant Breeders' Rights in demonstrating the distinctiveness, uniformity and stability (DUS) for each new cultivar (Mailer *et al.*, 1994; Law *et al.*, 1999). If there is uncertainty about cultivar origin and identity, then the extent of the gene pool for the species under investigation is not known (Bradley *et al.*, 1996). This lack of information about the gene pool may create difficulties in crop improvement and conservation programs. In this case, RAPD has been proven to be a powerful technique for genetically fingerprinting cultivars of economically important plant species such as broccoli and cauliflower (Hu and Quiros, 1991), apple (Mulcahy *et al.*, 1993), olive (Fabbri *et al.*, 1995) and garlic (Bradley *et al.*, 1996).

The power of RAPD as an identification tool provides a strong correlation to its application in plant taxonomy. Graham and McNicol (1995) point out that RAPD data in conjunction with other information on the species and cultivars can be used to improve taxonomic classification. In this regard, Vazquez *et al.* (1999) used RAPD to justify the taxonomic position of *Sideritis pusilla*, while Brunell and Whitkus (1997) re-evaluated subspecies delimitation in *Eriastrum densiflorum*. Fukuoka *et al.* (1992) demonstrated that the grouping of *Oryza sativa* accessions based on RAPD data corresponds to the ecospecies groupings. Some other examples on the use of RAPD in resolving taxonomic problems are the studies of Demeke *et al.* (1992), Kazan *et al.* (1993b), Tao *et al.* (1993), and Karihaloo *et al.* (1995).

2.4.2 Analysis of RAPD Data in Systematic Studies

The most common application of RAPDs in systematic studies is for the estimation of genetic similarities. For this purpose, the systematic characters are defined as the RAPD bands of a certain molecular size, while the character states are the presence or absence of these bands. For the construction of similarity or distance matrix the bands could be simply scored as presence or absence and then transferred into binary character of 1 or 0, respectively. There are some authors, however, who score the RAPD bands as multi-state characters by considering the differences in their intensity, such as Demeke *et al.* (1992) and Adams and Demeke (1993). They scored the bands as 7-state characters ranging from 0 to 6, with 0 being no band, and 6 being very bright band. In this case, they claimed that by scoring all bands (including the faint ones) the data become more informative.

In fact, the cause of the differences in band intensity is still in debate. It has been argued that the differences in band intensity could be due to mismatch or relative sequence abundance (Yang and Quiros, 1993; Ratnaparkhe *et al.*, 1995; Wolfe and Liston, 1998). The second possibility, however, has been countered by Thormann *et al.* (1994) and Weising *et al.* (1995) by claiming that RAPD marker intensity is not associated with the genomic copy number of the amplified sequences but it is more likely to be associated with the degree of homology between the primer and the DNA template. In this instance Williams *et al.* (1993) specifically point out that the magnesium ion concentration and the annealing temperature are the important factors that affect the relative intensity of the amplified bands, and therefore affect the reproducibility.

In dealing with the different band intensities, Pillay and Kenny (1995) argue that a decision should be made whether to include or exclude the faint bands in order to make a meaningful comparison of the amplified bands. The failure to do this could result in overestimation or underestimation of the genetic relationships. As has been stated by M'Ribu and Hilu (1994), the scoring of RAPD band is somewhat arbitrary, and different laboratories may use different criteria. In this case, Wolfe and Liston (1998) suggest that accurate scoring of RAPD band depends on the reliability of the amplification products, i.e. those that are reproducible between experiments and that can be scored unambiguously.

The next step following band scoring is the construction of a pair-wise similarity (or distance) matrix for the calculation of a similarity coefficient. Given the dominant nature of RAPD, the problem of homology, and the nature of data scoring, there is a debatable issue concerning the choice of similarity coefficient for RAPD data. The three most commonly used formulas are the Jaccard, Dice, and simple matching coefficients. They differ in the inclusion of the shared absence or double zero character and in giving weight on shared presence and shared absence characters (Sneath and Sokal, 1973). Simple matching coefficient includes all the character states, while Jaccard and Dice coefficients exclude the shared absence. The last two coefficients differ in weighting shared presence over shared absence characters, in which Jaccard's weighs the two equally whereas Dice's puts twice the weight on shared presence. In this regard, Wolfe and Liston (1998) argue that because band absence is more likely to be nonhomologous, the use of simple matching coefficient is not recommended, and suggest the use of Dice's coefficient. This argument is based on the fact that this coefficient gives more weight to shared presence than to shared absence. It has been claimed that this coefficient has a direct biological meaning by producing an estimate of the expected portion of amplified fragments shared by two samples due to inheritance from a common ancestor. In addition, Lamboy (1994) claims that Dice's coefficient is also less biased by the occurrence of artifactual bands, a problem that has been addressed by Pillay and Kenny (1995) and Skroch and Nienhuis (1995). In the case of shared absence character states for molecular data, Bowditch *et al.* (1993) argue that in systematics the absence of a RAPD band can potentially be the phenotype of many different alleles at a locus,

which means that the character state "band absence" may encompass many different character states such as inversion, different secondary structure, or any number of point mutations.

Another argument that has been raised for RAPD data is its suitability for phylogenetic analysis using parsimony. This is concerned with the assessment of band homology and independence. However, these two objections are equally applied to other fragment-based data such as amplified fragment length polymorphism (AFLP). When RAPD is to be used in assessing genetic parameters, two basic assumptions should be made: (1) that the interpretation of banding patterns can be accomplished in a completely unambiguous manner; and (2) each locus can be treated as a two-allele system (biallelic), with only one of the alleles being amplified (Lynch and Milligan, 1994). In this instance Clark and Lanigan (1993) set the criteria that should be met before employing RAPD data in the estimation of genetic parameters. While Backeljau *et al.* (1995) argue that RAPD is not suitable for parsimony analysis due to the problem of homology and independence, others are still considering some ways to overcome this problem. Wolfe and Liston (1998) in promoting the use of RAPD gave counter arguments to the limitations pointed out by Backeljau *et al.* (1995), and claimed that those limitations not only exist in RAPD but are also applicable to the other molecular marker such as DNA sequence data. In the case of assumption about the independence of RAPD data for use as systematic characters, Bowditch *et al.* (1991) and Gillies and Abbott (1998) point out that this

problem could be overcome by using a large number of primers and selecting only the strongly amplified and reproducable bands from each primer.

2.5 The Use of Herbarium Specimens for DNA-based Studies in Plant Systematics

Herbarium specimens are now gaining a more important role in systematic studies. Despite their traditional contribution to morphological studies, herbarium specimens at present are being increasingly used as an alternative to fresh material for molecular studies. Savolainen *et al.* (1995) evaluated the potential of herbarium specimens in phylogenetic studies, and argued that herbarium specimens represent an invaluable source of material for the molecular analysis of plant taxa. This increased emphasis is brought about by the invention of PCR technology that opens the possibility to recover ancient DNA from herbarium specimens and fossils. This particular application in plant molecular systematic studies was initiated when Rogers and Bendich (1985) reported the success of isolating DNA from herbarium specimens, and Golenberg *et al.* (1990) demonstrated the amplification of DNA from fossil of Miocene *Magnolia* leaves. This success then stimulated further research in finding the best way to preserve DNA from field-collected specimens, which in turn can be applied in preparing materials to be stored as herbarium specimens or to be used directly in molecular analysis. Both conventional and advanced preservation methods were explored, and the effect of various preserving media on DNA quality and integrity was investigated (Doyle and Dickson, 1987; Pyle and Adams, 1989; Liston and Rieseberg, 1990; Chase and Hills, 1991; Rogstad, 1992; Adam and Li, 1992; Harris, 1993; Flournoy *et al.*, 1996). Variable results were reported from these studies, from low yield and

poor quality to the successful recovery of DNA. Apart from the variable results, however, one common problem encountered in using herbarium specimens for DNA studies is the presence of contaminating compounds that could inhibit the PCR reaction. The most common contaminating compounds are usually from polysaccharides, proteins, phenolics and oxidised materials that co-precipitate with DNA (Wolfe and Liston, 1998).

Facing the problem of low yield and quality, contamination, and PCR inhibition on DNA originating from herbarium specimens, many studies have been conducted to overcome this problem. These include the improvement of DNA extraction protocols (Rogers and Bendich, 1985; Štorchová *et al.*, 2000), and manipulation of the PCR procedure to allow the amplification of ancient DNA through the so-called reconstructive PCR method (Golenberg *et al.*, 1996).

With the success of isolating DNA from herbarium specimens, these materials are now gaining more function and increasingly are being incorporated into plant molecular systematic studies. The most significant benefit to develop from accessibility to herbarium specimens is the increased range of taxa under study. This means that molecular studies have become comparable to morphological studies in their taxon coverage. Another advantage of using herbarium specimens is access to rare and endangered plant species or plants with restricted distribution in remote areas, in which case the sampling of fresh material is difficult. Some systematic studies using herbarium specimens include studies for *Datisca* (Liston *et al.*, 1992),

bryophytes (Mishler *et al.*, 1992), *Medusagyne oppositifolia* (Fay *et al.*, 1996), *Sophora toromiro* (Maunder *et al.*, 1999), and *Argemone* (Schwarzbach and Kadereit, 1999).

2.6 Morphological and Molecular Data in Systematic Studies

The development of molecular biology techniques that facilitate the generation of molecular data adds a new insight in systematic studies. The consequence of this is the possibility of resolving the debate concerning the reliability and importance of morphological versus molecular characters. Claimed as having more advantageous properties over more classical morphological data, there was a tendency to put more attention on molecular data. This point of view, however, has been countered by Donoghue and Sanderson (1992) who argue that it is not appropriate to ignore morphological data in favour of molecular data. In relation to the contribution of each data set in the study of systematics, it has been recognised that both morphological and molecular data have resolving power at any taxonomic level (Wolfe and Liston, 1998). In addition, each data set has its distinctive advantages. Hillis (1987), in a review of the contribution of morphological and molecular data in systematic studies, points out the advantages held by each data set. Morphological data have advantages in their applicability and accessibility to museum specimens and fossils, in the use of ontogenetic information, and in the cost involved in generating the data. Molecular data, on the other hand, have advantages in having much larger data sets available, covering different rates of evolutionary change, and being independent of environmental influence.

The underlying problem of the debate concerning the application of different data sets in systematic studies arises from the nature of the data themselves. There are four main criteria being used in considering the reliability of different data sets in systematics studies, namely the level of homoplasy, the neutrality of the data set and its and rate of evolution (different characters or DNA regions have different rates of evolution), independence, and the number of characters available from each data set (Donoghue and Sanderson, 1992).

When dealing with different data sets, one methodological issue is problematical: whether to analyse each data set separately or to combine data sets. Debates on this issue have been raised since the emergence of molecular-based studies and indeed are still a major concern among systematists, especially when the data are to be used in inferring phylogeny. Two options are available in dealing with this problem: "total evidence" and "taxonomic congruence" (Donoghue and Sanderson, 1992; Larson, 1994; Bremer, 1996; Peterson and Seberg, 1998). The first option starts with combining both morphological and molecular data into one matrix from which a phylogenetic tree is constructed. The second option is concerned with analysing each data set separately to construct phylogenetic trees, and then identifying points of congruence or conflicts between the resulting trees. Each option has its own advantages and disadvantages. Mayer and Soltis (1999) point out the advantageous property in each option, claiming that separate analysis allows the identification of heterogeneity or discord among partitions. Combining data sets, on the other hand, increases the number of

characters and thus provides a better opportunity to detect phylogenetic signals. In dealing with the two options, Larson (1994) argued that taxonomic congruence is more appropriate in phylogenetic evaluation, while most of the systematists however, support the total evidence option (Donoghue and Sanderson, 1992; Bremer, 1996).

Since the emergence of molecular systematics, many papers have been published to review the use of different data sets in systematic study (Hillis, 1987; Sytsma, 1990; Donoghue and Sanderson, 1992; Baker *et al.*, 1998). Many studies using both morphological and molecular data show that the combined analysis of different data sets (total evidence approach) has more support and is therefore a more useful method (Sytsma and Smith, 1992; Larson, 1994; Bremer, 1996). However, the total evidence approach has been reported as having potential problems due to the nature of each data set. Donoghue and Sanderson (1992) noted two major problems when the two data sets are to be combined: the difficulty in character weighting, and differences in the nature of the sampling of terminal taxa. Moreover, Peterson and Seberg (1998) argue that data sets could only be combined provided that they are sufficiently homogenous in their rates of evolution. The combination between slowly and rapidly evolving characters might result in an incorrect estimate of phylogeny. An assessment of the relative contribution, and the degree of support, provided by each data set has been investigated by Baker *et al.* (1998), who analysed 25 systematic studies previously conducted by different authors with combined morphological and molecular data. Despite the considerable incongruence between data sets, the

results indicated that both types of data contribute positively to the combined analysis. A study to evaluate the interaction of three independent data sets, anatomy/morphology, cytology, and molecular has been carried out by Dubuisson *et al.* (1998) in the genus *Trichomanes*. In this study, two approaches were proposed, namely "the conditional combination" and 'the empirical differential weighting". The first approach required the test of heterogeneity to be performed to evaluate the level of incongruence between different data sets. Ideally, when a high level of incongruence is found, the data sets are not suitable for a combined analysis. The second approach was applied to accommodate different evolutionary processes due to the existence of quantitatively unbalanced data sets (character size) between morphology and molecular data. In this study they found a high level of incongruence between different data sets, and the result of combined analyses were strongly conditioned by conflicts between data sets, which increases with the heterogeneity of the data.

When there are two or more different data sets available for any taxa under study, one has many options to analyse the data sets. An example of two different ways in analysing morphological and molecular data, to combine or to analyse separately, was the study of Bremer (1996) in the family Rubiaceae. In this study, it was found that there was much congruence, and also conflict, between the morphological and the molecular data sets. For reason of parsimony, the best phylogenetic hypothesis was obtained when the two data sets were combined in the analysis. Another approach in dealing with morphological and molecular data has been demonstrated by Sytsma

and Smith (1992) in the systematics of *Fuchsia* section *Skinnera*, in which four different methods of data analysis were used. In addition to the combined and separate methods of data analysis, the authors used two other methods: firstly only molecular characters were used to generate a cladogram and secondly morphological characters were overlaid onto the molecular cladogram, and vice versa. Using these methods it was revealed that combining data sets might be informative in studies in which either morphology or molecular data was each weak in clarifying different clades.

In this research study, individual specimens of *Drosera indica* will be subjected to assessments of morphological and genetic variability. The morphological variation will be assessed based on vegetative and floral characters, and micromorphology of seeds under scanning electron microscope (SEM). The genetic variability will be examined by DNA fingerprinting using random amplified polymorphic DNA (RAPD). These analyses will then be compared with one another to look for congruence in the patterns, which hopefully reflect morphs that are both morphologically and genetically distinctive.

Chapter Three

Seed Morphology of *Drosera indica* L.

3.1 Abstract

Seed morphology from 74 samples of *Drosera indica* was examined using a scanning electron microscope. Thirteen morphological characters were observed within five broad categories: seed shape, seed size, seed coat ornamentation pattern, shape of seed coat cells, and epicuticular waxes. Three seed types were defined, which provided evidence on the existence of within-species variation. Provided with some evident from other morphological data, the results suggest that there are five seed-based taxa in this species, and further study is required in order to formulate the taxonomic implications of the observed infraspecific variation.

3.2 Introduction

The importance of seed morphological characters in systematic studies has been the subject of investigation for decades. Davis and Heywood (1963) pointed out that seed morphological characters can be used for comparative or interpretative purposes in systematic studies. In many instances these characters have proven to be useful in verifying or resolving taxonomic positions and relationships (Sharma *et al.*, 1977; Rejdali, 1990; Sulaiman, 1995; Runyeon and Prentice, 1997; Koul *et al.*, 2000). Barthlott (1984) stated that differences in seed surface characters reflect both genetic and

phylogenetic differences. This valuable taxonomic attribute is mainly due to the conservative nature of seed surface characters (Whiffin and Tomb, 1972), and their apparent stability from environmental influence (Barthlott, 1984).

Previous studies indicate that seed morphological characters are often species-specific (Canne, 1979; Chance and Bacon, 1984; Juan *et al.*, 1994; Sulaiman, 1995; Koul *et al.*, 2000). In relation to this, Matthews and Levins (1986) point out the need to include seed coat morphological characters as a part of the description of a species. Despite having such considerable importance, however, seed characters are still far from being explored to their full extent, and this is in part due to the lack of suitable descriptive terminology (Barthlott, 1981; Rejdali, 1990).

The application of the scanning electron microscope (SEM) to seed morphology and ultrastructure revealed the potential use of these characters in resolving taxonomic problems (Sulaiman, 1995; Koul *et al.*, 2000). The use of SEM photomicrographs in obtaining morphological data in systematic studies is particularly important for the observation of small plant organs such as seeds (Stuessy, 1990; Sivarajan, 1991). SEM enables micromorphological features to be used as realistic and practicable taxonomic characters (Stace, 1989).

Most systematic studies on seed morphological characters have been at the generic level, such as those by Chuang and Heckard (1972), Canne (1979), Clark and Jernstedt (1978), Wofford (1981), Chance and Bacon (1984),

Matthews and Levins (1986), Rejdali (1990), Juan *et al.* (1994), Sulaiman (1995), Watanabe *et al.* (1999), and Bonilla-Barbosa *et al.* (2000). Studies at the family level (Whiffin and Tomb, 1972; Webb and Simpson, 1991; Chuang and Constance, 1992; Hufford, 1995; Juan *et al.*, 2000) and species level (Danin *et al.*, 1978; Wyatt, 1984; De Leonardis *et al.*, 1996) are less common. These studies indicate that seed morphology provides useful taxonomic information at any level of the systematic hierarchy.

The potential taxonomic value of seed morphological characters has been demonstrated in many taxa, ranging from familial to infraspecific level. Taxonomic implications derived from seed morphology at the familial level can either define or confirm the existence of tribes or sub tribes, and elucidate relationships among genera (Whiffin and Tomb, 1972; Juan *et al.*, 2000; Koul *et al.*, 2000). At the generic level, seed morphology has been proven to be useful in formulating taxonomy on sectional and subsectional ranks (Chuang and Heckard, 1972), and in the delimitation of subgenera (Bonilla-Barbosa *et al.*, 2000) and species (Chuang and Heckard, 1972; Clark and Jernstedt,1978; Wofford, 1981; Matthews and Levins, 1986; Sulaiman, 1995). In addition, it has been reported that seed morphological features are suitable for the identification of species (Juan *et al.*, 1999; Koul *et al.*, 2000), especially when other taxonomic information is lacking or contradictory (Clark and Jernstedt, 1978). At the specific level, seed morphology has been reported to be useful in distinguishing cultivars (Linskens *et al.*, 1977) and subspecies (Danin *et al.*, 1978).

In most cases, the grouping of taxa based on seed characters is in agreement with other characters (Webb and Simpson, 1991; Watanabe *et al.*, 1999; Koul *et al.*, 2000). For example, agreement between seed morphology and fruit morphology highlighted the need to reconsider the inclusion of genus *Ackama* within *Caldcluvia* in the family Cunoniaceae (Webb and Simpson, 1991). Watanabe *et al.* (1999) in their study on genus *Petunia,* found that seed morphology revealed the existence of three groups of species, and that this grouping related to the floral characters and crossability between the members of the groups. Similarly, Koul *et al.* (2000) suggested that the grouping of species in three subtribes of *Brassica* was consistent with earlier morphological, cytological, and molecular studies.

Another significant point revealed from studies on seed morphology is that taxonomic affinity deduced from seed morphology often shows conformity with that inferred from other data sets. Gopinathan and Babu (1985) found that the relationships between four species of *Vigna,* implied from seed morphology, was in accordance with relationships inferred from other morphological characters, the pattern of genetic variability, and isozymes. In a study on three species of *Cicer*, De Leonardis *et al.* (1996) reported that seed morphology supported karyological affinities, confirming the progenitor-derivative relationships of the taxa under study. Seed morphological data has also been reported to be in agreement with RAPD analysis in confirming the hybrid status of the taxon under study (Kumar *et al.*, 1999).

Some studies have suggested that seed morphology is related to adaptive significance to habitat preferences and dispersal (Chuang and Heckard, 1972; Clark and Jernstedt, 1978; Wyatt, 1984; Matthews and Levins, 1986). In this regard, Clark and Jernstedt (1978) reported evidence of seed coat ornamentation as an adaptation to dispersal by run-off water in genus *Eschscholzia*. In a study of infraspecific variation in seed morphology of *Arenaria uniflora*, Wyatt (1984) also found that variation in seed coat morphology was correlated to the habitat, with different characteristics of seeds was observed between plants from dry and wet sites.

The published literature on seed morphology refers mainly to families which are of economic importance, particularly the weedy and cultivated taxa, such as Leguminosae (Linskens *et al.*, 1977; Sharma *et al.*, 1977; Gopinathan and Babu, 1985; Chernoff *et al.*, 1992), Caryophyllaceae (Wofford, 1981; Wyatt, 1984), Scrophulariaceae (Chuang and Heckard, 1972; Canne, 1979; Juan *et al.*, 1994; Juan *et al.*, 2000), and Brassicaceae (Mulligan and Bailey, 1976; Koul *et al.*, 2000). Information on seed morphological characters in the family Droseraceae occurs in some floras (van Steenis, 1953; Ohwi, 1965; Obermeyer, 1970; Marchant *et al.*, 1982; Wheeler, 1992) as well as the study of seed development by Boesenwinkel (1989).

The micromorphology of seeds of *Drosera* has been reported as showing variations on the shape and pattern of exotestal cells and epicuticular waxes (Boesenwinkel, 1989). Moreover, based on the structural analysis of ovule and seed development, it is concluded that *Drosera* is the most advanced

genus in the family Droseraceae as indicated by very small and derived ovules and seeds, and the simple seed coat compared to the other three genera (Boesenwinkel, 1989; Gregory, 1998).

Structural features of *Drosera* seed, particularly the nature its of integument, were described by Corner (1976), however, there was no description of the ornamentation patterns, and to date there are no specific publications detailing the seed morphology of *D. indica*. The morphological features of the seed of this species commonly cited in floras are the seed shape and reticulate ornamentation pattern without any further detail on the infraspecific variation.

In this study the scanning electron microscope was used to determine the contribution of seed morphology in defining the infraspecific morphological variability noticed in *Drosera indica* L.

3.3 Materials and Methods

3.3.1 Materials

Seeds for this study were obtained from field collections of natural populations, herbarium specimens, and the seed bank of the Australian Carnivorous Plant Society (ACPS). Seeds from field collections were made available by courtesy of Mr. Allen Lowrie and Dr. John Conran. The 74 samples and their details used in this study are listed in Appendix I.

3.3.2 Methods

Seeds were cleaned of dust with a fine brush, mounted on the stubs using double-sided adhesive tape, and coated with gold-carbon in a vacuum evaporator. The examination of seed morphology was carried out using Philips XL-20 SEM at 10kV. In most samples ten seeds were examined, except those with few seeds and for which only three seeds were used. The thirteen morphological characters examined are listed in Table 3.1. The terminology of seed morphological characters follows Moore and Webb (1978), Stearn (1983), and Barthlott *et al.* (1998).

Table 3.1 Seed morphological characters examined

Code	Character	Units or states
SES	Seed shape	ovoid; ovoid-ellipsoid; ellipsoid
SEO	Seed coat ornamentation pattern	reticulate; foveolate; furrowed
SEL	Seed length	μm
SEW	Seed width	μm
PSH	Pore shape (outline of the seed coat cells)	elliptic; tetragonal; transversely hexagonal; longitudinally hexagonal; rectangular
NPL	Number of pores per seed length	count
NPW	Number of pores per seed width	count
PCU	Pore curvature (curvature of outer periclinal wall).	flat; concave; deeply concave
PFS	Pore floor sculpturing	smooth; undulate; wavy
SLR	Longitudinal ridges on the seed surface	absent; present
POL	Average pore length	μm
POW	Average pore width	μm
ECW	Epicuticular wax	rounded granules; irregular granules; rosettes

3.4 Results

The results of examination on seed morphological characters are listed in Appendix II. The seed of *Drosera indica* is apiculate, and varies from ovoid to ellipsoid. While a number of samples display one particular seed shape (ovoid or ellipsoid), almost half of the samples (34 out of 76) show a mix between the two, and therefore they are defined as having ovoid-ellipsoid seed shape. Seed length ranged from 262 to 912 μm.

Three classes of ornamentation pattern were identified: reticulate (Figure 3.1), foveolate (Figure 3.2 A), and longitudinally ridged or furrowed (Figure 3.2 B). There was no variation in the shape of seed coat cells in the foveolate and longitudinally ridged types. The shape of seed coat cells was elliptic and concave in the foveolate pattern, whereas the longitudinally ridged pattern was composed of rectangular and deeply concave (furrowed) cells. In contrast, reticulate patterned seeds showed variation in the shape of seed coat cells. Three different cell shapes and orientation were noted: tetragonal (Figure 3.1 A); transversely hexagonal (Figure 3.1 B); and longitudinally hexagonal (Figure 3.1 C).

Based on seed coat ornamentation patterns, three types of seed were recognised. Type I was the seed with reticulate ornamentation composed of either tetragonal or hexagonal cells, with seed shape varying from ovoid to ellipsoid. Regarding the variation in the shape of seed coat cells found in this type, each of these variants was designated as a subtype of Type I.

Seeds included in Type II were characterised by foveolate ornamentation, with seed coat cells that were elliptic, concave, and arranged in parallel lines along the seed length. Seed shape of this type varied from ovoid to ellipsoid.

Type III consisted of seeds with a pattern of longitudinal ridges or furrows, formed by rectangular and deeply concave cells, with a seed shape that was uniformly ellipsoid. The grouping of the seed samples into their types and subtypes is summarised in Table 3.2.

Table 3.2 Grouping of seed samples based on seed coat ornamentation patterns

Seed type	Samples
Type I subtype tetragonal	AL 261, AL 1108, AL 1164, AL 1694, AL 2225, AL 2242, AL 2299, AL 2302, AL 2525, FR s.n. 'A', Dro 24.
subtype transversely hexagonal	AL 962, AL 1031, AL 1322, Al 1352, AL 1697, AL 2216, AL 2219, AL 2461, CR, DEM 1812, FR s.n. 'B', JGC 738B, Dro 17, Dro 39, Dro 42, Dro 53.
subtype longitudinally hexagonal	AL 976, AL 1103, AL 1172, AL 1193, AL 1195, AL 1208, AL 1734, AL 2462, Dro 22, Dro 29, Dro 34.
Type II	AL 84, AL 1036, AL 1113, AL 1162, AL 1173, AL 1283,, AL 1284, AL 1289, AL 1325, AL 1716, AL 1749,, AL 2206, AL 2220, AL 2226, AL 2227, AL 2243,, AL 2249, AL 2255, AL 2273, AL 2304, AL 2508, AL 2542, JGC 738A, JGC 739, Dro 14, Dro 21, Dro 36, Dro 38.
Type III	AL 965, AL 1200, AL 1215, AL 1318, AL 1730, AL 2523, AL 2526, Dro 23.

In all seed samples it was apparent that the seed coat was covered by epicuticular wax deposits which varied in form and quantity. However, there was high variability in wax particle forms observed among the seeds from any one sample (Figure 3.3), so that no distinct wax particle characters could be defined. As a result, seed epicuticular wax characters were not included in this study.

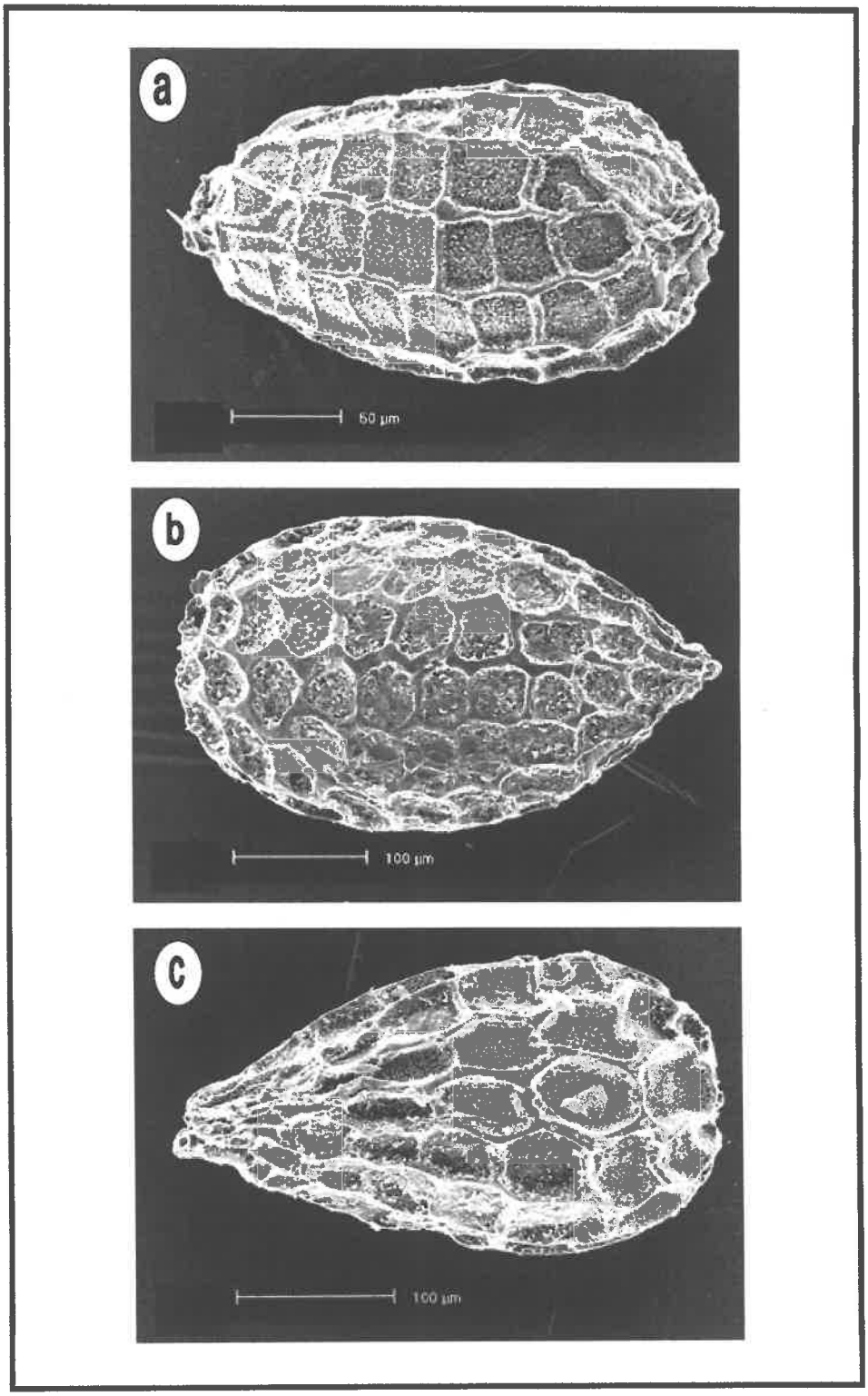

Figure 3.1 Scanning electron micrograph of *Drosera indica* seeds with reticulate ornamentation pattern (Type I), showing three different seed coat cell shapes:
a. tetragonal (AL2242); b. transversely hexagonal (AL2219); c. longitudinally hexagonal (AL1208).

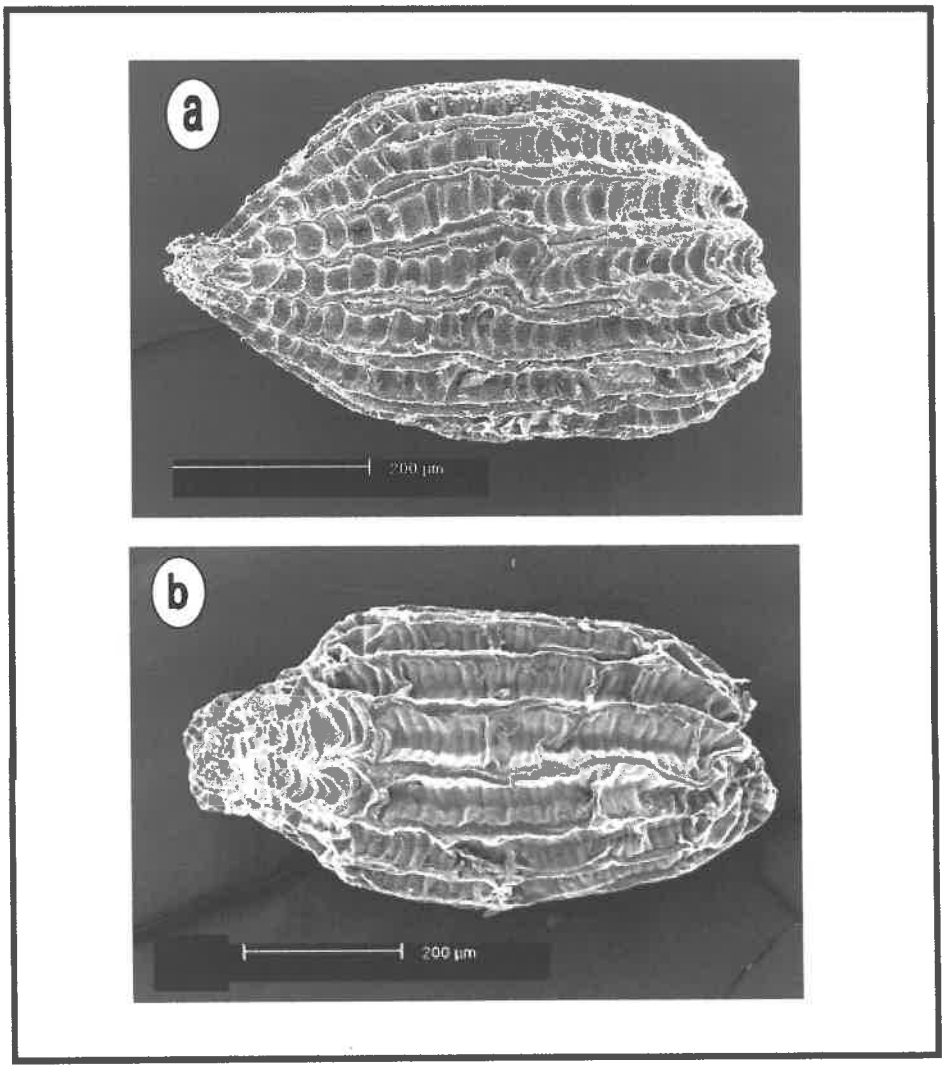

Figure 3.2 Scanning electron micrograph of *Drosera indica* seeds with (a) foveolate (Type II) ornamentation (AL1306), and (b) longitudinally ridged or furrowed (Type III) ornamentation (AL1730).

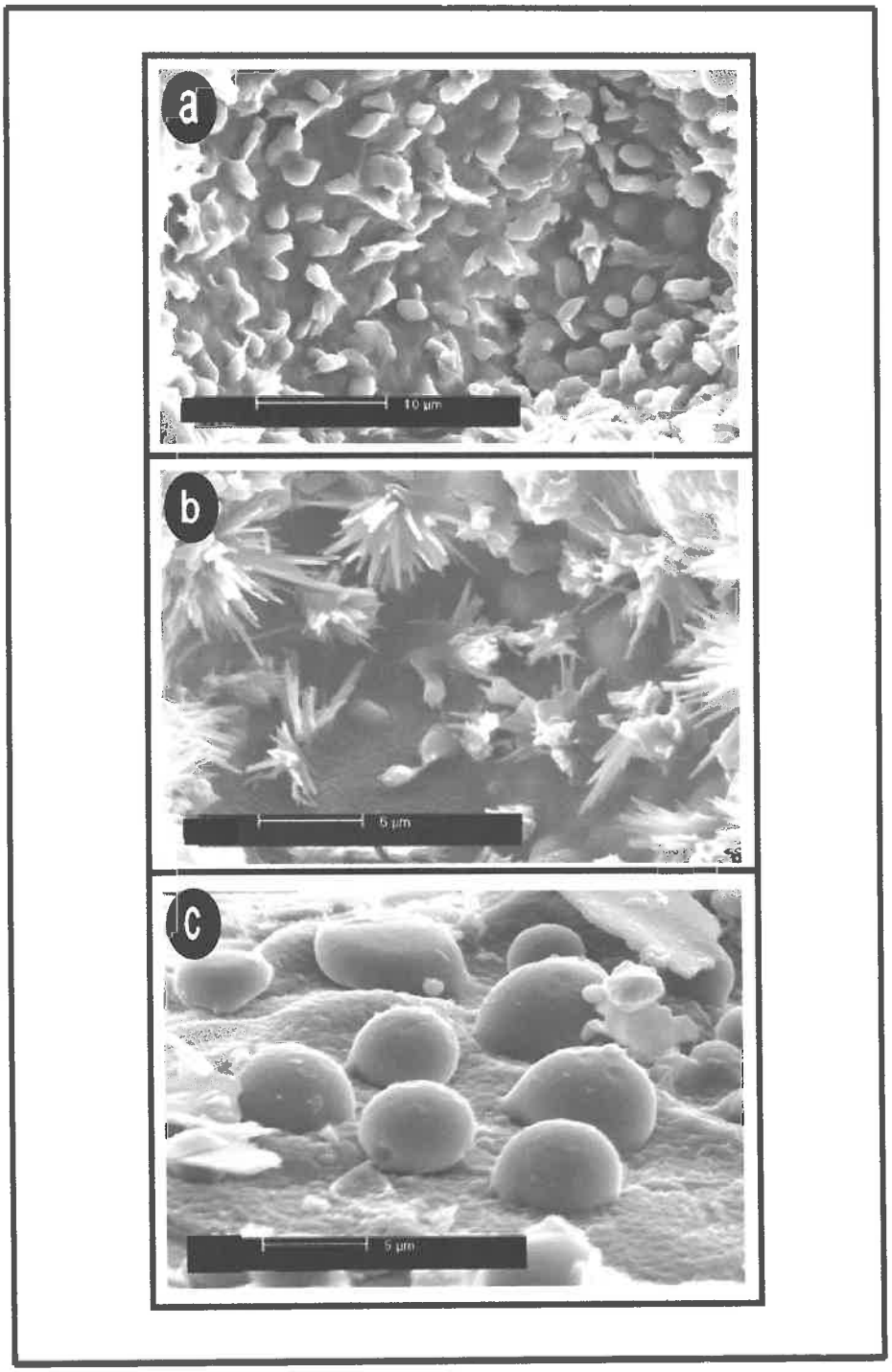

Figure 3.3 Scanning electron micrograph of epicuticular wax deposits on the surface of the seed coat, showing: (a). irregular granules ; (b). rosettes; (c). rounded granules. All forms are from seeds within the same sample (Dro42).

4.5 Discussion

From the thirteen seed morphological characters examined in this study, it was apparent that each character provided a different level of contribution toward delimitation of infraspecific variation in *D. indica*. SES, SEW, NPW, and POW were of least value in distinguishing seed types, as these characters varied within most samples, except for those seed samples classified as Type III, where all samples exhibited a uniformly ellipsoid seed shape. Seed length was distinctive, particularly for seeds from Type III which were characterised by seed length greater than 600 μm, and Type I, where with few exceptions, the seeds were mainly 240 – 400 μm long. SLR and NPL were also distinctive characters, with SLR absent in Type I, and NPL greater in Type II (16-26) than that in Type I (6-13, except for AL 261) and Type III (9-12).

PFS and PCU represented characters where one state was unique to a seed type, and the other shared by the other two types. In this case, PFS was wavy in Type III, while Type I and II exhibited either smooth or undulate features. In the same way, PCU uniquely distinguished Type III with deeply concave pore curvature, whereas Type I and II displayed either flat or concave pore curvature.

Seed coat cell shape (PSH) was associated with seed coat ornamentation pattern (SEO). Elliptic, concave cells were characteristic of seeds of Type II, rectangular and furrowed cells were distinctive for seeds of Type III, and tetragonal, and two variants of hexagonal, characterised seeds of Type I. Because differences in the shape of seed coat cells was a direct consequence of

the differences in seed coat ornamentation pattern, the taxonomic contribution of the shape of seed coat cells cannot be assessed independently. This was also the case on POL, in which its close association with SEO made its contribution cannot be evaluated independently.

Seed coat ornamentation pattern, on the other hand, clearly showed its significant contribution in the grouping of seeds of this species. In other words, seed coat ornamentation pattern displayed a taxonomic value as a diagnostic character. This result is in agreement with previous findings on the systematic value of seed coat ornamentation pattern (sculpture) in defining infraspecific variation (e.g. Rejdali, 1995; Koul *et al.*, 2000).

Scanning electron microscopy revealed that the seed surface of *D. indica* was covered with different forms of epicuticular wax deposits (Figure 3.3), agreeing with Boesenwinkel (1989), that epicuticular waxes in *Drosera* seeds varied in the shape and pattern. However, detailed examination of the micromorphology of epicuticular waxes from the 74 samples used in this study indicated that the form of the wax particles showed high inter- and intra-sample variability. Due to the high degree of variation observed, it was not possible to discern unique epicuticular wax characters and/or states for sample groups. Therefore the character of epicuticular waxes was not included in the grouping of seed types.

Seed morphology in *D. indica* was not related to the geographical distribution of the samples, and in most cases different samples collected from the same

locality possessed different seed types. An example of this was shown by the samples JGC 738A and JGC 738B. These two plants, collected from the same location, had the same flower colour but differed markedly in plant size. Scanning electron microscopy revealed that seeds of JGC 738A belonged to Type II whereas JGC 738B was of Type I, subtype transversely hexagonal. Another example of plants growing at the same location, but exhibiting different seed types was shown by the samples AL 2225, AL 2226, and AL 2227. These plants were collected from the same locality, as indicated by their geographic position (15° 176' 19" S; 128° 37' 07" E), but whereas AL 2225 displayed Type I seeds, the other two samples exhibited Type II seed morphology.

Drosera indica showed evidence of infraspecific variation in morphological characters such as plant size, plant colour, petiole feature, and flower colour as noted on their collection details (Appendix I). Two herbarium specimens exhibiting these morphological variations are shown in Appendix III. The recognition of three types of seed from SEM examination provides additional evidence for the existence of infraspecific variation in this species. The systematic significance of seed morphology in providing evidence for the identification of infraspecific variation has been suggested from studies on various plant taxa (Danin *et al.*, 1978; Hauptli *et at.*, 1978; Wyatt 1984). In the case of *D. indica*, the indication of variability on seed morphology, especially the seed coat ornamentation pattern, has emerged only recently (Lowrie, 1998), with descriptions of seed morphology in early floras and publications recognising only reticulate ornamentation (Diels, 1906; van

Steenis, 1953; Ohwi, 1965; Obermeyer, 1970). However, both of Diels (1906) and van Steenis (1953) described the seed coat ornamentation in *D. indica* as 'scrobiculate' (pitted or having numerous small shallow depressions or excavation), which was characteristic of the seed of Type I (reticulate) as defined here. A drawing in Diels (1906) clearly showed a reticulate pattern of the seed coat. Ohwi (1965), in *Flora of Japan,* characterised the seed coat ornamentation pattern as "longitudinal ribs with obsolete cross lines", but unfortunately there was no picture presented in this publication. This pattern seems to correspond to Type I subtype tetragonal, the seed type displayed by the specimen from Japan (FR s.n. 'A'). Obermeyer (1970), on the other hand, identified the seed coat of *D. indica* as having an ornamentation pattern that was coarsely reticulate. Recently, the existence of more than one seed type in *D. indica* has been identified by Lowrie (1998) where he figured the seed as deeply reticulate, but then noted that seeds from one sample collected from the Kimberley, Western Australia were of a larger size and different surface sculpture compared with the other samples seen by him.

Plants with seeds of Type III consistently shared a number of other morphological characters. All plants were green, had petiolate leaf bases, and had generally white or rarely pale pink flowers. The stamens in these plants were red to blood red, and showed a hooded, cobra-like form. The seeds of the eight samples seen representing this group were ellipsoid in shape, and larger than the other types (seed length ranged from 616 to 912 μm). The fact that this group of plants exhibits a repeated pattern of seed morphology and

other morphological characters not shared by the other samples, it strongly suggests that this group needs recognition as a distinct taxon.

Another group of plants that merit recognition as a distinct taxon is those with seeds Type II. All plants in this group had green stems and petiolate leaves. This group, however, obviously consisted of two smaller subgroups, differentiated by filament forms: one subgroup with deltoid filaments, and another one with filiform filaments. Examination on other morphological characters did not produce additional characters to support differentiation of these two subgroups. Based on this fact, therefore, at this stage it seems reasonable to keep this group of plants as a single seed-based taxon.

Unlike those that comprised groups of foveolate and longitudinally ridged seeds, accessions with reticulate seed type did not form a solid group, indicating by the occurrences of some deviating individual accessions. Most of the accessions belonged to Type I subtype tetragonal had red stems, filiform filaments, and leaves with tentacles on both upper surface and margin of the lamina. The deviating individuals in this group were AL 261, AL 2242, and FR s.n. 'A' because they had green stems. Moreover, AL 261 only had tentacles on the upper surface of the leaves, and AL 2242 had deltoid filaments. Type I subtype transversely hexagonal comprised of individuals with green stems, apetiolate leaves, and filiform filaments, except accessions FR s.n. 'B', Dro 39, and AL 1031. Accessions FR s.n. 'B' and Dro 39 had petiolate leaves, while AL 1031 has deltoid filaments. Type I subtype longitudinally consisted of individuals with small-sized seeds and red stems

(except AL 1195). Two filament forms (filiform and deltoid) were found in this group. Like in the case of Type II seeds, which was no additional character found to support separation of the two subgroups with different filament forms, this group was considered as one seed-based taxon. Due to the indisputable differentiation between three subtypes within the reticulate seeds, and that each of the subtype can be related to other morphological characters, it is obvious that there are three distinct seed-based taxonomic entities within Type I.

4.6 Conclusion

Seed morphology showed a taxonomic value in providing evidence on the existence of infraspecific variation in *D. indica*. Three seed types (one of them with three subtypes) were recognised, and this differentiation was mainly based on seed coat ornamentation patterns. Considering the fact that there was an apparent consistency between seed morphology and other morphological characters within seed types and subtypes, it could be argued that *D. indica* consist of five seed-based taxa.

Chapter Four

DNA Extraction Methods for *Drosera indica* L.

4.1 Abstract

Four different protocols were compared to extract DNA from various types of materials of the carnivorous plant *Drosera indica*. None of the protocols were successful in recovering DNA from air-dried herbarium specimens. The DNA from this type of material appeared to be highly degraded and contaminated with oxidised compounds. Silica sand used to preserve plant material during field collection failed to prevent DNA degradation due to the prolonged desiccation process. The use of silica gel, however, was found to be effective, facilitating rapid desiccation for DNA preservation in plant materials. DNA from fresh and water-preserved leaf tissue, and silica gel-dried tissue was successfully extracted with DNA of high molecular weight and adequate quality obtained using the technique of Bekesiova *et al.* (1999).

4.2 Introduction

In studies employing DNA-based assays, the availability of intact, high quality DNA in sufficient amount is an essential prerequisite. The use of PCR, which enables amplification from nanograms of DNA, eliminates the requirement for large amounts of DNA. However, the requirement for the DNA to be of high purity, and free from contaminating compounds that interfere in subsequent enzymatic reactions is a crucial starting point for the

subsequent DNA analysis. In this regard, even for techniques that were believed to be tolerant to low quality DNA, such as RAPD, the availability of intact and high quality DNA is a substantial factor affecting the success of the experiment and the reproducibility of the results. The process responsible for this is the DNA extraction method.

DNA extraction procedures generally consist of four steps: tissue disintegration, cell disruption, nucleic acid purification, and DNA precipitation. The most common method used for tissue disintegration is to grind the tissue, either in liquid nitrogen or in an extraction buffer. The following step is cell disruption, which is accomplished by adding a detergent in high concentration, facilitating the lysis of cell membranes. This step allows the release of nucleic acid into the extraction buffer. Antioxidants and Mg^{++} chelating agents are usually included in this step to prevent phenolic oxidation and nucleic acid degradation, respectively. The nucleic acid is then purified from the unwanted substances, such as plant pigments and protein, by the addition of an organic solvent, such as chloroform or phenol. Additional purification steps are usually performed to eliminate contaminating polysaccharides. RNA can be removed from the nucleic acid preparation by digestion with RNase A. After the removal of those undesirable substances, the purified DNA is then precipitated using either isopropanol or ethanol.

Various protocols for plant DNA extraction have been published since the discovery of plant genome analysis in molecular biology. All these protocols

have a common aim in obtaining DNA of high yield, high molecular weight, and high quality. The most widely used protocol is based on the hot CTAB (hexadecytrimetyl ammonium bromide) method (Murray and Thompson, 1980), from which some modifications were made. The purpose of the modifications is to find an efficient method for extracting DNA that is applicable for various plants, especially the recalcitrant plant species, from which the pure DNA preparation is notoriously difficult to obtain due to the high content of polyphenolics and polysaccharides (Baker *et al.*, 1990; Maliyakal, 1992; Lodhi *et al.*, 1994; Maguire *et al.*, 1994; Kim *et al.*, 1997; Peterson *et al.*, 1997; Porebski *et al.*, 1997; Tel-Zur *et al.*, 1999). These two compounds have been recognised as the most common contaminants in DNA preparations (Loomis, 1974; Newbury and Possingham, 1977; Murray and Thompson, 1980; Katterman and Shattuck, 1983; Fang *et al.*, 1992; Rogers and Bendich, 1988; Do and Adams, 1991; Manning, 1991).

Contaminating polyphenolics and polysaccharides that co-precipitate with, and are difficult to separate from, DNA have been known to inhibit the activity of enzymes used in molecular biology procedures. They have been known to inhibit ligase, restriction endonuclease, and polymerase enzymes resulting in unrestrictable and unamplifiable DNA (Fang *et al.*, 1992; Lodhi *et al.*, 1994; Mannerlof and Tenning, 1997). Despite inhibiting enzyme activity, this contamination could be interfering in the quantification of DNA using UV absorbance-based techniques (Gilmore *et al.*, 1993).

There have been many studies conducted to overcome the problem of low quality DNA due to phenolics and polysaccharides. The addition of antioxidation agents and phenolic-binding compounds, such as polyvinylpyrrolidone (PVP), β-mercaptoethanol, sodium metabisulfite, diethyldithiocarbamate and activated charcoal, have been reported to eliminate polyphenolics during the DNA extraction process (Hattori *et al.*, 1987; Manning, 1991; Maliyakal, 1992; Vroh Bi *et al.*, 1996; Peterson *et al.*, 1997). It was claimed that the oxidation product of phenolic compounds that co-precipitate with the DNA was, in part, responsible for reducing the purity of DNA preparations. Bendich *et al.* (1979) reported that in the case of diethyldithiocarbamate, the mechanism for preventing phenolic oxidation is that this antioxidation agent will compete for copper with the oxygen needed for the activation of phenoloxidase enzyme. In the case of polysaccharides, various purification agents have been added in the DNA extraction procedures in an attempt to overcome the interfering polysaccharides. This includes CsCl density gradient centrifugation (Bendich *et al.*, 1979; Murray and Thompson, 1980), Elutip-d (RPC-5 type resin) and phenol/chloroform wash (Do and Adams, 1991), high salt precipitation (Manning, 1991), and affinity chromatography-based purification using column concanavalin A linked to Sepharose (Edelman, 1975).

DNA extraction from *Drosera indica* is particularly difficult due to the presence of phenolic compounds and mucilaginous polysaccharides. The major phenolic compound found in this species is plumbagin (Zenk *et al.*, 1969), a naphthoquinone known as having antispasmatic and antibiotic

activity (Perica and Berljak, 1996). The presence of this phenolic compound can be observed from the brownish colour of the DNA that precipitates during the extraction process. The second source of contamination in the DNA of *D. indica* is acidic polysaccharides from the mucin secreted by talked glands on the leaf surface. Based on the observation of *D. capensis* and *D. binata*, Rost and Schauer (1977) reported that the mucin secreted from sundews was acidic (pH 5), high molecular weight polysaccharides that consisted of xylose, mannose, galactose, glucuronic acid, and ester sulphate. The acidic polysaccharides were reported as being more inhibitory to the restriction enzyme (Do and Adams, 1991) and to the PCR reaction (Demeke and Adams, 1992) than the neutral ones.

In the present study, three different kinds of material were available for DNA analysis of *Drosera indica*: fresh, preserved, and air-dried herbarium leaves. While the first two materials could be extracted using the protocol of Bekesiova *et al.* (1999) and Wang *et al.* (1996), DNA extraction from herbarium specimens was found to be more difficult. Facing this difficulty, the herbarium specimens were subjected to four DNA extraction protocols.

4.3 Materials and Methods

4.3.1 Plant material

Fresh leaf tissue for DNA extraction was obtained from tissue culture and greenhouse-grown plants. Both groups of plants were produced from seeds collected from different populations around Australia, and one sample from Taiwan. Materials preserved either in water (i.e. live plants collected into

vials of water and posted before they degraded) or silica sand (Moisture-Gone™) were provided by Mr. Allen Lowrie, who also supplied the herbarium specimens. A total of 38 samples, consisting of 15 fresh, 19 preserved and 4 air-dried herbarium samples were used in this study (Table 4.1).

Table 4.1 List of materials used in this study

Collection	Locality	Type of material
dro14	east of Drysdale River, Kimberley, WA	fresh, tissue culture
dro17	Merry Creek, Kimberley, WA	fresh, tissue culture
dro21	Honeymoon Beach, Kimberley, WA	fresh, tissue culture
dro22	Barnett Station, Kimberley, WA	fresh, tissue culture
dro23	Barnett Station, Kimberley, WA	fresh, glasshouse
dro24	Gibb River, Kimberley, WA	fresh, tissue culture
dro29	Grevillea Creek, Kimberley, WA	fresh, tissue culture
dro34	Beverley Springs, WA	fresh, tissue culture
dro36	Cairns, Queensland	fresh, tissue culture
dro38	Darwin, NT	fresh, tissue culture
dro39	Darwin, NT	fresh, tissue culture
dro42	Taiwan	fresh, glasshouse
dro53	Kimberley, WA	fresh, glasshouse
JGC738A	500 m east of Fogg Dam, NT	fresh, tissue culture
JGC738B	500 m east of Fogg Dam, NT	fresh, tissue culture
AL2461	Crossing Falls, Kimberley, WA	silica sand-preserved
AL2462	Crossing Falls, Kimberley, WA	silica sand-preserved
AL2470	Yampi Ren, Kimberley, WA	water-preserved
AL2471	11 km north east of Keriwell Yard, Kimberley, WA	water-preserved
AL2487	Yampi Ren, Kimberley, WA	water-preserved
AL2504	Yampi Ren, Kimberley, WA	silica sand-preserved
AL2505	Yampi Ren, Kimberley, WA	water-preserved
AL2507	Yampi Ren, Kimberley, WA	water-preserved
AL2508	Yampi Ren, Kimberley, WA	water-preserved
AL2509	Yampi Ren, Kimberley, WA	water-preserved
AL2511	15 km from Derby, WA	water-preserved
AL2512	15 km from Derby, WA	water-preserved
AL2522	Phillips Range, Kimberley, WA	silica sand-preserved
AL2523	Phillips Range, Kimberley, WA	water-preserved
AL2524	Phillips Range, Kimberley, WA	water-preserved
AL2525	Adcock Creek, Kimberley, WA	water-preserved
AL2526	Adcock Creek, Kimberley, WA	water-preserved
AL2531	Bamboo Creek, Kimberley, WA	water-preserved
AL2542	Lily Creek, Kimberley, WA	water-preserved
AL1322	Merry Creek crossing, Mt. Elizabeth Station, Kimberley, WA	air-dried herbarium specimen
AL2219	about 129 km west of Fitzroy Crossing, Kimberley, WA	air-dried herbarium specimen
AL2242	Cave Spring, Kununurra, WA	air-dried herbarium specimen
DEM1812	80 km north of Tennant Creek, NT	air-dried herbarium specimen

4.3.2 Sample preparation

Sample preparation procedures were applied to the preserved and herbarium materials, whereas the fresh tissue from tissue culture and glasshouse-grown plants could be used directly for DNA extraction without any special treatment. Upon arrival at the laboratory, the water-preserved material was drained on absorbent paper. The leaves then were cleaned of trapped insects and dirt. The samples were then placed in a zip-lock plastic bag and frozen in liquid nitrogen. The leaves from silica sand-preserved materials were cleaned in the same way as the water-preserved ones. These leaves were then placed in a zip-lock plastic bag containing silica gel to foster the desiccation process.

The cleaning procedure for the air-dried herbarium specimens was done in two steps. First, the leaves were cleaned of trapped insects under a magnifier lamp (3x magnification). Glandular hairs were removed when necessary. Secondly, the cleaned leaves were checked under a compound dissecting microscope with 40x magnification to ensure that there were no traces of insect tissue or foreign material attached.

4.3.3 DNA extraction protocols

Four DNA extraction protocols were performed in this study. The procedure of Steenkamp *et al.* (1994) was used after scaling down to facilitate the extraction of DNA from milligrams of material. The protocols of Wang *et al.* (1996), and Bekesiova *et al.* (1999) were followed with minor modifications, while the QIAGEN® DNeasy™ Plant Mini Kit (QIAGEN Pty Ltd, Clifton Hill,

Vic.) DNA extraction procedure was carried out according to the manufacturer's protocol.

A. Modified Steenkamp *et al.* (1994) protocol:

Twenty mg of air-dried leaf was placed in a 2 mL Eppendorf tube and frozen in liquid nitrogen. The tissue was then ground into a fine powder using a stainless steel rod. The tissue powder was kept in liquid nitrogen until all samples were ground. Then 700 µL of extraction buffer [3% (w/v) CTAB, 1.4 M NaCl, 20 mM EDTA, 1.0 M Tris-HCl, pH 8.0], 1.5 mg of sodium metabisulphite and 1.5 mg of PVP-40T was added. The mixture was incubated at 60°C for 30 min during which time the tubes were inverted every 10 minutes. The same volume (700 µL) of chloroform / isoamylalcohol (24:1) was added, and mixed gently for 10 min at room temperature. The mixture was centrifuged at 8000 rpm for 20 min at room temperature, and the aqueous phase was transferred to a sterile 1.5 mL Eppendorf tube. 0.66 volume of ice cold isopropanol was added, the mixture was mixed gently and then incubated on ice for 20 min. The DNA was pelleted by centrifuging the mixture at 8,000 rpm for 20 min. The DNA pellet was then washed in 1.2 mL of wash buffer (76% ethanol, 10 mM NH_4Ac) for 30 min and dissolved in 100 µL of TE buffer (10 mM Tris HCl, 0.1 mM EDTA, pH 8.0). RNA was removed by adding 0.5 µL of DNase-free RNase A (Amresco®, Solon, Ohio) (10 mg/mL) and incubating the samples at 37°C for 30 min. After cooling, 200 µL of TE buffer and 100 µL 7.5 M NH_4Ac were added, and the mixture was left on ice for 20 min at 4°C, then centrifuged at 14,000 rpm for 20 min. The aqueous phase was transferred to a sterile 1.5 mL Eppendorf tube, and 1 mL of cold

ethanol was added. The mixture was incubated on ice for 10 min, and then centrifuged at 8,000 rpm for 20 min at 4°C. The pellet was recovered, and washed with 100 μL of wash buffer and then centrifuged at 8,000 rpm for 5 min. The washing procedure was repeated using 100 μL of 70% ethanol. The DNA pellet was then dissolved in 100 μL of TE buffer.

B. QIAGEN® DNeasy™ Plant Mini Kit protocol:

Twenty mg fresh or hydrated plant tissue was ground into a fine powder in the presence of liquid nitrogen. The powder was then transferred into a 1.5 mL Eppendorf tube, and 400 μL of Buffer AP1 and 4 μL of RNase A (QIAGEN®) stock solution (100 mg/mL) were added. The mixture was vortexed vigorously, incubated for 10 min at 65°C, during which time the tubes were inverted 2-3 times and then 130 μL of Buffer AP2 was added, followed by incubation on ice for 5 min. After being centrifuged for 5 minutes at 14,000 rpm, the supernatant was applied to the QIAshredder spin column sitting in a 2 mL collection tube, and then centrifuged for 2 min at maximum speed. The flow-through fraction was then transferred to a new tube without disturbing the cell-debris pellet. Half volume of Buffer AP3 and one volume of 100% ethanol were added to the lysate and mixed by pipetting. Then 650 μL of the mixture (including any precipitate that may have been formed) was applied to the DNeasy mini spin column sitting in a 2 mL collection tube. The mixture was then centrifuged for 1 min at 8000 rpm and the flow-through was then discarded. This step was repeated with the remaining sample. In the next step the DNeasy column was placed in a new 2 mL collection tube, and 500 μL Buffer AW was added to the column. After centrifuging the

mixture for 1 min at 8,000 rpm, the resultant flow-through was discarded. Using the same collection tube from the previous step, the washing was repeated by adding 500 µL Buffer AW to the DNeasy column and the mixture was centrifuged for 2 min at maximum speed to dry the membrane. The DNeasy column was then transferred to a 1.5 mL Eppendorf tube and 100 µL of preheated (65°C) Buffer AE was added directly to the DNeasy membrane. The column was incubated for 5 min at room temperature and then centrifuged for 1 min at 8000 rpm to elute. The elution step was repeated once as described.

C. Wang *et al.* (1996) protocol:

Twenty mg of silica gel- and silica sand-dried leaves were ground in a mortar and pestle together with 42 mg PVP powder. The powder was then transferred into an Eppendorf tube, to which 700 µL ice-cold extraction buffer I (0.25 M NaCl, 0.2 M Tris-HCl pH 8, 50 mM EDTA pH 8) and 2% β-mercaptoethanol were added. The mixture was mixed well and incubated on ice for 10 min, then centrifuged at 7,000 rpm for 10 min at 4°C. After discarding the supernatant, 350 µL hot (65°C) extraction buffer II (100 mM Tris-HCl pH 8, 20 mM EDTA pH 8, 2% (w/v) CTAB, 1.4 M NaCl) and 0.2% β-mercaptoethanol were added to the nuclear pellet, and the mixture incubated at 65°C for 40 min. Following the addition of one volume of chloroform / isoamylalcohol (24:1), the mixture was centrifuged at 7,000 rpm for 10 min at 4°C. The aqueous phase was transferred to a new tube, and one volume of chloroform/isoamylalcohol (24:1) was added. The mixture was then centrifuged at 7,000 rpm for 10 min at 4°C. Two-third volume of ice-cold

isopropanol was added to the aqueous phase, incubated for 30 min at –20°C, and centrifuged at 10,000 rpm for 5 min at 4°C. The pellet was resuspended in 1x TE buffer (10 mM Tris HCl, 0.1 mM EDTA, pH 8.0) at room temperature. Half volume of 5 M NaCl was added and the mixture was mixed gently before adding 2 volumes of ice-cold ethanol. The nucleic acid was precipitated by incubating the mixture for 30 min at –20°C. DNA was pelleted by centrifuging at 10,000 rpm for 5 min at 4°C, washed with 70% ethanol twice, air-dried, and redissolved in 0.1x TE buffer. RNA was eliminated by adding 0.5 µL of 10 mg/mL RNase A and incubating the samples at 37°C for 30 min.

D. Bekesiova *et al.* (1999) protocol:

Fresh leaves (100 mg) or air-dried leaves (20 mg) were collected in a 2 mL Eppendorf tube and frozen in liquid nitrogen. The sample was ground into a fine powder using a stainless steel rod. Then 300 µL of Buffer I [0.35 M sorbitol, 0.1 M Tris-HCl, 5 mM EDTA (all adjusted to pH 7.5) and 0.02 M sodium bisulfite (added just before use)] and 300 µL Buffer II [(0.2 M Tris-HCl (pH 7.5), 50 mM EDTA (pH 7.5), 2 M NaCl, 2% CTAB)] were added to the powder. The mixture was shaken vigorously for a few seconds at room temperature. 120 µL of 5% sodium N-lauryl sarcosine was added and the mixture was shaken vigorously again and then incubated at 65°C for 20 min. After cooling the mixture for 5-10 min at room temperature, the lysate was extracted with 700 µL chloroform/isoamylalcohol (24:1). The mixture was then centrifuged at maximum speed for 10 min at 4°C. DNA was precipitated by adding 800 µL of precipitation mixture (8 volume of 96% ethanol, 2

volumes of 1 M sodium acetate, pH 5.2). The tube was inverted several times and left for 5 min at room temperature. The DNA was pelleted by centrifuging the mixture at 14,000 rpm for 10 min at 4°C. The pellet was then washed twice with 70% ethanol and air-dried before dissolving it in TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)]. RNA as removed by adding 0.1 volume of RNase A (10 mg/mL) and incubating the samples at 37°C for 30 min.

4.3.4 Spectrophotometry

The yield and quality of the DNA was determined using dual beam UV spectrophotometer analysis. Fifty μL of DNA sample was diluted in 1mL of sterile water and the mixture was subjected to spectrophotometry on a Shimadzu UV-160A UV-visible spectrophotometer. The UV absorbance at 260 nm (A260) was used to determine DNA concentration, while the A260/280 and A260/230 ratios were used to estimate the DNA purity from contaminating protein and polysaccharides/phenolics compounds, respectively.

4.3.5 Gel electrophoresis of DNA

Observations on DNA integrity were done by gel electrophoresis. Five μL of DNA samples and one μL of 10x loading buffer (500 μL gycerol, 150 μL 0.5 M EDTA, 340 μL sterile water, 10 μL 20% SDS, 1 mg bromophenol blue, 1 mg xylene cyanol FF) were loaded on 1% agarose and run in 1x TBE (89 mM Tris-borate, 2 mM EDTA pH 8.0) at 80 mA for 60 min. The DNA was then stained with ethidium bromide (0.5 μg/mL), and visualised under UV

illumination. The gel image was captured using "Video Capture-Composite", CAPTV Program (Tekcap V 1.0, Tekram Corporation), and saved as a JPEG file using Paint Shop Pro 5.0 (Jasc Software, Inc.).

4.4 Results

The yield of DNA extracted from fresh leaf tissue ranged from 367 μg/g to 907 μg/g fresh weight, while the A260/280 and A260/230 ratios ranged from 1.43 to 1.79 and 1.09 to 1.64, respectively. The DNA obtained from water-preserved materials had A260/280 values ranging from 1.43 to 1.91, and A260/230 from 1.07 to 1.78. These materials yielded DNA ranging from 205 μg/g to 2.8 mg/g dry weight. Silica sand-preserved leaves yielded DNA ranging from 370 μg/g to 3.2 mg/g dry weight with A260/280 and A260/230 ratios ranging from 1.43 to 1.83 and 0.63 to 1.87, respectively. The DNA yield from air-dried herbarium specimens varied considerably between different protocols. The quality of this DNA varied between protocols for the A260/230 ratios but showed a narrower range of A260/280 ratios. The spectrophotometer analysis of DNA preparations from air-dried herbarium specimens was shown in Table 4.2.

The DNA extracted from fresh leaves was intact and of high molecular weight as indicated by the presence of discrete bands on the agarose gel. In contrast, the DNA from preserved and herbarium materials showed some degree of degradation. This can be observed from the presence of smearing in the DNA preparations run on the gel. Figure 4.1. shows the DNA integrity from various source of materials. The degree of degradation varied from minor to

severe, with the latter being mainly observed on the silica sand-preserved and air-dried herbarium DNA.

Table 4.2. Comparisons of DNA yield and quality from accessions extracted using four different protocols.

Sample	Yield (μg/g DW)				Quality parameter							
					A260/A280				A260/A230			
	STK	QIA	WWZ	BNM	STK	QIA	WWZ	BNM	STK	QIA	WWZ	BNM
AL 1322	82	235	964	2,348	1.33	1.50	1.69	1.66	0.80	0.55	1.35	1.38
AL 2219	180	185	900	2,165	1.56	1.67	1.69	1.73	0.64	0.46	1.35	1.35
AL 2242	222	313	1,200	2,710	1.50	1.43	1.78	1.62	0.75	0.56	1.66	1.40
DEM 1812	256	375	429	1,966	1.11	1.15	1.64	1.43	0.63	0.48	0.92	1.15

DW: dry weight; STK: modified Steenkamp *et al.* (1994); QIA: QIAGEN® DNeasy™ Plant Mini Kit; WWZ: Wang *et al.* (1996); BNM: Bekesiova *et al.* (1999).

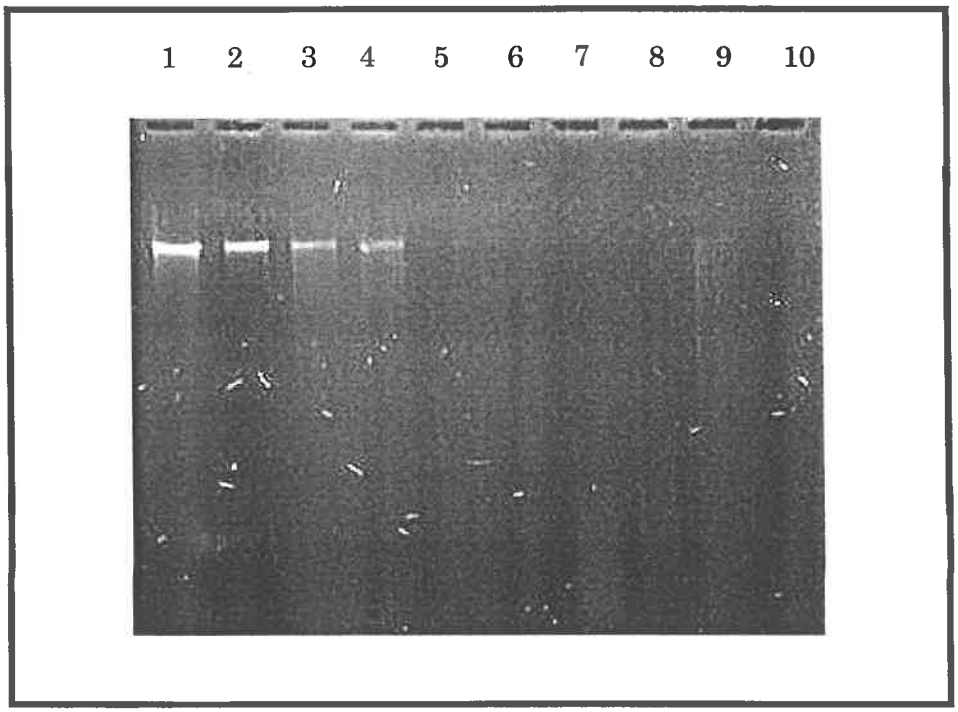

Figure 4.1 Comparison of DNA obtained from different materials. 1-2: fresh tissue; 3-4: water-preserved tissue; 5-6: silica sand-preserved tissue; 7-10: herbarium specimens.

4.5 Discussion

The results indicated that, of the four extraction protocols used in this study, the protocol of Bekesiova *et al.* (1999) produced DNA with a higher yield and better quality than the other three. Although the A260/230 ratio for some samples was still low (as low as 1.09) these DNA samples were amplifiable and generated reproducible RAPD bands (results shown in separate chapter). The success of the Bekesiova *et al.* (1999) protocol for extracting DNA from *D. indica* was brought about by the composition of the extraction buffer used. The use of sorbitol extraction buffer in the first step gives the benefit of releasing polyphenolics and polysaccharides from the tissue into the extraction buffer at the very first stage of the procedure (Štorchova *et al.*, 2000). These two compounds are the most common source of contamination in DNA preparations, which are also known to inhibit enzymatic reactions (Fang *et al.*, 1992; Lodhi *et al.*, 1994; Mannerlof and Tenning, 1997). The addition of sodium bisulphite in extraction buffer I (sorbitol extraction buffer) was aimed at preventing the oxidation of phenolic compounds released following cell disruption. The second extraction buffer (2% CTAB) resulted in the lysis of cell membranes which facilitate the release of nucleic acids from the cells. The addition of 5% sodium lauroyl sarcosine (sarkosyl), a strong detergent, following the two extraction buffers has been claimed as the critical point in the success of the DNA extraction procedure for carnivorous plants which are rich in mucilagineous polysaccharides (Bekesiova *et al.*, 1999). The presence of two detergents (CTAB and sarkosyl), which is not common in other protocols could also be a determining factor for the effective

release of nucleic acids from the tissue. This could explain the high yield of DNA obtained with this protocol when it was used for herbarium specimens.

The observable degradation of DNA extracted from the silica sand-preserved materials may be due to the desiccation procedure used. In this case, the amount of Moisture-Gone™ was insufficient relative to the amount of tissue, and also the leaves were not cut into small pieces to shorten the drying process. The lack of efficient desiccation was clearly observed when the plant material arrived at the laboratory. The plant tissue failed to be fully dehydrated after several days kept in a tube containing Moisture-Gone™. When these materials were transferred into silica gel, they were completely dry within 24 hours, whereas those that were kept longer in Moisture-Gone™ suffered from severe fungal infection. There is also the possibility that because unlike silica gel, Moisture-Gone™ does not have colour indication to show the degree of hydration, that the particles may not have been completely dry when the plant tissue was added, further reducing its efficiency as a desiccant.

A basic requirement for the success of using desiccants for the preservation of DNA in plant specimens is rapid drying. Savolainen *et al.* (1995) and Štorchová *et al.* (2000) stated that rapid drying is essential to prevent DNA from degradation by limiting endogenous hydrolytic damage (Eglinton and Logan, 1991). In the case of DNA preservation in dried tissue, rapid desiccation prevents the cleavage of phosphate links in the nucleic acid chain that could result in breaking DNA into shorter strands. In this regard,

Savolainen *et al.* (1995) argue that a desiccant, such as silica gel, denatures DNases during rapid desiccation. Prolonged desiccation periods will induce injuries which results in the production of phenolic compounds and free radicals that may influence DNA extraction and/or the amplification process (Savolainen *et al.*, 1995). These injuries include extreme water stress, shortage of nutrients, and wounding.

The use of silica gel for desiccation of leaves of *D. indica* appears to be essential to preserve DNA, provided the leaves are completely dried after 24 hours (personal observation). DNA extracted from this material using the protocol of Wang *et al.* (1996) was of high yield and good quality. When the DNA was examined after agarose gel electrophoresis, a bright, wide discrete band was observed, indicating high integrity with a relatively minor degree of degradation.

Unlike silica sand-preserved materials, water-preserved tissue produced DNA of high quality and integrity (Fig. 4.1). Although there was some degree of degradation observed as smearing of the DNA on the gel, the presence of a discrete band was indicative of intact, high molecular weight DNA. This result showed the potential of the water-preservation technique, in contrast to the study of Pyle and Adams (1989) where water-preserved tissue was rotting and infected by fungi after three days of storage, so that the DNA could not be recovered. The difference between these two results is thought to be due to the moist habitat in which *D. indica* grows, preadapting it to short term inundation, as well as the more favourable condition of the tissue in the

present study compared to the delayed desiccation reported by Pyle and Adams (1989).

None of the four extraction protocols used for herbarium specimens produced DNA with high molecular weight. Although spectrophotometric analysis showed that both yield and absence of contaminant was adequate, the DNA appeared to be highly degraded (Fig. 4.1). The Steenkamp *et al.* (1994) protocol produced the lowest yield, followed by the QIAGEN® DNeasy™ Plant Mini Kit, the protocol of Wang *et al.*, (1996), and the highest yield was obtained with the protocol of Bekesiova *et al.*, (1999). In term of DNA quality, the ratio of A260/280 between the four protocols did not show significant differences. This means that there was no significant problem with protein contamination, as the A260/280 is usually used to detect the presence of contaminating protein in nucleic acid preparation (Manning, 1991). In contrast, the A260/230 ratio varied greatly between protocols. This ratio provides an indication of the contamination by polysaccharides/phenolics (Manning, 1991). The presence of contaminating phenolic compounds can also be detected from visual observation, by the brown to dark-brown colour of DNA (Savolainen *et al.*, 1995; Maunder *et al.*, 1999).

When dealing with DNA from herbarium specimens, it has been recognised that two major problems are generally encountered: the extremely low amount of DNA, and difficulties with PCR (Savolainen *et al.*, 1995; Golenberg, 1991). While the first problem could be overcome by increasing the starting material for extraction, the second problem needs more

attention. Pääbo (1989) argued that dried herbarium samples continue to undergo oxidative damage, which affects the quality of the DNA and subsequently inhibits the PCR amplification process (Golenberg, 1991). Savolainen *et al.* (1995) in their study using herbarium specimens between 2 to 151 years old reported that at least three classes of PCR inhibitors with different properties were present in the DNA extracted from herbarium specimens. In dealing with this problem it has been reported that there are some possible way to reduce these inhibitors by using inhibitor–binding substances such as PVP, gelatin and bovin serum albumin, by purifying the extracted DNA, or by diluting the DNA extracts (Golenberg, 1991; Savolainen *et al.*, 1995). However, these procedures were not successful with all samples (Savolainen *et al.*, 1995).

From various studies conducted using herbarium specimens for DNA analysis (Liston *et al.*, 1992; Mishler *et al.*, 1992; Fay *et al.*, 1996, Schwarzbach and Kadereit, 1999; Maunder *et al.*, 1999), it is apparent that the success of DNA preservation and its subsequent amplification has no correlation with the age of the specimens. There are some factors that determine the successful amplification of herbarium DNA. These include: the chemical particularities of the species, the developmental stage of the collected tissue, the drying method used for preserving the material, and the duration and condition of preparation of the specimens (Savolainen *et al.*, 1995).

4.6 Conclusion

The protocol of Bekesiova *et al.* (1999) was most effective for the extraction of DNA of high quality from both fresh and water-preserved leaf tissue of *Drosera indica.* None of the protocols used in this study produced DNA of adequate quality from herbarium specimens for subsequent molecular analysis. DNA from leaf tissue desiccated in silica gel was successfully extracted using the protocol of Wang *et al.* (1996), but the use of silica sand (Moisture-Gone™) as a drying agent resulted in degradation of the DNA.

Chapter Five

Morphological Variation in the *Drosera indica* Complex

5.1 Abstract

The patterns of morphological variation in *Drosera indica* complex were studied using multivariate numerical analysis i.e. cluster analysis, multidimensional scaling (MDS) and principal component analysis (PCA). Sixty-two accessions were measured and scored for 26 morphological characters, including 5, 9, and 12 characters from vegetative, floral, and seed features respectively. The results indicated that the morphological data differentiated the accessions into six morphotypes based mainly on seed and floral characters. Examination of the geographic distribution of accessions under study suggests that these morphotypes did not exhibit distinctive geographical and ecological patterns. Considering the degree of morphological differences between morphotypes, these morphotypes might represent different varieties within *D. indica*, or possibly even distinct species.

5.2 Introduction

Drosera indica is an annual, tropical carnivorous plant that grows in poor, sandy soils, and acid, swampy areas. Field observations reveal that this species exhibits noticeable morphological variation in plant size, stem colour, flower

colour, filament form, and seed features (Lowrie, 1998; Russell Barrett, pers. comm.; Robert Gibson, pers. comm.). The distribution area of the species includes South Africa, Asia, and Australia. In Australia, this species is mainly found in the Kimberley region of Western Australia, from where most of the specimens in this study were collected. This region is located between 14° and 19° S latitude, and 125° and 130° E longitude, and covers an area of approximately 320,000 km^2 (Fitzgerald, 1983).

The traditional taxonomic approach recognises only a single species of *D. indica* without any infraspecific taxa (van Steenis, 1953; Ohwi, 1965; Obermeyer, 1970; Marchant *et al.*, 1982), although there are some synonyms designated to infraspecific categories reflecting morphological variations of the species (Walker, 1994). So far, there is no published study on the variability within the species. In this present study the pattern of morphological variation within the species, and its taxonomic implications, will be examined using multivariate numerical analysis methods.

5.2.1 Morphology in taxonomy

Morphology has been, and is still, the main character used for recognition and description of taxa (Davis and Gilmartin, 1985; Stuessy, 1994; Sattler and Rusthauser, 1997). This is due to the ease of assessment, the large amount of variation, the availability of well-established descriptive terminology, and access to herbarium and fossil collections (Sivarajan, 1991; Stuessy, 1994). Despite

those advantages, however, some morphological data should be used with care due to the possibility of a high level of within-species polymorphism, and the influence of environmental factors (Quicke, 1993). In addition, Stuessy (1990) pointed out that because the level of critical evaluation and comparison of morphological data may vary considerably from one researcher to another, careful scrutiny in assessing morphological variation is required.

Morphology is applicable at all levels of the taxonomic hierarchy (Stuessy, 1990). It is particularly important for categories below species level, since morphology is the first evidence to be considered for the recognition of infraspecific taxa. If no morphological differences occur among populations within a species, then no formal designation should be provided, even though there are differences revealed from other data sets (Stuessy, 1990). An example of the important role of morphological data in infraspecific studies has been reported for the *Cardamine pratensis* group (Marhold, 1996), in which groups of populations with characteristic chromosome numbers and geographical distribution were not distinguishable morphologically, and thus could not be differentiated taxonomically.

5.2.2 Infraspecific variation and classification in plant

It has been recognised that variation below species level is complex, and usually continuous rather than discrete (Prentice, 1986; Brunell and Whitkus, 1999). An understanding of infraspecific variation is important in defining taxonomic groupings at the species level (Snaydon, 1973). Moreover, it has been argued

that patterns of within-species variation should be defined to understand the evolutionary processes of speciation (Krauss, 1996).

After the infraspecific taxa have been recognised, the next step is to determine the taxonomic level to which the identified taxa should be placed, that is, to define the infraspecific category. Krauss (1996) claimed that this is not a straightforward process, i.e., one which can be deduced from phenetic analysis alone, and that there are no universally recognised criteria. Nevertheless, there is an agreement among authors that morphological distinctness and geographical or ecological coherence is generally necessary for the recognition of infraspecific taxa (Eckenwalder, 1996; Chiapella, 2000; Wendt *et al.*, 2000). The importance of geographical data in this instance is in the delimitation of infraspecific taxa, particularly subspecies and varieties (Stuessy, 1990; Hamilton and Reichard, 1992; Krauss, 1996; Nicole and Conran, 1999; Chiapella, 2000; Wendt *et al.*, 2000). Data on the geographical distribution of plant taxa are also valuable for showing possible sympatry of species and infraspecific taxa, as are data on their overall ecological requirements, and spatial reproductive isolation (Stuessy, 1990).

The practise of creating formal infraspecific classifications to describe within-species variations has been a debate for years, with two contrasting arguments whether to adopt or to abandon the recognition of infraspecific categories (Burtt, 1970; Hawkes, 1986; Prentice, 1986. Stace, 1986; Hamilton and Reichard, 1992). Those who support the formal recognition of infraspecific categories claim that

the purpose of this practice is to help in the description of the diversity within the species, and to provide a system of reference to the important variants (Burtt, 1970; Prentice, 1986). In addition, Stace (1986) argues that the availability of infraspecific categories is important in order to express the degree of relationships among taxa.

An argument for not formulating formal classifications for infraspecific variation is to avoid overloading the formal nomenclature (Burtt, 1970). In fact, the construction of formal classification is difficult because infraspecific variations are numerous, continuous, and have different directions (Hawkes, 1986). In this regard, it has been argued that there is instability in the practice of defining taxonomic categories for infraspecific classification (Prentice, 1986; Stace, 1986), and that the choice of infraspecific categories appears largely arbitrary (Brunell and Whitkus, 1999). Nevertheless, there are widely accepted criteria for the delimitation of subspecies and varieties (Hamilton and Reichard, 1992). The term subspecies is usually applied to morphologically distinguishable groups with distinct geographical and ecological properties, while the term variety is applied to groups that are morphologically but not geographically distinct (van den Berg and Spooner, 1992; Stuessy, 1994; Thiele and Ladiges, 1994).

While the subspecies and variety categories are the two commonly used infraspecific categories, this is not the case with the category of form ("forma") (Hamilton and Reichard, 1992; McDade, 1995). The infraspecific category of form is applied to the occurrences of plants with unusual morphological features

growing near individuals with more "typical" morphology (Stuessy, 1990). In this case Stuessy (1990) gave a recommendation that forms should not be used in a formal nomenclatural sense except perhaps for groups with strong economic value or for wild relatives of cultivated plant species with good potential for inclusion in breeding programs.

5.2.3 Phenetic analysis in taxonomy

Phenetics is a method that uses the overall resemblances among organisms to assess relationships and to develop classifications based on characters assumed to be heritable (Sokal, 1986; Abbott *et al.*, 1985; Quicke, 1993; Stuessy, 1994). In practice, phenetics only considers similarities and differences, regardless of the evolutionary content of the characters reflected, and thus do not provide evidence of evolutionary relationships (Duncan and Baum, 1981; Stuessy, 1990; Quicke, 1993).

In its application in taxonomy, phenetic analysis was hoped to be able to discern if recognisable taxa exist, and to distinguish "taxonomic signals", i.e., characters useful in classification and identification, from "taxonomic noise", i.e., characters with variable features within taxa (Kephart *et al.*, 1999). In the practice of phenetic analysis, the term phenetic taxonomy emerges as a system of classification that uses numerical methods to make classifications of objects based on the overall similarity of the objects being classified (Romersburg, 1984; Sokal, 1986).

The advantage of numerical methods for the assessment of variation is that they allow the rapid, simultaneous, accurate, and repeatable analysis of many samples and characters (Prentice, 1979). In addition, Eckenwalder (1996) pointed out that using simultaneous consideration of many characters, multivariate analysis could overcome the varying deficiencies or biases of individual characters. In its application in systematic study, multivariate phenetic analysis, or numerical taxonomy, has been used successfully for solving various taxonomic problems, including defining species boundaries, delimiting difficult taxa, and assessing the status of hybrids (Tyteca and Dufrêne, 1994; Spooner *at al.*, 1995; Dibble *et al.*, 1998; Hess and Stoynoff, 1998).

While numerical taxonomy is applicable at all taxonomic levels, it is particularly important in infraspecific studies, where the nature of variation may be complex. It has been argued, therefore, that the variation in infraspecific taxa provides an ideal area for the application of multivariate analysis for the recognition of patterns of variation (Stuessy, 1990). An example of this is the use of numerical phenetic analysis of morphological data to determine the appropriate taxonomic level for *Quercus acerifolia*, a taxon exhibiting a considerable degree of characters overlapping with its relatives (Hess and Stoynoff, 1998). Numerical taxonomy can also be used to evaluate classifications produced by the practice of classical taxonomy. One example, based on morphoqualitative analysis, is that by Sahuquillo *et al.* (1996) which revealed the inability of classical taxonomy to define infraspecific variation in *Triticum aestivum*, due to instability and

inconstancy of characters used, and consequently, the three previously known varieties in *T. aestivum* were abandoned.

The present study employs two kinds of multivariate phenetic methods: cluster analysis, and ordination. Duncan and Baum (1981) claimed that when the result of phenetic taxonomy is to be presented in a hierarchical non-overlapping form, then it is suggested to use sequential agglomerative hierarchical non-overlapping cluster analysis in combination with ordination analysis. In this study the ordination is carried out using two techniques: multidimensional scaling (MDS) and principal component analysis (PCA).

5.2.3.1 Cluster analysis

Cluster analysis is a class of numerical techniques for defining groups of related samples (called Operational Taxonomic Units) based on similarity coefficients (Sokal and Sneath, 1963). By this definition, the members of a cluster are linked by some sort of internal relationship, and they are separated by some comparable negative relationships from all other elements (Williams, 1971). The result of cluster analysis is displayed in the form of dendrogram, that is, a two-dimensional tree-like diagram illustrating the fusions or partitions of objects and object groups (Dillon and Goldstein, 1984).

The clustering technique used in this study is an hierarchical agglomerative method. This method performs a series of progressive fusions of related objects, so that every cluster obtained at any stage is a merger of clusters at a previous

stage (Williams, 1971; Gnanadesikan, 1977; Dillon and Goldstein, 1984). When unweighted pair-group method with arithmetic average (UPGMA) is employed, the one used in this study, during each clustering cycle the correlation between OTUs is recomputed based on the initial resemblance matrix (Sokal and Sneath, 1963). Since cluster analysis will always generate a dendrogram regardless of the underlying relationships, it is important to have a measure of the reliability of the resulting tree. Such a measure can either be in the form of a cophenetic value (Romersburg, 1984; Rohlf, 1998) or a bootstrap value (vander Kloet and Paterson, 2000).

5.2.3.2 Ordination

Ordination is a map of objects in low dimensional space, usually two or three, in which distances between points represent the dissimilarity between the objects (Abbott *et al.*, 1985; Clarke and Warwick, 1994). The objects can be plotted in either two- or three-dimensional space formed by components, that is, the set of axes produced from transformation of the original variables. This technique is used to provide a visually displayed simplification of the variation pattern, and to produce the most distortion-free reproduction of variation, i.e., the one which best preserves the rank order of magnitudes of the similarities or distances (Abbott *et al.*, 1985). The two most commonly used ordination techniques are multidimensional scaling (MDS) and principal component analysis (PCA).

MDS is a class of techniques that aims to compute coordinates for a set of points in space such that the distances between pairs of points fit as closely as possible

the dissimilarities between samples (Kruskal and Wish, 1978). In non-metric multidimensional scaling, as used in this study, these coordinates are calculated by monotonic regression. The configuration of samples in a low dimensional space involves an iteration procedure, whereby the relative positions of points are successively refined until they satisfy the dissimilarity relations between samples (Clarke and Warwick, 1994; Krauss, 1996). MDS is usually carried out in an attempt to make the data accessible to visual inspection and exploration (Borg and Groenen, 1997).

PCA was developed as an ordination technique to reduce the dimensionality of multivariate data by removing intercorrelations among variables (Broschat, 1979). If some or all of the variables are measured using different units, then the data should be standardised. Following this, a matrix of simple correlation is calculated, and then the variables are transformed into principal components (PCs), which are the linear combinations of the standardised variables. Gil and Cubero (1993) pointed out that PCA is useful in identifying the characters that contribute most to the total variability of the individual group by evaluating the magnitude of loadings of individual characters on each principal component. Nevertheless, PCA has a number of underlying numerical assumptions relating to data structure that are often violated by the type of characters used in taxonomic studies (Shi, 1994) and its use is often discouraged in favour of MDS which has fewer data-dependent assumptions (Belbin, 1994; Faith *et al.*, 1987).

5.2.4 Studies in species complexes using phenetic methods

The application of numerical phenetics in plant systematics has been reviewed by Duncan and Baum (1981), from which it was revealed that studies at the species, species complex, or generic level have predominated in the application of numerical phenetic methods. These kinds of studies dealing with species complexes for solving taxonomic problems are of particular relevance to the present study, as shown by the following examples.

Francisco-Ortega *et al.* (1993) reported the use of multivariate phenetic analysis to support the recognition of seven morphological types in *Chamaecytisus proliferus* complex. Thompson and Lammers (1997) used quantitative methods to determine the existence of discrete clusters of populations that are morphologically distinguishable and geographically coherent in an attempt to clarify phenetic relationships, and to propose a classification for the *Lobelia cardinalis* complex, concluding that the complex consisted of a single species that cannot be divided into infraspecific taxa. Baatout (1995) reported the use of phenetic methods to assess variability in morphological and physiological characters to evaluate the taxonomic status of *Hedysarum spinosissimum* species complex. Two morphologically distinct groups previously assigned as subspecies, were reassigned as two separate species. Chiapella (2000) demonstrated the use of numerical analysis of morphological data, in combination with geographical data, to clarify the taxonomic status of the *Dechampsia cespitosa* complex.

The purpose of this study is to assess the pattern of morphological variation in *D. indica* complex. Multivariate numerical analysis methods will be used to determine if there are morphologically distinguishable groups, and to determine characters that are taxonomically diagnostic in defining morphs. Taxonomic implications of the morphological variability will be formulated by considering geographical and ecological data.

5.3 Materials and Methods

Seventy-nine air-dried herbarium specimens were examined for recordable morphological characters on vegetative and reproductive organs. Of these, fifty-nine specimens were selected and, together with three plants grown from seeds in a glasshouse, comprised the sixty-two accessions that were measured and scored for 26 morphological characters. The accessions used, and their localities, are listed in Appendix I. Each accession was treated as an operational taxonomic unit (OTU). The accessions were named according to their collection number, except for those with prefix "dro-", which were arbitrarily named from the seed collections.

Of the 26 morphological characters examined, 10 were quantitative and 16 were qualitative, including 5, 9, and 12 characters from vegetative, floral, and seed features, respectively (Table 5.1). Leaf and flower characters were examined under an Olympus CH-BI45-T-2 microscope at 40 x or 100 x magnifications, and measurements carried out using a calibrated ocular micrometer. The procedure for scanning electron microscopy of seeds was as described in Chapter Three.

The original scores and measurements of the accessions are tabulated in Appendix IV

Table 5.1 Morphological characters examined

Code	Characters	Units or states
PLC	Plant colour	0: green; 1: orange-red, red, maroon-red
LPT	Petiole	0: absent; 1: present
LFG	Leaf flat or grooved	0: flat; 1: grooved
LRS	Red stripe on abaxial surface of the leaf	0: absent; 1: present
LTD	Distribution on tentacles on the leaf	0: surface only; 1: surface and margin
FSL	Sepal length	mm
FPL	Petal length	mm
FPC	Petal colour	0: white; 1: orange; 2: pink, mauve, cerise, purple
FFF	Filament form	0: filiform; 1: deltoid; 2: cobra-like
FFL	Filament length	mm
FAL	Anther length	mm
FAP	Anther position on the filament	0: top; 1: margin
FAE	Anther erect or hooded	0: erect; 1: hooded
FAC	Anther colour	0: yellow; 1: red
SES	Seed shape	0: ovoid; 1: ovoid-ellipsoid; 2: ellipsoid
SEO	Seed coat ornamentation pattern	0: reticulate; 1: foveolate; 2: furrowed
SEL	Seed length	0: 250-475 μm; 1: 476-700 μm; 2 > 700 μm
SEW	Seed width	0: 150-265μm; 1: 266-375 μm; 2 > 375 μm
PSH	Pore shape (outline of the seed coat cells	0: elliptic; 1: tetragonal; 2: transversely hexagonal; 3: longitudinally hexagonal; 4: rectangular
NPL	Number of pores per seed length	count
NPW	Number of pores per seed width	count
PCU	Pore curvature (curvature of outer periclinal wall)	0: flat; 1: concave; 2: deeply concave
PFS	Pore floor sculpturing	0: smooth; 1: undulate; 2: wavy
SLR	Longitudinal ridges on the seed surface	0: absent; 1: present
POL	Average pore length	0: 14-45 μm; 1: 46-77 μm; 2: 78-109 μm; 3 > 109 μm
POW	Average pore width	0: 25-43 μm; 1: 44-62 μm; 3 > 62 μm

The assessment of morphological variability was carried out by employing cluster analysis and two ordination techniques: PCA and MDS. Cluster analysis was carried out to generate a dendrogram using the SAHN clustering procedure and UPGMA method (NTSYS-pc version 2.0) (Rohlf, 1998). The 62 x 26 data matrix was standardised, i.e. by subtracting the character mean from each sample and then dividing by the character standard deviation, prior to cluster analysis to reduce distortion that could result from the use of different scales of measurement and from differences in the ranges of character values (Romersburg, 1984). The cophenetic value was calculated as a measure of the amount of distortion between the distance matrix and the dendrogram generated from cluster analysis (Sokal and Rohlf, 1962; Romersburg, 1984). MDS ordination was performed to reveal the trend of each character toward the grouping of accessions. The cluster analysis and ordination were carried out based on Gower's metric distance coefficient (Gower, 1971), which is suitable for mixtures of quantitative and qualitative data (Brown and Wiecek, 1996). Principal canonical correlation (PCC) was also performed to calculate the correlation coefficient between each character and the ordination axis scores of the individual OTUs (Nicolle and Conran, 1999). The MDS and PCC analyses were accomplished using PATN version 3.5 (Belbin, 1994). PCA was performed to estimate the contribution of each character to the total variability that discriminated the groups (Romero *et al.*, 2000). The use of PCA for comparison to MDS has been reported by Duretto and Ladiges (1997). PCA was carried out using SPSS version 8.0 (SPSS Inc., 1998).

5.4 Results

5.4.1. Cluster Analysis

The cophenetic correlation value between the dendrogram and the distance matrix was 0.90, indicating a good fit between the two, which means that the groupings produced by this cluster were unlikely to be a random event (Rohlf, 1998).

The dendrogam of morphological data (Figure 5.1) displays three major clusters: **A**, **B**, and **C** at a taxonomic distance of 1.92. These three clusters correspond to the three seed types of *D. indica* noted previously in Chapter Three. Cluster **A** consisted of individuals characterised by seed Type II, stems green, and petiolate leaves. Most of the plants in this group (77%) also possessed leaves with tentacles only on their adaxial surface of the lamina (lack of tentacles on the leaf margin). Two subclusters were recognised within cluster **A**, assigned as **A1** and **A2**, and these differed from each other in their filament form: group **A1** consisted of individuals with normal (filiform) filaments; those of **A2** with deltoid filaments.

Cluster **B** comprised individuals with reticulate seeds, and, with a few exceptions (accessions 2, 55, 56, and 39), apetiolate leaves. This cluster consisted of two big subclusters (**B1** and **B2**), and two distantly related small groups each consisting of two individuals at the upper and lower part of cluster **B** (accessions A2 and A55, and A24 and A39, respectively). Individuals in subcluster **B1** are characterised by green stems, normal filaments, and with a few exceptions

(accessions A2, A55, and A56), exhibit reticulate subtype transversely hexagonal seeds. Subcluster **B2**, on the other hand, consisted of individuals with red stems, and reticulate seeds of the longitudinally hexagonal and tetragonal subtypes. This subcluster can be further divided into two groups that differ from each other by filament form. Group **B2-a** consisted of individuals with deltoid filaments, whereas those with normal filaments belonged to group **B2-b**.

The four OTUs that do not belong to any group in cluster **B** (accessions 2, 55, 24, and 39) are those exhibiting some degree of deviation from either typical subcluster **B1** or **B2**. Accessions A2 and A55 exhibit reticulate subtype tetragonal seeds and filiform filament form, but differ from the other members of group **B2b** by having green stems, and this explains their closer position to subcluster **B1** rather than to subcluster **B2**. Accession A39 also has reticulate subtype tetragonal seeds and green stems, but unlike accessions A2 and A55, this OTU is placed close to the rest of subcluster **B2** members due to its deltoid filament form, which is characteristic of group **B2a**. Accession A24 has reticulate subtype transversely hexagonal seeds, which is typical of subcluster **B1**, but its maroon-red stems and deltoid filament form make this OTU significantly different from the members of subcluster **B1**, and place it closer to subcluster **B2** than to subcluster **B1**.

The third cluster (marked as **C**), was clearly separated from the other two major clusters, as indicated by the distance coefficient of 1.92, and consisted of

individuals with a number of distinctive characters, such as furrowed seeds, red, hooded, cobra-like filaments, and leaves with a red stripe on the abaxial surface.

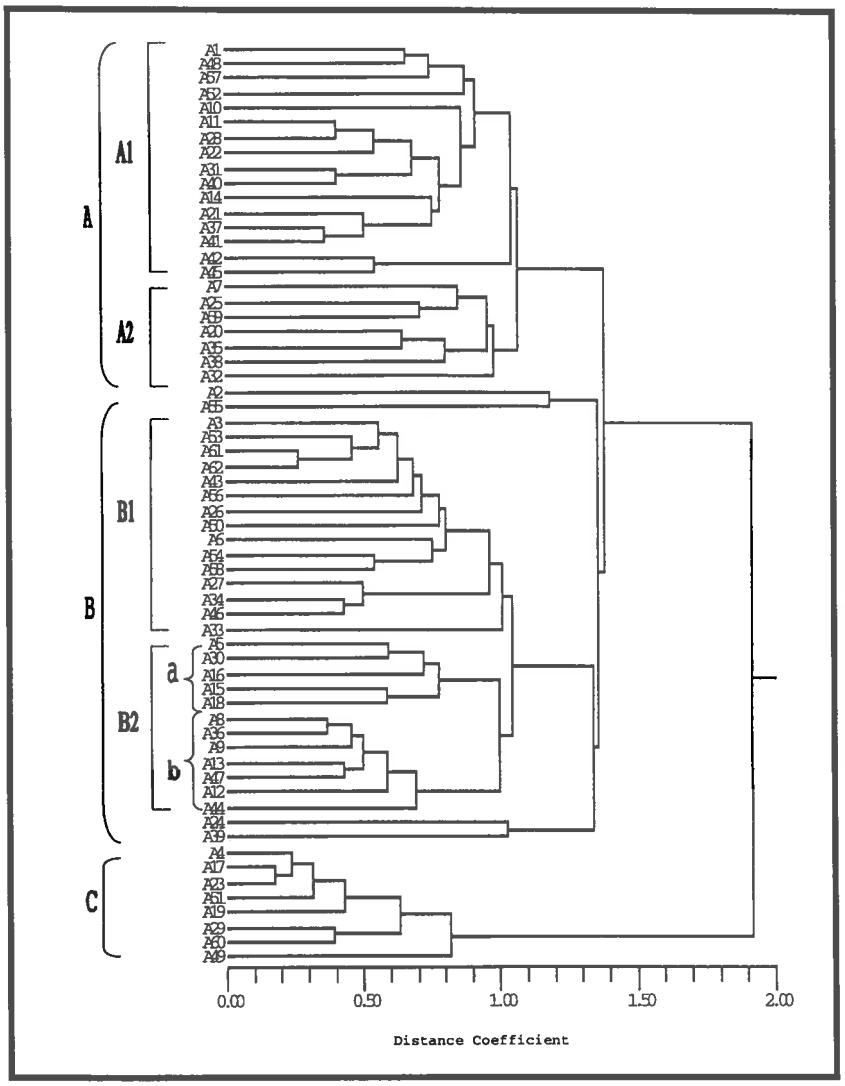

Figure 5.1 UPGMA dendrogram of 62 accessions of *Drosera indica* based on morphological data, showing six morphological groups. A1: plants with foveolate seeds, green stems, petiolate leaves, and filiform filaments; A2: plants with foveolate seeds, green stems, petiolate leaves, and deltoid filaments; B1: plants with reticulate subtype transversely hexagonal seeds, green stems, apetiolate leaves, and filiform filaments; B2a: plants with reticulate subtype longitudinally hexagonal or tetragonal seeds, red stems, apetiolate leaves, and deltoid filaments; B2b: plants with reticulate subtype longitudinally hexagonal or tetragonal seeds, red stems, apetiolate leaves, and filiform filaments; C: plants with furrowed seeds, green stems, petiolate leaves with a red stripe on the back, cobra-like filaments, and red, hooded anthers.

Considering the scattered geographical distribution of the accessions (OTUs) within each of the clusters, it is apparent that these morphological groups do not exhibit distinctive geographical patterns. Furthermore, in most cases, OTUs from the same site did not cluster together, and most clusters comprised OTUs from different areas.

5.4.2. Multidimensional Scaling Analysis

The result of the MDS analysis (Figure 5.2) supports the existence of the six dendrogram groups as discrete entities. To provide consistency in defining groups, the term supergroups **A** and **B** are applied, referring to clusters **A** and **B** in the dendrogram (Figure 5.1). The four accessions that do not belong to any group in the cluster analysis are marked as outliers in the two-dimensional MDS plot (Figure 5.2). In this MDS the characters are plotted onto two-dimensional space based on their best-fit direction, allowing the examination of trends of characters across the ordination space.

Axis 2 of the MDS separates group **C** from supergroups **A** and **B**. Twenty out of twenty six (77%) characters contributed to this separation. These characters, located at the bottom of the MDS plot, are mainly those from seed and flower morphology, such as SES, SEL, SEO, SEW, FSL, FFF, FAE, and FAC (Table 5.2).

Despite the seed type, another distinctive feature of group **C** is the occurrence of a red stripe on the abaxial surface of the leaf (LRS; Appendix V), which is absent

in both supergroups **A** and **B**. This character was a distinguishing feature of group **C**, and both its consistency, and environmentally-independent nature were confirmed in plants reared in the glasshouse.

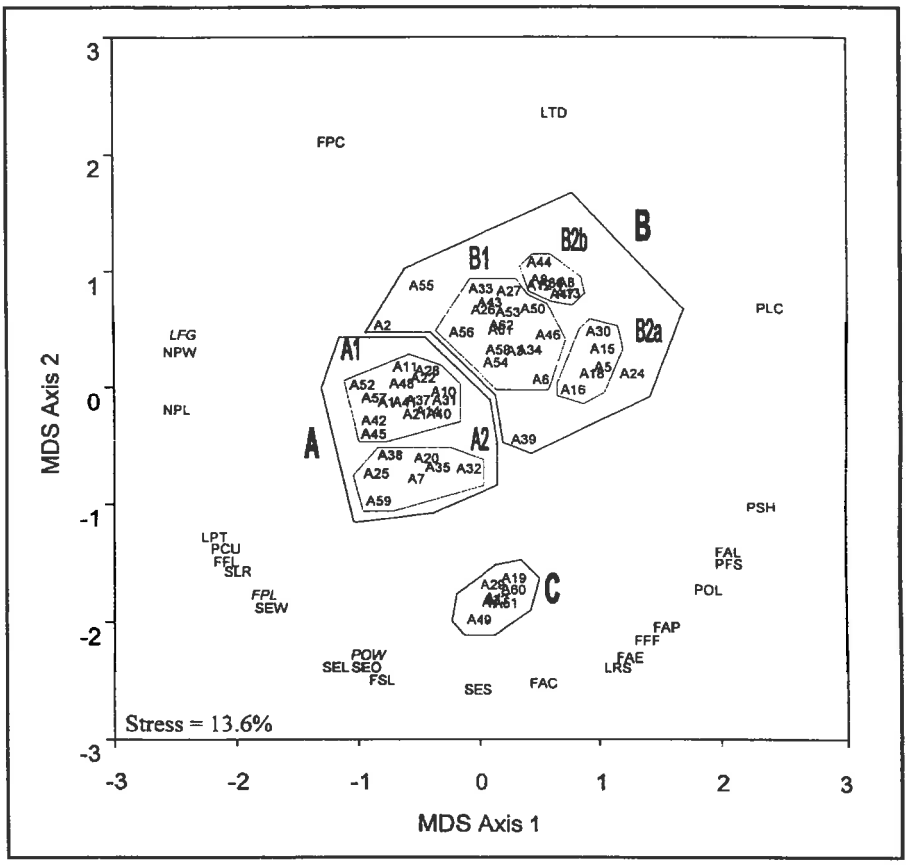

Figure 5.2 The MDS analysis of *Drosera indica* accessions using Gower's metric, showing the groups from the dendrogram in Figure 5.1. Character names indicate the direction of positive increase for that character's score after correlation with the MDS axes using PCC analysis. Character names in italics represent non-significant ($p > 0.05$) trends only. Group names correspond to those in the dendrogram Figure 5.1.

Axis 1 of the MDS tends to differentiate supergroup **A** from supergroup **B**. Characters that show a positive contribution in this differentiation are NPL, NPW, PLC, PSH, and LPT.

The stress value of the MDS, as a measure of fit (Kruskal and Wish, 1978), is 13.6 %, indicating that this MDS gives a potentially useful two-dimensional representation of the rank order of dissimilarities among the accessions (Clarke and Warwick, 1994).

5.4.3. Principal Component Analysis

The result of the PCA is summarised in Table 5.2. From the 26 morphological characters analysed, six principal components (PCs) with an eigenvalue greater than one were extracted. The first three PCs account for 36.9, 21.4, and 9.4% of the variance, respectively. Collectively, they explain 67.7% of variance for the 26 morphological characters. Since the successive PCs only give a slight increase in the total variance, they were not used in the analysis.

The two-dimensional scatter-plot of accessions projected in the first two PCs of the PCA (Figure 5.3) confirms the clustering of accessions into six groups, with group **C** placed at a considerable distance from the others. On the contrary, supergroups **A** and **B** showed some degree of overlapping in the placement of the individual accessions. Nevertheless, the result corresponds to those for the MDS analysis (Figure 5.2) and the cluster analysis (Figure 5.1).

Table 5.2 Coefficient loadings of characters on the first three components of PCA

	Component		
	1	2	3
SES	0.065	-0.021	-0.053
SEO	0.097	0.026	-0.042
SEL	0.092	0.021	-0.035
SEW	0.085	0.058	-0.092
PSH	0.030	-0.158	0.069
NPL	0.028	0.155	-0.026
NPW	0.006	0.088	-0.106
PCU	0.068	0.090	-0.074
PFS	0.047	-0.118	0.003
SLR	0.076	0.094	-0.043
POL	0.064	-0.124	0.023
POW	0.031	0.018	0.071
FSL	0.074	0.047	0.138
FPL	0.013	0.096	0.305
FPC	-0.048	0.072	0.074
FFF	0.083	-0.068	0.141
FFL	0.055	0.057	-0.126
FAL	-0.007	0.056	0.342
FAP	0.061	-0.040	0.224
FAE	0.089	-0.086	-0.024
FAC	0.086	-0.038	0.017
PLC	-0.047	-0.097	0.046
LPT	0.073	0.102	-0.063
LFG	-0.004	0.068	0.168
LRS	0.089	-0.086	-0.024
LTD	-0.056	-0.043	-0.103
Eigenvalues			
Total	9.608	5.555	2.432
% of variance	36.952	21.366	9.353
% cumulative	36.952	58.318	67.671

The characters contributing most to the separation of group **C** from the other groups are those with the high positive loadings on PC1 (Table 5.2). These characters are: SEO, SEL, FAE, LRS, FAC, SEW, FFF, and FSL. The second principal component (PC2) differentiates supergroup **B** from **A** based mainly on NPL and LPT characters, both with loadings greater than 1.0. This result

demonstrates the supplementary function of PCA to the cluster analysis, in which PCA provides information on the usefulness of the characters in defining groups (Dias *et al.*, 1993).

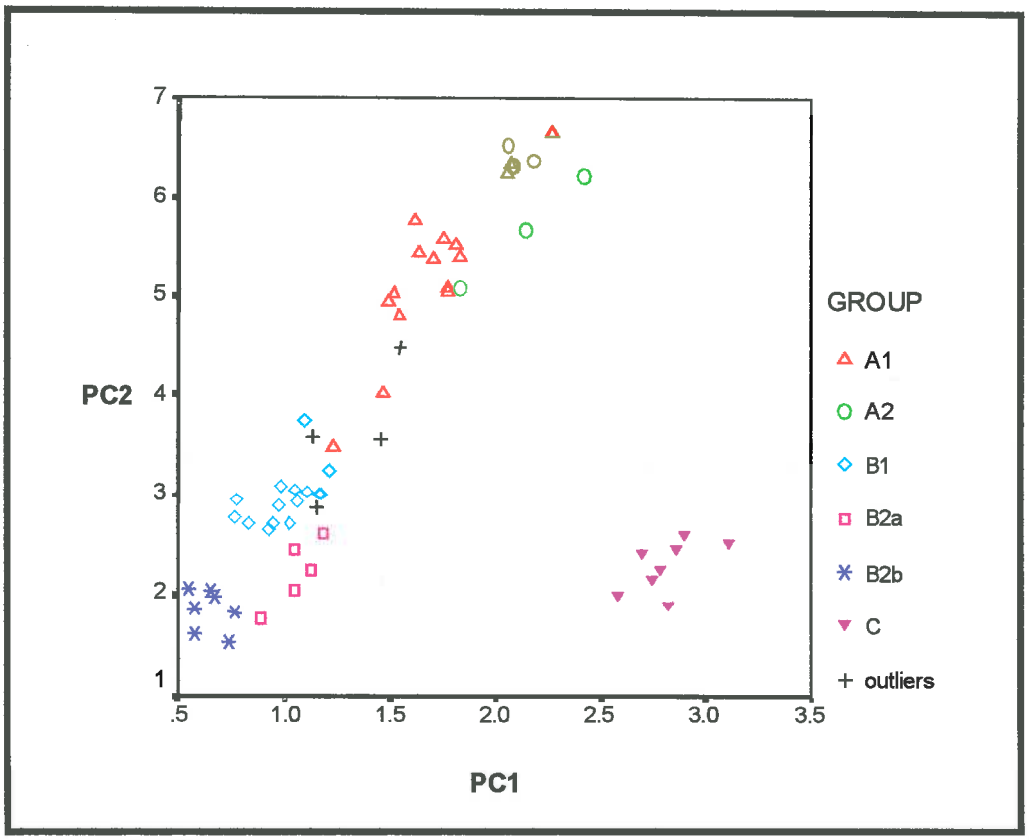

Figure 5.3 Two-dimensional scatter-plot of *Drosera indica* accessions projected in PC1 and PC2, showing six morphological groups and outliers. Group names correspond to those in the dendrogram (Figure 5.1).

5.5 Discussion

Cluster analysis and the two ordination techniques employed on morphological data revealed the same grouping on 62 accessions of *D. indica* under study. Six morphological groups were recognised, each displaying a distinctive combination of characters in seed type, filament form, and floral and vegetative features. The agreement between cluster analysis and ordination gives the confidence to assign the groups as six different morphotypes. For the purpose of ease of recognition in the following discussion, these morphotypes will be designated as morphotype **A1**, **A2**, **B1**, **B2a**, **B2b**, and **C**, following the division in Figure 5.1.

Based on the dendrogram of morphological data (Figure 5.1), morphotype **C** forms a solid, distinct, and a distantly related group to the five other morphotypes. The distinctness of morphotype **C** is due to the occurrence of a number of unique characters that are not found in the other morphotypes. These characters are (excluding seed type): cobra-like filaments, red and hooded anthers, and the presence of a red stripe on the abaxial surface of the leaf. The existence of this red stripe is marked by glandular hairs with red pigment, short stalks, and globular heads (diameter 10-14 μm), which are easily recognised even in air-dried herbarium specimens. The presence of these glandular hairs, however, is less obvious in the water- or ethanol-preserved materials, due to the discoloration of the leaf, and a consequent loss of the distinctive red colour of these glandular hairs. This leaf character (LRS) appears to be a reliable distinguishing character, since it occurs in glasshouse-grown plants as well as in plants collected from wild populations. The consistency of LRS indicates that it is

attributable to the genotype, and is not observably affected by environmental factors (Wendt *et al.*, 2000; Olfelt, 2001).

Differences between morphotypes **A1** and **A2** (supergroup **A**), and **B1**, **B2a**, and **B2b** (supergroup **B**), on the other hand, are not as sharp as those that distinguish morphotype **C**. The MDS and PCA plots (Figs. 5.2 and 5.3) clearly portray this case, where these five groups are placed at a relatively close distance from one to another. In addition, there is an overlapping area between them occupied by some accessions from each group. In fact, only a few characters positively contribute toward the separation between them (Table 5.2), the most important being micromorphological seed characters.

Results of cluster analysis and ordination suggest that the most contributing character in defining the grouping of accessions into six morphotypes is seed micromorphology, followed by floral and vegetative characters. This result confirms the taxonomic importance of seed micromorphological characters in defining infraspecific variation in *D. indica,* as described in Chapter Three.

Once the pattern of variation is revealed, the taxonomic implications of the variation to the taxa under study can be generated. In general, the best interpretation of taxonomic relationships is that for which the majority of characters correlate, thereby suggesting the same group (Stuessy, 1990). In addition, it has been suggested that the constancy and cohesion of characters responsible for grouping is a requirement for the delimitation of the entities

(Naranjo *et al.*, 1990). In an attempt to determine the taxonomic implications of significant morphological differences within a species, an examination of the extramorphological integrity is needed (Hamilton and Reichard, 1992; Krauss, 1996). Extramorphological integrity (geographic, ecological, and phylogenetic) should be considered to enable a decision to be made whether any infraspecific taxa can be granted to the morphologically distinguishable groups. Of these three components of extramorphological factors, geographic distribution is of particular significance, as this is the one that should be considered along with the analysis of similarities and differences of morphological characters as a basis for evaluating relationships among taxa (Radford *et al.*, 1974). The importance of geographic distribution data as an additional aid in the recognition of infraspecific taxa has been pointed out by Chiapella (2000) and Wendt *et al.* (2000). Moreover, Eckenwalder (1996) used geographical coherence as a criterion in determining whether the observable phenetic pattern represents a taxonomic signal or taxonomic noise.

In the case of *D. indica*, the accessible extramorphological data are their geographical distribution (Figure 5.4), coupled with the information on the two ecological factors: the geology and the average annual rainfall (Appendix VI and Appendix VII). Due to the lack of information on soil type in the region, the present study used the geology of bedrock to portray the soil characteristic. In this case, Sivarajan (1990) pointed out that bedrock geology can affect the distribution of plants, and that it relates to the development of soils. From the geographic distribution data of selected accessions (Table 5.3), it is obvious that

there are five cases where some accessions from different morphotypes were found growing at the same site. Accessions AL 1283 and AL 1325 (morphotype **A2**), AL 1284 and AL 1289 (morphotype **A1**), and AL 1322 (morphotype **B1**) were collected from the same site. Another example is accessions AL 1193, AL 1195, and AL 1208 (morphotype **B1**), and AL 1200 and AL 1215 (morphotype **C**). Based on this geographical evidence, it is clear that these morphotypes occur sympatrically. When the geographical distribution of the accessions was mapped onto the geological and rainfall data, it was apparent that the morphotypes collected did not show distinct geographical and ecological patterns. In other words, there is no geographical and ecological coherence displayed by these morphotypes of *D. indica*. A lack of geographical pattern has also been reported in studies of other species (van den Berg and Spooner, 1992; Krauss, 1996; Thompson and Lammers, 1997; Brunell and Whitkus, 1999; Speer and Hilu, 1999), and Speer and Hilu (1999) argued that this could be the consequence of higher intra-population than inter-regional variation in the morphological characters.

Figure 5.4 Distribution of the accessions used in this study, grouped into their 3 main seed morphotypes.

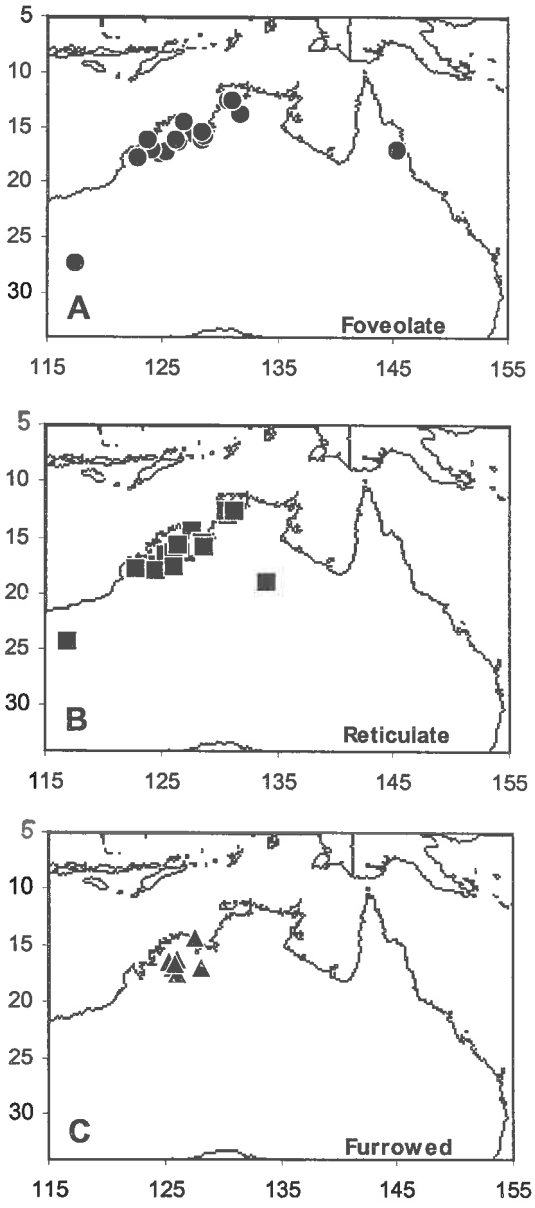

Table 5.3 Geographic positions where different morphotypes were found growing together in a mixed population in Kimberley region, Western Australia

Geographic position (S: South; E: East)	Accessions	Morphotype
17° 25' 09" S; 124° 43' 08" E	AL 1162	A1
	AL 1164	B2b
17° 10' 28" S; 125° 21' 25" E	AL 1172	B2b
	AL 1173	A1
16° 29' 23" S; 125° 21' 11" E	AL 1193	B1
	AL 1195	B1
	AL 1200	C
	AL 1208	B1
	AL 1215	C
16° 15' 18" S; 126° 06' 11" E	AL 1283	A2
	AL 1284	A1
	AL 1289	A1
	AL 1322	B1
	AL 1325	A2
15° 46' 19" S; 128° 37' 07" E	AL 2225	B2b
	AL 2226	A1
	AL 2227	A2

Considering the evidence that the six morphotypes defined here did not exhibit distinct geographical and ecological patterns, and following the definition of variety as groups that are morphologically distinct but not geographically (Stuessy, 1994; van den Berg and Spooner, 1992; Thiele and Ladiges, 1994), it could be argued that these morphotypes might represent varieties within *D. indica*.

There is also a possibility that these morphotypes might represent distinct species. The aforementioned discussions indicate that although these morphotypes, each with distinctive combination of morphological characters, occur sympatrically, they appear to retain their identity, and thus suggesting the existence of isolating mechanisms. In this case, it is likely that there is a reproductive, rather than geographic isolation mechanism, and this could be a basis for recognising them as separate species. When morphologically distinct populations systems occur sympatrically, they might represent reproductively isolated populations, and hence could be good species (Stuessy, 1990). Nevertheless, because the available evidence on the sympatric occurrences of different morphotypes only came from a local scale (Kimberley region), the possibility of recognising them as species should be carefully accounted, otherwise it could lead to the establishment of non-dimensional species (Mayr, 1969), i.e those that have no dimensions of time and space, and consequently do not represent the range of variations within their limits. In order to avoid this, examination of specimens from a wider region covering as much as possible the distribution of the given taxa is needed. This is particularly significant in case *D. indica*, a species with a wide range of distribution across different habitats and continents.

In addition, the morphological differences between morphotypes were of comparable degree to those used to separate some other species in Droseraceae from their nearest relatives, as shown by the following examples. Lowrie (1994) differentiate *D. ordensis* from *D. lanata* based on petiole shape, petiole width,

lamina size, and pedicel length. More recently, Lowrie (1996) described that lamina shape and size were sufficient to differentiate *D. kenneallyi* from three other members of *D. petiolaris* complex, e.g. *D. falconeri, D. petiolaris*, and *D. dilatato-petiolaris*. Lowrie and Conran (1998) distinguished *Byblis aquatica* from *B. liniflora* based on differences in their habit, flower colour, leaves colour, seed length, and seed ornamentation patterns; whereas *B. liniflora* differed from its sympatric congeneric *B. filifolia* in plant size and anther/filament length ratio.

So far, morphological data suggest that the six defined morphotypes might represent taxa, either as varieties or species. However, in this case a justifiable taxonomic conclusion could not be made until other supporting evidence from other data sets become available, such as breeding systems and the occurrences of intermediate forms between putative taxa. It has been recognised that a detailed consideration on breeding systems is important taxonomically, since breeding systems may define the pattern of variation, and hence the delimitation of taxa (Kay, 1984; Stace, 1989). Similarly, the occurrence of intermediate forms as an indication of hybridisation is needed to justify whether the suspected taxa are reproductively isolated and could be recognised as distinct species (Lowrie and Conran, 1998). In this case, further examinations are needed to reveal the breeding system and also the existence of intermediate forms between morphotypes of *D. indica*, in order to formulate implications of the morphological patterns. Before justifiable taxonomic conclusion could be reached, it is

suggested therefore, that the term *D. indica* complex be used to refer to the existence of the six defined morphotypes.

5.6 Conclusion

Drosera indica comprised six morphotypes, each displaying a distinctive combination of seed, floral, and vegetative characters. These morphotypes occur sympatrically with no geographical and ecological patterns, and, the available morphological data suggest that they might represent different varieties or even species.

Chapter Six

Morphological and Genetic Variation in the *Drosera indica* Complex

6.1 Abstract

The pattern of morphological and genetic variation in the *Drosera indica* complex was studied using the methods of multivariate numerical analysis. Fifteen accessions from tissue culture- and glasshouse-grown plants germinated from seeds were used in this study. Morphological analysis was carried out based on 16 characters, while genetic variability was evaluated using random amplified polymorphic DNA (RAPD). Morphological and molecular data were analysed separately using cluster analysis and multidimensional scaling analysis. On the basis of morphological data, the accessions were differentiated into their respective morphotypes. RAPD analysis confirmed that the complex is indeed inbreeds, and showed evidence of reproductive isolation. It is suggested, therefore, that the morphotypes could be considered as distinct species.

6.2 Introduction

An accurate characterisation of the pattern of morphological and genetic variation within species is important and very useful in assisting the process of decision making for the management strategy for conservation, the evaluation of potential inclusion in breeding programs, germplasm management, and for understanding evolutionary processes (Krauss, 1996;

Brunell and Whitkus, 1999; Ontivero *et al.*, 2000; Cole and Kuchenreuther, 2001; Olfelt *et al.*, 2001).

6.2.1 Molecular data in taxonomy

Molecular markers have been widely used to estimate the level of genetic variation between and within groups of plants, which is the basis for detecting gene flow and genetic drift among populations (Cambecèdes *et al.*, 1999; Cole and Kuchenreuther, 2001; Olfelt *et al.*, 2001). In studies of within-species variation, molecular data can be used to evaluate infraspecific patterns of variation, through the detection of the degrees of genetic similarities and differences (Stuessy, 1990). In this regard, a low level of genetic difference indicates taxonomic unity of populations within the species, with no differentiation into infraspecific taxa.

The use of molecular data in taxonomic studies is particularly important when morphological data cannot reliably differentiate taxa. Molecular data can provide an additional tool for assessing variation, the characterisation of varieties in crop plants, and to elucidate taxonomic positions (Orozco-Castillo *et al.*, 1994; Wilson, 1989; Law *et al.*, 1999; vander Kloet and Paterson, 2000; Ontivero *et al.*, 2000; Cole and Kuchenreuther, 2001).

The need to evaluate both morphological and molecular data in taxonomy has been demonstrated in various plant taxa. Komaki *et al.* (1998) confirmed the taxonomic status of *Ipomoea batatas* and *I. trifida* as one taxon based on morphological and RAPD data. Cambecèdes *et al.*, (1999) used a combination

of morphological and RAPD data to solve the taxonomic status of *Centrolepis paludicola*. Olfelt *et al.* (2001) also demonstrated that, by using morphological and RAPD data, the distinctive nature of *Sedum integrifolium* ssp. *leedyi* could be recognised, confirming that this taxon is endangered and thus merits protection.

A molecular analysis was carried out to assess whether a dendrogram based on DNA fingerprints would coincide with the morphological groups. The RAPD analysis provided a measure of genetic similarities among accessions under study based on the binary matrix prepared from the amplified DNA fragments. The requirement for the assessment of genetic diversity among phenotypically differentiated groups has been stated by Plague *et al.* (2001), that the genetic characteristic of individuals from different phenotypes should be assessed to determine whether the phenotypes belong to one or more genetically distinct groups.

6.2.2 Species concepts in plant systematic study

The importance of defining the species concept adopted in a particular study has been pointed out by McDade (1985), Tyteca and Dufrêne (1994), Luckow (1995), and Wendt *et al.* (2000). This is especially significant in studies aiming to evaluate the taxonomic status of a species complex or to identify the existence of infraspecific taxa within a species, and to assign taxonomic ranks to the identified taxa. Snaydon (1984) argued that the magnitude and pattern of infraspecific variation within a species depends upon the definition

of species, in which the broad definition of species will intensify the appearance of infraspecific variation.

Stuessy (1990) recognised seven species concepts, from which only three are commonly used in taxonomic studies: morphological species; biological species; and evolutionary species. The morphological species concept was defined by du Rietz (1930) as the smallest natural populations permanently separated from each other by a distinct discontinuity in the series of biotypes. Biological species concept, which is the most widely used framework for defining species (Stuessy, 1990; Wendt *et al.*, 2000), was defined by Mayr (1942) as a group of interbreeding populations that are genetically isolated from other groups by reproduction isolating mechanisms. Simpson (1961) formulated the evolutionary species concept as a lineage (an ancestral-descendant sequence of populations) evolving separately from others and with its own unitary evolutionary role and tendencies.

The role of morphological data in taxonomy, and the use of multivariate numerical analysis methods for the assessment of within-species variability have been described in Chapter Five. The contribution of molecular data in systematic studies, particularly the use of DNA-based molecular markers, has been described in Chapter Two. This chapter will, therefore, deal with the importance of integrating morphological and molecular data in resolving taxonomic problems. The aim is to investigate the pattern of morphological and genetic variability within *Drosera indica* complex using a combination of

morphological analysis and DNA fingerprinting technique using random amplified polymorphic DNA (RAPD).

6.3. Materials and Methods

6.3.1 Morphological analysis

Fifteen accessions of *D. indica*, those which produced DNA of good quality for RAPD analysis (Table 6.1), were examined for 16 morphological characters, including twelve, one (FPC), and three (PLC, LPT, and LRS) characters from seeds, flowers, and vegetative features, respectively, as defined in Chapter Five. Materials used in this study were tissue culture- and glasshouse-grown plants germinated from seeds. Because most of the materials were harvested before flowering time for DNA analysis, the only available floral character was flower colour based on information provided by the seed collectors.

The multivariate numerical analysis on morphological data was carried out with cluster analysis and multidimensional scaling (MDS) analysis using NTSYS-pc version 2.0 (Rohlf, 1998). The cluster analysis was performed following the procedure outlined in Chapter Five.

Table 6.1 Accessions of *Drosera indica* used in morphological and RAPD analysis

Accession	Place of original collection	Source of material
dro-14	east of Drysdale River, Kimberley, WA	TC
dro-17	Merry Creek, Kimberley, WA	TC
dro-21	Honeymoon Beach, Kimberley, WA	TC
dro-22	Barnett Station, Kimberley, WA	TC
dro-23	Barnett Station, Kimberley, WA	GH
dro-24	Deep Creek, Kimberley, WA	TC
dro-29	Grevillea Creek, Kimberley, WA	TC
dro-34	Beverley Springs, Kimberley, WA	TC
dro-36	Cairns, QLD	TC
dro-38	Darwin, NT	TC
dro-39	Darwin, NT	TC
dro-42	Taiwan	GH
dro-53	Kimberley, WA	GH
JGC738A	500 m east of Fog Dam, NT	TC
JGC738B	500 m east of Fog Dam, NT	TC

Note: TC = tissue culture
GH = glasshouse

6.3.2 RAPD analysis

Fifteen accessions of *D. indica* (Table 6.1) were used for the assessment of genetic variation using random amplified polymorphic DNA (RAPD). The DNA was obtained from fresh leaf tissue extracted using the protocol of Bekesiova *et al.* (1999) as described in Chapter Four.

Thirty decamer oligodeoxynucleotide primers from Operon Technologies were screened, and, based on the number of polymorphic bands produced and the reproducibility of the bands, six primers were chosen: OPA01, OPA03, OPA08, OPA13, OPA18, and OPB07. The RAPD reaction was carried out in a volume of 20 μL, containing 200 ng DNA, 1.5 mM $MgCl_2$, 1 x PCR buffer (Gibco BRL®, Life Technologies), 0.25 μM decamer oligodeoxynucleotide primer (Operon Technologies), 1 unit *Taq* DNA polymerase (Gibco BRL®, Life

Technologies), and 200 μM each of dGTP, dATP, dTTP, and dCTP (Gibco BRL®, Life Technologies). The reaction mixture was then overlaid with two drops of mineral oil. The amplification reaction was performed in an MJ Research Programmable Thermal Cycler, using a temperature profile consisting of an initial denaturation of 2 min at 95°C, followed by 39 cycles of 1 min at 95°C, 10 s 50°C, 15 s at 45°C, 20 s at °C, 1 min at 35 °C, 30 s at 45 °C, 1 min 45 s at 72 °C, and a final extension step of 5 min at 72 °C (Mekuria *et al.*, 1999). Each RAPD reaction was carried out twice to check the reproducibility of amplification fragments.

The amplified DNA fragments were separated electrophoretically on 1.5% agarose gels. Eight μL of each DNA sample was mixed with two μL of 10 x loading buffer (500 μL glycerol, 150 μL 0.5 M EDTA, 349 μL sterile water, 10 μL 20 % SDS, 1 mg bromophenol blue, 1 mg xylene cyanol FF), and loaded on 1.5% agarose (SeaKem®). A 100-bp ladder (GeneWorks) was included in the gel as a molecular weight standard. The gel was run in 1 x TBE (89 mM Tris-borate, 2 mM EDTA pH 8.0) at 80 mA for 90 min, stained with ethidium bromide (0.5 μg/mL), and visualised under UV illumination. The gel image was captured using "Video Capture-Composite", CAPTV Program (Tekcap V 1.0, Tekram Corporation), and converted to a JPEG image file using Paint Shop Pro 5.0 (Jasc Software, Inc.). An example of a typical gel is given in Appendix VIII.

The molecular weight of amplified DNA fragments (RAPD bands) was determined using Gel-Pro® Analyzer version 3.1 (Media Cybernetics, Silver

Spring, Maryland, USA). Only fragments that were present in both replicates and those that could be scored unambiguously were used in the analysis. The bands were scored as 1 for present and 0 for absent. The binary matrix of these bands was then subjected to cluster analysis and multidimensional scaling analysis using NTSYS-pc version 2.0 (Rohlf, 1998). The dendrogram was constructed using UPGMA based on the Dice coefficient of similarity (S_D). This coefficient excludes any shared absence (0-0 matches), and puts a double weighting on shared presence (1-1 matches) (Sokal and Sneath, 1963; Rohlf, 1998).

6.4. Results

6.4.1. Morphological analysis

The dendrogram of morphological data (Figure 6.1) differentiates the accessions into four clusters: **A**, **B1**, **B2**, and **C**. Each cluster displays a distinctive combination of seed and vegetative characters. Cluster **A** consisted of plants with foveolate seeds, green stems, and, except for dro 38, they had petiolate leaves. Cluster **B1** comprised of plants with reticulate subtype transversely hexagonal seeds, green stems, and, except for dro 39, they had apetiolate leaves. Cluster **B2** composed of individuals with reticulate subtypes longitudinally hexagonal and tetragonal, orange-red or red stems, and petiolate leaves. Cluster **C** only consisted of one accession, dro 23, with furrowed seeds, green stems, and petiolate leaves. The cophenetic correlation value of 0.93 indicates that the clusters were unlikely to be a random event.

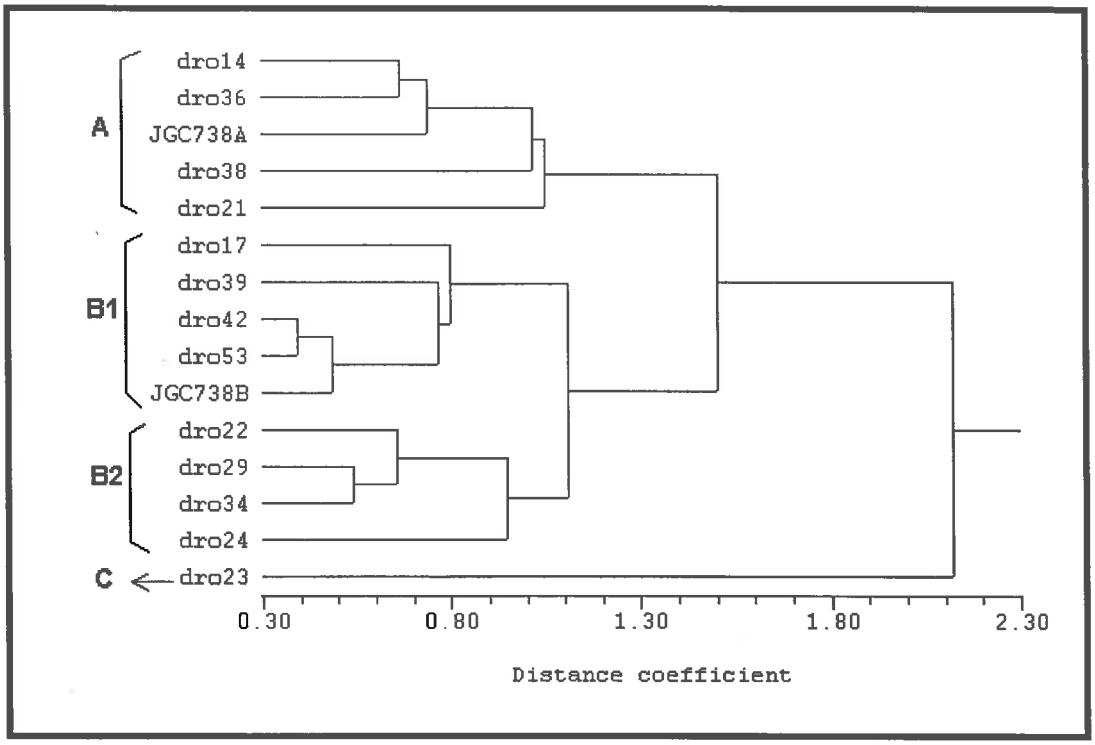

Figure 6.1 UPGMA dendrogram of 15 accessions of *Drosera indica* based on 16 morphological characters, showing the grouping of accessions into four groups. A: plants with foveolate seeds, green stems, and petiolate leaves; B1: plants with reticulate subtype transversely hexagonal seeds, green stems, and apetiolate leaves; B2: plants with reticulate subtypes longitudinally hexagonal or tetragonal seeds, orange-red or red stems, and apetiolate leaves; C: plant with furrowed seeds, green stems, and leaves with petiole and a red stripe on the back.

The result of MDS analysis is shown in Figure 6.2. The grouping of accessions into four groups is in agreement with, and corresponds to that from cluster analysis. The spatial arrangement of accessions in this two-dimensional plot corresponds to that shown in the dendrogram (Figure 6.1), where dro 23 is positioned some distance from the other groups, and groups **B1** and **B2** are relatively close to each other. The stress coefficient of 0.08 suggests that the distances between objects in this two-dimensional ordination give a good representation of dissimilarities between accessions.

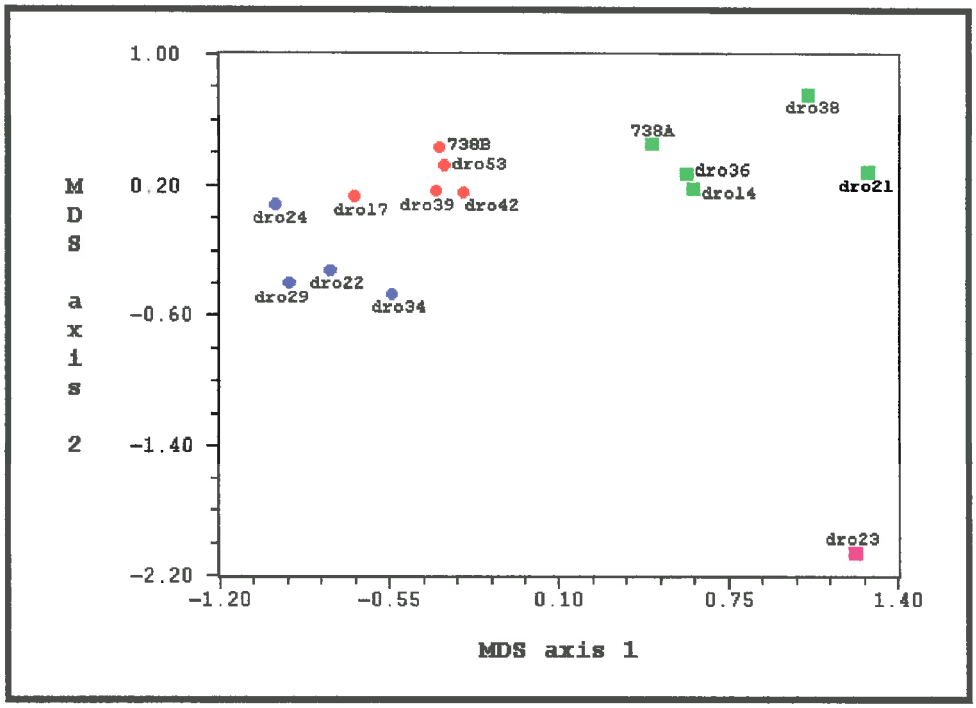

Figure 6.2 The MDS analysis of 15 accessions of *Drosera indica* based on 16 morphological characters, showing the grouping of accessions into four groups.

■: plants with foveolate seeds, green stems, and petiolate leaves;
●: plants with reticulate subtype transversely hexagonal seeds, green stems, and apetiolate leaves;
●: plants with reticulate subtypes longitudinally hexagonal or tetragonal seeds, orange-red or red stems, and apetiolate leaves;
■: plant with furrowed seeds, green stems, and leaves with petiole and a red stripe on the back.

6.4.2. RAPD analysis

The result of the cluster analysis on RAPD data is presented in Figure 6.3, where the accessions were grouped into three clusters. The cluster analysis indicates the high genetic variability among the fifteen *D. indica* accessions under study. The first cluster, in the upper part of the dendrogram, consisted of three accessions (dro 14, dro 36, and dro 17) which grouped together at a similarity coefficient of 0.08. The second cluster comprised of ten accessions

joined together at a similarity coefficient of 0.05. Accessions 738A and 738B formed the third cluster at a similarity coefficient of 0.1. The cophenetic coefficient of 0.94 indicates that the grouping of accessions in this dendrogram is a very good representation of similarities between accessions based on RAPD banding patterns, and is unlikely to be a random event.

When the grouping of accessions based on morphological data was superimposed onto this dendrogram (shown as bold letters in Figure 6.3) it can be seen that two small clusters on the upper and lower part of the dendrogram consisted of individuals from groups **A** and **B1**. The large cluster in the middle of dendrogram, on the other hand, consisted of individuals from four morphological groups: **A**, **B1**, **B2**, and **C**.

The two-dimensional plot of accessions, generated from the MDS analysis of RAPD data, is shown in Figure 6.4. The placement of accessions in this MDS plot shows the same pattern as that in Figure 6.3, particularly for those accessions that had a high genetic similarity, such as dro 42 and dro 53 (S_D = 0.96), and dro 22 and dro 29 (S_D = 0.76).

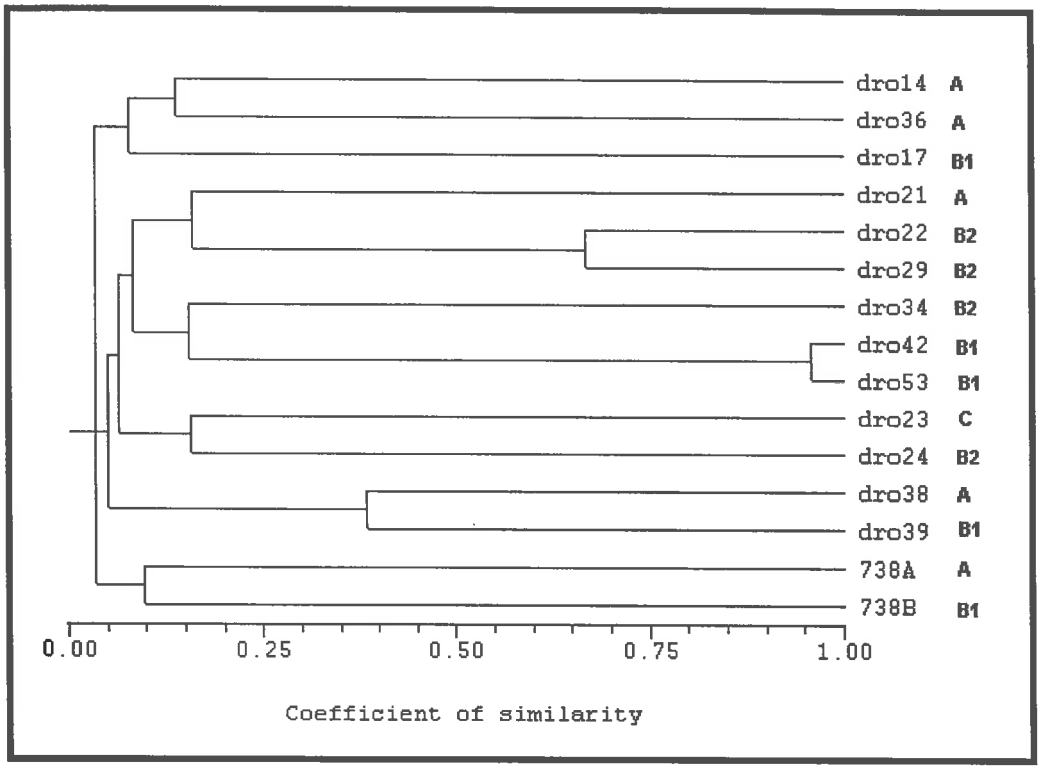

Figure 6.3 UPGMA dendrogram of 15 accessions of *Drosera indica* based on RAPD data. The bold letters indicate morphological groups. A: plants with foveolate seeds, green stems, and petiolate leaves; B1: plants with reticulate subtype transversely hexagonal seeds, green stems, and apetiolate leaves; B2: plants with reticulate subtypes longitudinally hexagonal or tetragonal seeds, orange-red or red stems, and apetiolate leaves; C: plant with furrowed seeds, green stems, and leaves with petiole and a red stripe on the back.

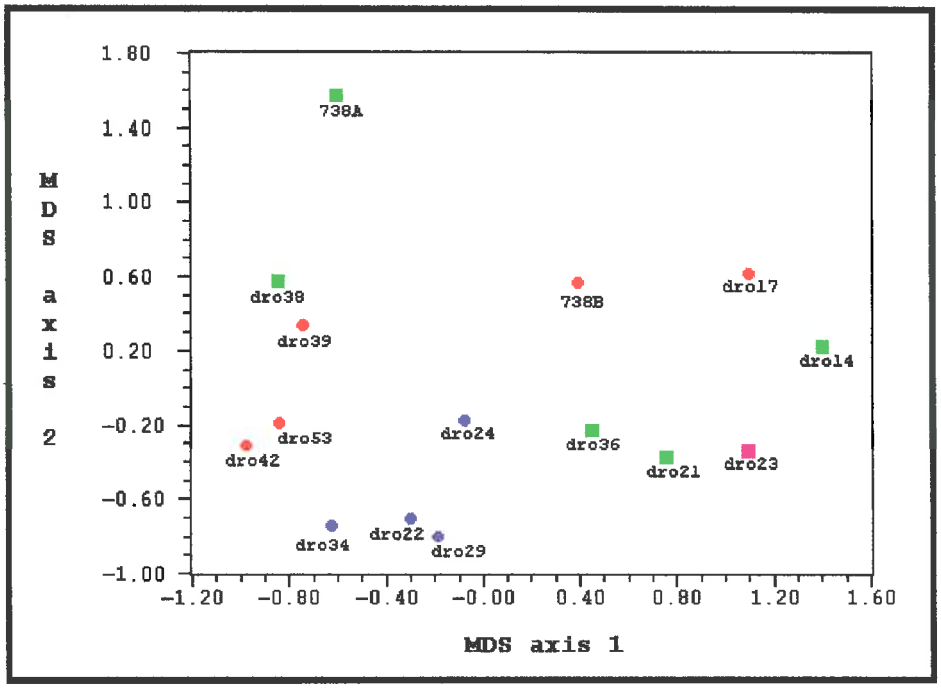

Figure 6.4 The MDS analysis of 15 accessions of *Drosera indica* based on RAPD data.

■: plants with foveolate seeds, green stems, and petiolate leaves;
●: plants with reticulate subtype transversely hexagonal seeds, green stems, and apetiolate leaves;
●: plants with reticulate subtypes longitudinally hexagonal or tetragonal seeds, orange-red or red stems, and apetiolate leaves;
■: plant with furrowed seeds, green stems, and leaves with petiole and a red stripe on the back.

6.5 Discussion

The four groups of accessions revealed from cluster analysis and ordination analysis based on 16 morphological characters are in general agreement with the defined morphotypes of *D. indica*, except that the analysis result failed to distinguished two morphotypes in cluster **A**, and two in cluster **B2**. This was due to the fact that the morphological data used in the present study lacked floral characters that differentiated accessions into six morphotypes as defined in Chapter Five, i.e. filament form. This result, therefore, verifies the

importance of floral characters in defining infraspecific variation in *D. indica*. In this case, the inclusion of floral characters has proven to produce a refinement in the delimitation of morphological variation. The refinement process has been argued as important in taxonomy for giving confidence that the observed pattern is stable (Abbott, 1985).

The dendrogram of 15 accessions of *D. indica* based on RAPD data (Fig. 6.3) shows that three clusters were formed with a low genetic similarity coefficients, i.e. 0.08, 0.05, and 0.1, respectively. This result, thus, indicates a low degree of genetic similarity between accessions, except for dro 42 and dro 53, and dro22 and dro 29, which joined at similarity coefficient of 0.96 and 0.67, respectively.

In an attempt to determine whether the grouping of accessions produced by DNA fingerprints was in agreement to that generated from morphological data, the defined morphotypes were plotted onto the dendrogram and two-dimensional plot of the MDS of RAPD analysis (Figs. 6.3 and 6.4). From these two diagrams, it was apparent that the RAPD grouping did not coincide with the morphological grouping. In most cases, accessions from different morphotypes were intermixed, reflecting the lack of genetic differentiation on the defined morphological groups. Nevertheless, from the dendrogram (Figure 6.3) there are three pairs of accessions that show an agreement between morphological and RAPD grouping, i.e. dro 14 and dro 36 (group **A**), dro 22 and dro 29 (group **B2**), and dro 42 and dro 53 (group **B1**). This means that although morphology and RAPD data do not reveal the same pattern of

differentiation, the two data sets are in agreement in detecting the similarities between pairs of accessions.

The high degree of genetic dissimilarity between accessions revealed from RAPD data analysis confirms the inbreeding nature of *D. indica*. In this case, it seems that selfing is a mechanism that responsible for maintaining the morphological and genetic identity of each individual accession, and thus explaining the high genetic diversity between them. This result supports the uniformity of seeds from each type, even when different morphotypes co-exist, for example dro 22 and dro 23, and JGC 738A and JGC 738B. More importantly, this result provides evidence on the existence of reproductive, rather than geographical or ecological isolation mechanisms that prevent co-occurring populations to exchange genes. The existence of reproductive isolation between sympatric populations suggests that morphotypes could be considered as distinct species. It has been recognised that sympatry is the test for biological species, i.e. sympatric populations that retain their identity and remain distinct from each other are regarded as separate species (Sivarajan, 1990). Furthermore, Sivarajan (1990) argued that reproductive isolation that prevents populations from exchanging genes provides some objective basis for the species category in sexually reproducing organisms.

Comparing the results of morphological and RAPD analysis, it is apparent that there is an incongruence between the two data sets in the grouping of accessions, thus reflecting different pattern of variation. It has been recognised, however, that this incongruence between morphological and

molecular data is a normal rather than exceptional case (Prentice, 1986; Jasienski *et al.*, 1997; Heaton *et al.*, 1999).

Some possible causes of the discordant patterns in morphology and molecular data have been pointed out by many authors, which can be summarised into two factors (Davis and Gilmartin, 1985; Wilson, 1989; Quicke, 1993; Krauss, 1996; Black-Samuelson *et al.*, 1997; Brunell and Whitkus, 1997). The first possibility is that morphological and molecular change or differentiation may proceed differently, due to differences in modes and rates of change, especially the concept of phenotypic plasticity (Davis and Gilmartin, 1985; Quicke, 1993; Krauss, 1996). Secondly, it has been argued that morphology is affected by environment (Wilson, 1989; Black-Samuelson *et al.*, 1997; Brunell and Whitkus, 1997). Whereas, apart from changes in methylation, DNA is not influenced by differences in either developmental stages of the plant or environmental changes (Wolff *et al.*, 1995; Fanizza *et al.*, 1999).

Another explanation for the discrepancy between the pattern of variations detected from morphology and molecular analysis is the presence of non-coding region in the genomic DNA. Because RAPD primers amplify randomly across the entire genome, there is great possibility that non-coding regions will also be amplified (Black-Samuelson *et al.*, 1997; Wen and Hsiao, 1999). The amount of non-coding region has been estimated up to 90 – 95 % of the nuclear genome in some plants (Flavell, 1980; Crawford, 1990). The amount of non-coding DNA in *D. indica* is not known but can be assumed to be a significant proportion of the genome. In a similar way, Wolf *et al.* (1995)

claimed that because only a small portion of the genome is sampled by RAPD, some regions that influence the phenotype may not be detected. In addition, it has been argued that many of the polymorphisms revealed by RAPD could be neutral markers that are not correlated or linked to differences in phenotype or adaptation to habitat (Heaton *et al.*, 1999).

It has been recognised that RAPD can provide an estimation on genetic variation in natural population which is useful in providing a measure of gene flow and also in detecting hybridisation and recombination patterns (van Heusden and Bachmann, 1992b; Crawford, 2000). It has been pointed out that the estimation of genetic variation is associated with breeding systems (Nybom, 1993). In this regard, RAPD can provide information on who breeds with whom (Karp *et al.*, 1997). In the case of *D. indica*, however, due to the inbreeding nature of this species, this kind of information is lacking. This may explain the incongruence between results of morphological and RAPD data.

Before any taxonomic conclusion can be made on the infraspecific variation, it should be clear what species or taxon concept is applied in any taxonomic study (Tyteca and Dufrêne, 1994; Luckow, 1995; McDade, 1995; Wendt *et al.*, 2000). This concept will be referred to when making a decision on the taxonomic status of taxa. In this regard, the present study employs the biological species concept.

Considering the evidence that the six defined morphotypes showed a considerable degree of morphological differences, and that these differences were maintained between co-occurring populations by some sorts of reproductive isolation, it could be argued that morphotypes in *D. indica* complex may warrant recognition as distinct species. Nevertheless, regarding the wide area of distribution of *D. indica* across different habitats and continents, detail examinations of representative specimens worldwide covering as much as morphological variations is required before a justifiable taxonomic conclusions could be made.

6.6 Conclusion

Morphological and genetic variation patterns suggest that the morphotypes in the *D. indica* complex could be considered as distinct species. Further investigations are needed in order to justify the suggested taxonomic implications revealed in this study.

Chapter Seven

General Discussion

Drosera indica is a species known to exhibit a wide range of morphological variability in both vegetative and reproductive organs. The most obvious variations occur in plant size and flower colour, and these have been the basis for assigning synonyms at the infraspecific level, including *D. indica* L. forma *robusta* F.M. Bail., *D. indica* forma. *albiflora* Makino, *D. indica* var. *albiflora* (Makino) Makino, and *D. indica* L. forma *rosea* Makino. Variability in seed morphology, such as size, ornamentation patterns, and epicuticular waxes, has also been identified (Boesenwinkel, 1989; Lowrie, 1998). The phenotypic expression of *D. indica* morphotypes is consistent for plants grown under natural, glasshouse, or *in vitro* conditions, and therefore appears to be a function of the genotype.

In an attempt to determine the taxonomic implication of the observed morphological variation, the examination on the geographic distribution and ecological data was carried out on selected accessions collected from Kimberley region of Western Australia. It has been claimed that morphological distinctness and geographical or ecological coherence is considered necessary for the recognition of infraspecific taxa (Eckenwalder, 1996; Chiapella, 2000; Wendt *et al.*, 2000).

D. indica is mainly found in remote areas in the north west of Western Australia. Due to the seasonal growth and the remote areas where the natural populations found, the most accessible material to conduct this research was seeds and air-dried herbarium specimens.

Scanning electron microscopy on 13 micromorphological characters of 74 seed samples indicated that the seeds of *D. indica* can be differentiated into three types based on their seed coat ornamentation patterns, i.e. the reticulate, foveolate, and longitudinally ridged or furrowed types. The reticulate seed type can be divided into three subtypes based on the differences in the shape of seed coat cells, i.e. tetragonal, transversely hexagonal, and longitudinally hexagonal subtypes. This finding shows the taxonomic importance of seed coat ornamentation pattern as a diagnostic character in defining infraspecific variation, as has been suggested by Rejdali (1995) and Koul *et al.* (2000). Other micromorphological characters vary in their contribution toward the grouping of seeds into their types, and none of them are considered as showing comparable role as the ornamentation pattern in distinguishing seed types.

Detailed examination on the epicuticular waxes on seed surface indicated that the form of the wax particles showed high inter- and intra-sample variability, and therefore this character was not included in the analysis. Observation on other morphological characters, e.g. stem colour, filament form, and the presence or absence of petiole, resulted in the recognition of five seed-based taxa, each representing a distinctive combination of seed types or

subtypes with other morphological characters. This result provides evidence on the existence of infraspecific variability in *D. indica* complex.

Examination on the 26 morphological characters from vegetative, floral, and seed features on 62 accessions of *D. indica* using multivariate analysis methods revealed the differentiation of accessions into six morphological groups. The agreement between the results of cluster analysis and the two ordination techniques (multidimensional scaling and principal component analysis) gave confidence to assign the groups as six different morphotypes. Each of the morphotypes shows a characteristic combination of morphological features. These morphotypes occurred sympatrically, and did not show distinct geographical or ecological patterns, and therefore it could be argued that they represented different varieties (van den Berg and Spooner, 1992; Stuessy, 1994; Thiele and Ladiges, 1994). However, there is also a possibility that they might represent sympatric species, as indicated by the co-existence of different morphotypes in some localities. In fact, the extent of morphological differences between morphotypes was of comparable degree to those used to differentiate species in other members of Droseraceae (Lowrie, 1994, 1996; Lowrie and Conran, 1998). However, to be able to formulate conclusive taxonomic implications on the morphological variation patterns of *D. indica*, further studies are needed especially those examining the breeding systems to determine whether populations of different morphotypes interbred. Similarly, examination of the occurrence of intermediate forms is required to detect the hybridisation between morphotypes to justify whether

they are indeed could be recognised as distinct species (Lowrie and Conran, 1998).

The assessment of genetic variation was carried out to determine whether the six morphologically distinguishable groups are genetically distinct. Herbarium specimens failed to produce DNA of sufficient amounts and quality to be used in the molecular analysis using DNA fingerprinting technique. Four DNA extraction protocols were tested (Steenkamp *et al.*, 1994; Wang *et al.*, 1996; Bekesiova *et al.*, 1999; and Qiagen® DNeasy™ Plant Mini Kit protocols), and none of them were successful in recovering DNA from the herbarium specimens. DNA from these materials were highly degraded and contaminated with oxidised compounds. The prolonged desiccation process is believed to be responsible for the degradation of these DNA. The same result was observed in plant materials preserved in silica sand (Moisture-Gone™). The fresh, water-preserved, and silica gel-dried materials, on the other hand, produced DNA of high molecular weight and adequate quality when extracted using the technique of Bekesiova *et al.* (1999). This result highlights the requirement of rapid drying procedure to prevent the degradation of DNA by limiting endogenous hydrolytic damages (Eglinton and Logan, 1991; Savolainen *et al.*, 1995; Štorchová *et al.*, 2000), and supports the finding of Chase and Hills (1991) in the use of silica gel as a drying agent for preservation of plant materials for DNA analysis.

The method of DNA fingerprinting using random amplified polymorphic DNA (RAPD) technique was used for the estimation genetic variation. In order to

make a reasonable comparison between morphological and genetic variations, the same set of sample (accessions) were used. Fifteen accessions of *D. indica,* which produced good quality DNA, were used for the analysis the pattern morphological and genetic variation. These materials were obtained from tissue culture- and glasshouse-grown plants germinated from seeds. Because most of the tissue culture plant materials were harvested before flowering, the only available floral character for analysis was flower colour, as noted in seed collection detail.

Cluster analysis and multidimensional scaling ordination technique were performed on 16 morphological characters, from which four morphological groups were identified. In general, these groups correspond to the defined morphotypes, but the lack of filament form character makes the differentiation between highly similar morphotypes cannot be made, and this explains the different result in the grouping of accessions. This result shows the importance of floral characters in defining the differentiation of *D. indica* accessions into their respective morphotypes.

Morphology and RAPD data are in general agreement in detecting the degree of similarity between accessions. In this regard, there are two cases where pair of accessions from the same morphotypes cluster together at high similarity coefficient in dendrogram generated from RAPD data. They are accessions dro 42 and 53, and accessions dro 22 and dro 29, which cluster at the similarity coefficient of 0.96 and 0.67, respectively.

The grouping of accessions based RAPD data was different from that generated based on morphological data. The three groups generated from cluster analysis and multidimensional scaling ordination of molecular data did not correspond to the defined morphotypes. In this case, one cluster or group consisted of different morphotypes, and there was no consistent pattern on the grouping of individual accessions. This result indicates differences in the pattern of morphological and genetic variation. It has been claimed, however, that the incongruence between results of morphological and molecular data is a normal case (e.g. Prentice, 1996; Jasiénski *et al.*, 1997; Heaton *et al.*, 1999). There are some factors that may cause the discordant pattern in morphology and molecular data. First, the differences in the mode and rate of change between morphology and molecules, so that their change may proceed differently under similar selective pressure (Davis and Gilmartin, 1985; Quicke, 1993; Krauss, 1996). The second factor is the nature morphology which, unlike DNA, is susceptible to the environmental conditions, such as differences in habitat and period of the growing season (Wolff *et al.*, 1995; Black-Samuelson *et al.*, 1997; Brunell and Whitkus, 1997; Fanizza *et al.*, 1999). In the case of this study, the property of RAPD itself could be another factor that causes the discrepancy between the patterns of morphological and genetic variations. It has been known RAPD primers amplify randomly across the entire genome, and that a large proportion of the plant genome composed of non-coding region, there is possibility that the non-coding regions of the genome will have a greater chance to be amplified (Black-Samuelson *et al.*, 1997; Wen and Hsiao, 1999). If this is the case, it seems reasonable that differences in RAPD banding pattern do not correlate

to differences in the morphology. This argument has been suggested by Heaton *et al.* (1999) in which polymorphisms revealed by RAPD could be neutral markers, i.e. those that do not correlate to differences in phenotype or adaptation to habitat.

Despite the above-mentioned explanations, there is another characteristic of RAPD that could explain the observed incongruence between the patterns of variation in morphology and RAPD. It has been recognised that one of the most widely used applications of RAPD in population studies is in the detection of gene flow, hybridisation, and recombination patterns by providing an estimation of genetic variation (van Heusden and Bachmann, 1992b; Karp *et al.*, 1997; Crawford, 2000). While such information could be obtained from outcrossing species, this is not the case for inbreeders. Selfing facilitates the maintenance of pure inbred lines, and repeated selfing results in the complete homozygosity in a few generations (Briggs and Walters, 1984; Stace, 1989; Richards, 1997). As a consequence, the phylogenetic information based on the extent and distribution of genetic variation could not be deduced from RAPD when it was used in the study of inbreeding species. The problem on the application of RAPD for the estimation of genetic variation for inbreeding species has been pointed out by Huff *et al.* (1993).

Analysis of RAPD data indicates the high degree of genetic dissimilarity between *D. indica* accessions, thus confirms its inbreeding nature. Results also suggest that each individual accession has maintained its inbred line, which explains the consistent separation of accessions into their respective

seed types. It seems that selfing is favourable for *D. indica* by providing advantages that facilitate the survival of this taxon, i.e. the reproductive assurance and the short-term adaptive advantages as an annual plants growing in less favourable habitats, e.g. poor sandy or stony soils or acid swampy areas (Levin, 1971; Jain, 1976; Briggs and Walters, 1984; Richards, 1997).

The evidence that co-occurring morphotypes remained distinct from each other indicated the existence of reproductive isolating mechanisms, which further supports the recognition of distinct species. However, considering the worldwide distribution of *D. indica*, detailed examination of specimens covering as much as possible its range of geographic distribution, habitat, and morphological variations is required to make a justifiable taxonomic conclusion, otherwise it could lead to the recognition of non-dimensional species described by Mayr (1969). Before any conclusive taxonomic treatment could be made, it is suggested that the six morphotypes defined here be considered as members of *D. indica* complex.

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APPENDICES

Appendix I. Accessions used in this study

Accession	Code [a]	Locality	Specimen details [b]
AL 84	A1	South end of Driver Avenue, Palmerston, NT	Plant green, petiolate; flowers pink
AL 261	A2	Chewko, near Mareeba, North Queensland	Plant green, petiolate; flowers white
AL 962	A3	Durack River crossing, on Gibb River Road, Kimberley, WA	Plant green, apetiolate; flowers pink
AL 965	A4	3 km north of King Edward River crossing, Kimberley, WA	Plant green, petiolate; flowers white, stamens blood-red and cobra-like
AL 976	A5	2.5 km north of King Edward River crossing, Kimberley, WA	Plant reddish, apetiolate; flowers dark pink, large
AL 1031	A6	Russ Creek, east of Drysdale River Station, Kimberley, WA	Plant green, apetiolate; flowers pink
AL 1036	A7	Durack River Station, Kimberley, WA	Plant green, petiolate; flowers magenta
AL 1103	A8	Parry Lagoon, west of Kununurra, WA	Plant reddish, small, apetiolate; flowers pink
AL 1108	A9	15 km west of Kununurra, WA	Plant red, small, apetiolate; flowers dark pink
AL 1113	A10	15 km west of Kununurra, WA	Plant green, petiolate; flowers pink
AL 1162	A11	Gibb River Road, 119 km from Derby, WA	Plant green, petiolate; flowers cerise, stamens yellow
AL 1164	A12	Gibb River Road, 119 km from Derby, WA	Plant red, apetiolate; flowers pale pink
AL 1172	A13	Bell Creek, Silent Grove, WA	Plant red, apetiolate; flowers pale pink, small
AL 1173	A14	Bell Creek, Silent Grove, WA	Plant green, petiolate; flowers pink
AL 1193	A15	Grevillea Creek, Beverley Springs Station, WA	Plant orange-red, apetiolate; flowers apricot, stamens yellow
AL 1195	A16	Grevillea Creek, Beverley Springs Station, WA	Plant green, apetiolate; flowers mauve, stamens yellow
AL 1200	A17	Grevillea Creek, Beverley Springs Station, WA	Plant green, petiolate; flowers white, stamens red, cobra-like, large
AL 1208	A18	about 500 m from Grevillea Creek, Beverley Springs Stn, WA	Plant orange-red, apetiolate; flowers orange, stamens yellow
AL 1215	A19	north side of Grevillea Creek, Beverley Springs Station, WA	Plant green, petiolate; flowers white, stamens red, cobra-like, large
AL 1283	A20	Merry Creek crossing, Mt. Elizabeth Station, Kimberley, WA	Plant green, petiolate; flowers cerise, stamens red, deltoid
AL 1284	A21	Merry Creek crossing, Mt. Elizabeth Station, Kimberley, WA	Plant green, petiolate; flowers light pink
AL 1289	A22	Merry Creek crossing, Mt. Elizabeth Station, Kimberley, WA	Plant green, petiolate; flowers pale mauve
AL 1318	A23	Mt. Elizabeth Station, 3 km from Hann River, Kimberley, WA	Plant green, petiolate; flowers white, stamens red, large, cobra-like
AL 1322	A24	Merry Creek crossing, Mt. Elizabeth Station, Kimberley, WA	Plant maroon-red, apetiolate; flowers purple, large
AL 1325	A25	Merry Creek crossing, Mt. Elizabeth Station, Kimberley, WA	Plant green, petiolate; flowers magenta, stamens red, large, deltoid
AL 1352	A26	Nallam Dam, 19 km north of Cue, WA	Plant green, petiolate; flowers white to very pale pink
AL 1694		Taylors' Lagoon, Broome, WA	Plant red, apetiolate; flowers pink
AL 1697	A27	About 1 km from Taylors' Lagoon, Broome, WA	Plant green, apetiolate; flowers pink, stamens yellow
AL 1716	A28	about 114 km from Derby, Kimberley, WA	Plant green, petiolate; flowers cerise
AL 1730	A29	about 30 km south of Barnett Roadhouse, Kimberley, WA	Plant green, petiolate; flowers pale pink, stamens blood-red
AL 1734	A30	Drysdale Mound Spring, Kimberley, WA	Plant red, green leaves, apetiolate; flowers pink
AL 1749	A31	Kimberley, WA	Plant green, petiolate; flowers pink, large
AL 2206	A32	entrance to Taylors' Lagoon, Broome, WA	Plant green, petiolate; flowers cerise with red centre, stamens red, deltoid
AL 2216	A33	about 129 km west of Fitzroy Crossing, Kimberley, WA	Plant green, apetiolate; flowers pink, stamens yellow
AL 2219	A34	about 129 km west of Fitzroy Crossing, Kimberley, WA	Plant green, apetiolate; flowers pink, stamens yellow
AL 2220	A45	about 250 km north of Halls Creek, Kimberley, WA	Plant green, petiolate; flowers cerise, stamens red, deltoid
AL 2225	A36	west of Kununurra, WA	Plant reddish, small, apetiolate; flowers dark pink

Appendix I (continued)

Accession	Code [a]	Locality	Specimen details [b]
AL 2226	A37	west of Kununurra, WA	Plant green, petiolate; flowers **pink**
AL 2227	A38	west of Kununurra, WA	Plant green, petiolate; flowers cerise, stamens cerise, deltoid, large
AL 2242	A39	Cave Spring, Kununurra, WA	Plant green, petiolate; flowers cerise, stamens yellow
AL 2243	A40	Cave Spring, Kununurra, WA	Plant green, petiolate; flowers cerise, stamens yellow
AL 2249		about 31.8 km south of Kununurra, WA	Plant green, petiolate; flowers pink, small
AL 2255	A41	Stuart Highway, about 85 km north of Katherine, NT	Plant green, petiolate; flowers pink
AL 2273	A42	Howard Spring Lagoon, NT	Plant green, petiolate; flowers cerise
AL 2299	A43	Elrundie Road, opposite 'Dry Dump', Palmerston, NT	Plant green, small, apetiolate; flowers pink, stamens yellow
AL 2302	A44	end of Owston Ave, near drain Palmerston, NT	Plant red, small, apetiolate; flowers white
AL 2304	A45	Jenkin Road, about 2 km from Stuart Highway, NT	Plant green, petiolate; flowers cerise
AL 2461	A46	Crossing Falls, Kimberley, WA	Plant green, apetiolate; flowers pink
AL 2462	A47	Crossing Falls, Kimberley, WA	Plant red, apetiolate; flowers dark pink
AL 2508	A48	Yampi Ren, Kimberley, WA	Plant green, petiolate; flowers pink
AL 2523	A49	Phillips Range, Kimberley, WA	Plant green, petiolate; flowers white, stamens red, cobra-like
AL 2525	A50	Adcock Creek, Kimberley, WA	Plant red, apetiolate; flowers orange
AL 2526	A51	Adcock Creek, Kimberley, WA	Plant green, petiolate; flowers white, stamens red, cobra-like
AL 2542	A52	Lily Creek, Kimberley, WA	Plant green, petiolate; flowers cerise
CR	A53	Mt. Augustus, WA	Plant green, apetiolate; flowers pink
DEM 1812	A54	80 km north of Tennant Creek, NT	Plant green, apetiolate; flowers light pink, stamens yellow
FR s.n. 'A'	A55	Tsukuba, Tokyo area, Japan	Plant green, petiolate; flowers white
FR s.n. 'B'	A56	Goa, India	Plant green, petiolate; flowers pink
JGC 738A	A57	500 m east of Fogg Dam, NT	Plant green, petiolate; flowers mauve/pink, stamens yellow
JGC 738B	A58	500 m east of Fogg Dam, NT	Plant green, apetiolate; flowers mauve/pink, stamens yellow
JGC 739	A59	on Darwin-Katherine highway, NT	Plant green, petiolate; flowers bright orange, stamens red, deltoid
Dro 14		east of Drysdale River, Kimberley, WA	Plant green, petiolate, up to 60 cm high; flowers pink, large
Dro 17		Merry Creek, Kimberley, WA	Plant maroon-red, apetiolate; flowers purple, large
Dro 21		Honeymoon Beach, Kimberley, WA	Plant green, petiolate; flowers cerise
Dro 22		Barnett Station, Kimberley, WA	Plant red, large, petiolate; flowers orange
Dro 23	A60	Barnett Station, Kimberley, WA	Plant green, robust, petiolate; flowers pale pink, stamens red, cobra-like
Dro 24		Gibb River, Kimberley, WA	Plant reddish, petiolate; flowers pale pink
Dro 29		Grevillea Creek, Kimberley, WA	Plant orange-red, petiolate; flowers apricot-orange, stamens yellow
Dro 34		Beverley Springs, WA	Plant orange-red, petiolate; flowers orange
Dro 36		Cairns, Queensland	Plant green, petiolate; flowers white-blushed pink
Dro 38		Darwin, NT	Plant green, apetiolate; flowers orange
Dro 39		Darwin, NT	Plant green, petiolate; flowers lilac
Dro 42	A61	Taiwan	Plant green, apetiolate; flowers pale pink, stamens yellow
Dro 53	A62	Kimberley, WA	Plant green, apetiolate; flowers pale pink, stamens yellow

[a] Accession code only given for those used in morphological study (Chapter Five)
[b] Details as shown in specimen label, except for the presence/absence of petiole

Appendix II. Results of examination on seed morphological characters

Collection	SES	SEO	SEL	SEW	PSH	NPL	NPW	PCU	PFS	SLR	POL	POW	ECW a)
AL 84	ovoid - ellipsoid	foveolate	572	310	elliptic	20	7	deeply concave	undulate	present	20.5	44.7	igr; ros
AL 261	ovoid	reticulate	510	334	tetragonal	18	5	deeply concave	smooth	absent	22.1	66.3	ros
AL 962	ovoid - ellipsoid	reticulate	429	264	transversely hexagonal	10	5	concave	undulate	absent	35.5	51.3	igr; ros
AL 965	ellipsoid	furrowed	736	390	rectangular	12	5	deeply concave	wavy	present	93.9	59.0	igr
AL 976	ovoid - ellipsoid	reticulate	361	238	longitudinally hexagonal	8	6	flat	undulate	absent	49.5	33.4	igr; ros
AL 1031	ovoid	reticulate	446	267	transversely hexagonal	9	5	concave	smooth	absent	35.9	58.6	igr; ros
AL 1036	ovoid	foveolate	558	411	elliptic	24	5	deeply concave	smooth	present	23.8	51.8	rgr
AL 1103	ovoid	reticulate	262	187	longitudinally hexagonal	7	5	flat	undulate	absent	48.9	31.7	igr; ros
AL 1108	ovoid - ellipsoid	reticulate	273	170	tetragonal	7	5	flat	undulate	absent	44.1	25.1	igr
AL 1113	ovoid - ellipsoid	foveolate	433	273	elliptic	10	6	deeply concave	undulate	present	18.6	48.1	igr; ros
AL 1162	ovoid	foveolate	463	349	elliptic	20	5	deeply concave	smooth	present	20.8	43.4	igr
AL 1164	ovoid	reticulate	266	157	tetragonal	6	5	flat	undulate	absent	45.6	24.7	igr
AL 1172	ovoid - ellipsoid	reticulate	308	175	longitudinally hexagonal	6	4	flat	undulate	absent	58.8	33.4	ros
AL 1173	ellipsoid	foveolate	467	282	elliptic	18	5	deeply concave	undulate	present	22.5	41.2	igr
AL 1193	ovoid	reticulate	393	250	longitudinally hexagonal	8	5	flat	undulate	absent	56.1	47.3	igr; rgr
AL 1195	ovoid - ellipsoid	reticulate	349	199	longitudinally hexagonal	9	5	concave	undulate	absent	51.2	30.0	ros
AL 1200	ellipsoid	furrowed	738	427	rectangular	10	5	deeply concave	wavy	present	101.9	60.5	igr; rgr
AL 1208	ovoid - ellipsoid	reticulate	333	228	longitudinally hexagonal	7	5	flat	undulate	absent	44.4	53.1	rgr
AL 1215	ellipsoid	furrowed	694	357	rectangular	9	5	deeply concave	wavy	present	80.7	49.5	igr; rgr
AL 1283	ovoid - ellipsoid	foveolate	607	380	elliptic	24	6	deeply concave	smooth	present	22.6	45.5	igr; rgr
AL 1284	ovoid - ellipsoid	foveolate	534	297	elliptic	16	5	deeply concave	smooth	present	27.4	52.0	igr; ros

Appendix II. (continued)

Collection	SES	SEO	SEL	SEW	PSH	NPL	NPW	PCU	PFS	SLR	POL	POW	ECW [a)]
AL 1289	ovoid – ellipsoid	foveolate	473	273	elliptic	16	5	deeply concave	smooth	present	24	47.1	igr
AL 1318	ellipsoid	furrowed	912	432	rectangular	11	5	deeply concave	wavy	present	96.4	44.0	igr; ros
AL 1322	ovoid	reticulate	354	257	longitudinally hexagonal	8	5	flat	undulate	absent	49.5	64.6	igr; rgr
AL 1325	ovoid – ellipsoid	foveolate	532	330	elliptic	24	7	deeply concave	smooth	present	16.9	34.6	igr; rgr; ros
AL 1352	ovoid – ellipsoid	reticulate	466	277	transversely hexagonal	12	6	concave	undulate	absent	27.6	53.0	igr; ros
AL 1694	ovoid – ellipsoid	reticulate	351	198	longitudinally hexagonal	7	6	flat	undulate	Absent	30.0	47.0	igr
AL 1697	ovoid – ellipsoid	reticulate	375	232	transversely hexagonal	9	5	flat	undulate	absent	30.7	46.7	igr; ros
AL 1716	ovoid	foveolate	443	293	elliptic	16	5	deeply concave	undulate	present	30.4	62.5	rgr; ros
AL 1730	ellipsoid	furrowed	755	402	rectangular	10	6	deeply concave	wavy	present	99.6	62.5	igr
AL 1734	ovoid – ellipsoid	reticulate	314	175	longitudinally hexagonal	7	5	flat	undulate	absent	50.9	31.9	igr
AL 1749	ovoid – ellipsoid	foveolate	415	279	elliptic	20	5	concave	undulate	present	25.1	45.9	igr
AL 2206	ovoid	foveolate	540	350	elliptic	16	6	deeply concave	undulate	present	24.4	41.6	ros
AL 2216	ellipsoid	reticulate	470	270	transversely hexagonal	13	7	flat	undulate	absent	30.1	40.5	ros
AL 2219	ovoid – ellipsoid	reticulate	406	255	transversely hexagonal	10	5	concave	undulate	absent	35.6	53.7	igr; rgr; ros
AL 2220	ellipsoid	foveolate	699	389	elliptic	20	6	deeply concave	undulate	present	22.6	47.5	rgr
AL 2225	ovoid	reticulate	290	183	tetragonal	6	5	flat	undulate	absent	53.3	31.3	igr; ros
AL 2226	ovoid	foveolate	476	323	elliptic	12	5	deeply concave	smooth	present	19.5	45.7	ros
AL 2227	ovoid – ellipsoid	foveolate	538	365	elliptic	24	7	concave	smooth	present	19.3	54.0	igr; rgr
AL 2242	ovoid	reticulate	508	316	tetragonal	7	5	deeply concave	undulate	absent	23.9	46.5	igr
AL 2243	ovoid – ellipsoid	foveolate	478	267	elliptic	20	5	concave	undulate	present	22.1	49.0	igr
AL 2249	ovoid – ellipsoid	foveolate	392	227	elliptic	18	5	concave	undulate	present	22.1	49.0	igr; ros

Appendix II. (continued)

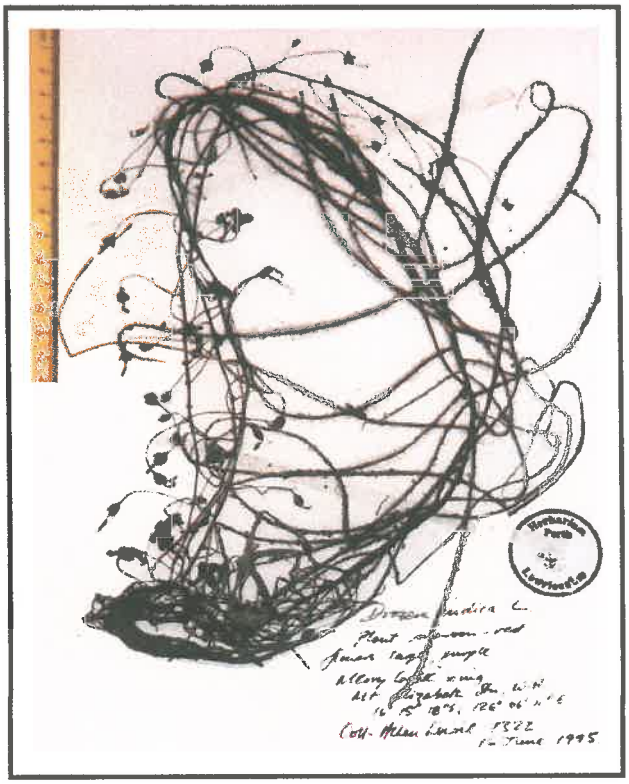
Collection	SES	SEO	SEL	SEW	PSH	NPL	NPW	PCU	PFS	SLR	POL	POW	ECW [a]
AL 2255	ovoid	foveolate	585	372	elliptic	20	6	deeply concave	smooth	present	24.1	54.0	igr; ros
AL 2273	ellipsoid	foveolate	639	405	elliptic	24	8	deeply concave	undulate	present	14.3	32.4	igr; rgr
AL 2299	ovoid – ellipsoid	reticulate	466	320	tetragonal	9	6	flat	undulate	absent	40.5	68.8	rgr
AL 2302	ovoid	reticulate	312	213	tetragonal	8	5	flat	undulate	absent	39.9	38.6	igr
AL 2304	ellipsoid	foveolate	853	482	elliptic	26	7	deeply concave	undulate	present	21.5	40.4	igr; rgr
AL 2461	ovoid	reticulate	396	234	transversely hexagonal	10	5	flat	undulate	absent	30.1	48.5	igr; ros
AL 2462	ovoid	reticulate	349	213	longitudinally hexagonal	7	5	flat	undulate	absent	54.0	34.3	igr
AL 2508	ovoid	foveolate	441	307	elliptic	16	6	deeply concave	undulate	present	22.8	41.9	ros
AL 2523	ellipsoid	furrowed	805	404	rectangular	9	5	deeply concave	wavy	present	92.5	78.9	igr
AL 2525	ovoid - ellipsoid	reticulate	391	238	tetragonal	9	5	flat	undulate	absent	50.8	39.8	igr; rgr
AL 2526	ellipsoid	furrowed	806	399	rectangular	9	5	deeply concave	wavy	present	109.2	50.2	igr
AL 2542	ellipsoid	foveolate	573	346	elliptic	24	6	deeply concave	undulate	present	19.8	41.7	rgr
CR	ovoid	reticulate	346	234	transversely hexagonal	10	6	concave	undulate	absent	27.7	44.5	ros
DEM 1812	ovoid	reticulate	433	295	transversely hexagonal	9	6	deeply concave	undulate	absent	31.8	54.3	ros
FR s.n. 'A'	ovoid	reticulate	527	353	tetragonal	14	8	flat	smooth	absent	34.8	52.0	igr
FR s.n. 'B'	ovoid - ellipsoid	reticulate	408	271	transversely hexagonal	11	6	deeply concave	undulate	absent	28.4	51.7	igr; rgr; ros
JGC 738A	ovoid	foveolate	477	326	elliptic	16	6	deeply concave	smooth	present	25.9	49.4	ros
JGC 738B	ovoid	reticulate	406	274	transversely hexagonal	8	5	concave	smooth	absent	43.6	59.7	igr
JGC 739	ovoid – ellipsoid	foveolate	709	448	elliptic	24	6	deeply concave	smooth	present	24.0	58.3	igr
Dro 14	ovoid – ellipsoid	foveolate	486	299	elliptic	18	7	concave	undulate	Present	21.1	43.3	ros
Dro 17	ovoid – ellipsoid	reticulate	354	258	transversely hexagonal	8	5	flat	undulate	Absent	31.3	46.6	igr; rgr

Appendix II. (continued)

Collection	SES	SEO	SEL	SEW	PSH	NPL	NPW	PCU	PFS	SLR	POL	POW	ECW [a]
Dro 21	ellipsoid	foveolate	508	308	elliptic	24	7	deeply concave	smooth	present	16.5	33.8	igr
Dro 22	ovoid - ellipsoid	reticulate	363	232	longitudinally hexagonal	8	5	flat	undulate	absent	54.1	50.8	igr
Dro 23	ellipsoid	furrowed	912	399	rectangular	10	5	deeply concave	wavy	present	136.9	55.6	igr
Dro 24	ovoid	reticulate	291	169	longitudinally hexagonal	7	5	flat	smooth	absent	42.8	28.0	igr; ros
Dro 29	ovoid - ellipsoid	reticulate	364	230	longitudinally hexagonal	7	5	flat	undulate	absent	52.4	40.7	igr
Dro 34	ovoid - ellipsoid	reticulate	399	271	longitudinally hexagonal	10	6	concave	undulate	absent	58.8	38.8	igr; rgr
Dro 36	ovoid - ellipsoid	foveolate	595	392	elliptic	20	6	deeply concave	undulate	present	25.1	57.9	igr; ros
Dro 38	ovoid - ellipsoid	foveolate	801	517	elliptic	26	7	deeply concave	smooth	present	19.5	43.3	igr; ros
Dro 39	ovoid - ellipsoid	reticulate	456	270	transversely hexagonal	9	5	concave	smooth	Absent	36.6	62.0	igr; ros
Dro 42	ovoid - ellipsoid	reticulate	405	273	transversely hexagonal	9	5	concave	undulate	absent	36.8	61.6	igr; rgr; ros
Dro 53	ovoid	reticulate	404	297	transversely hexagonal	9	5	concave	undulate	absent	28.3	61.5	igr; rgr; ros

[a] Code for ECW: irg – irregular granules
rgr – rounded granules
ros – rosettes

Appendix III. Herbarium specimens of *Drosera indica* L. showing variation in morphology (plant size, stem colour, and flower colour)



Appendix IV. Scores and measurements of morphological data

Collection	PLC	LPT	LFG	LRS	LTD	FSL	FPL	FPC	FFF	FFL	FAL	FAP	FAE	FAC
AL 84	green	present	grooved	absent	surface only	3.4 – 3.9	7.5 – 8.4	pink	filiform	4.3 – 4.8	0.68 – 0.83	top	erect	yellow
AL 261	green	present	grooved	absent	surface only	4.5 – 4.6	5.5 – 5.9	white	filiform	2.4 – 2.5	0.85 – 0.90	top	erect	yellow
AL 962	green	absent	flat	absent	surface only	4.1 – 4.3	7.0 – 7.5	pink	filiform	3.2 – 3.5	1.0 – 1.15	top	erect	yellow
AL 965	green	present	flat	present	surface only	4.9 – 5.1	6.9 – 7.5	white	cobra-like	4.5 – 4.8	0.70 – 0.88	margin	hooded	red
AL 976	reddish	absent	flat	absent	surface only	3.3 – 4.0	11.0 – 11.5	pink	deltoid	1.9 – 2.3	1.8 – 1.9	margin	erect	yellow
AL 1031	green	absent	flat	absent	surface and margin	4.6 – 5.0	8.5 – 8.9	pink	deltoid	3.3 – 3.5	1.1 – 1.3	margin	erect	yellow
AL 1036	green	present	flat	absent	surface only	4.7 – 5.1	11.3 – 12.3	magenta	deltoid	3.8 – 4.3	1.8 – 1.9	margin	erect	red
AL 1103	reddish	absent	flat	absent	surface and margin	2.4 – 2.8	5.5 – 6.0	pink	filiform	1.5 – 1.8	0.50 – 0.60	top	erect	yellow
AL 1108	red	absent	flat	absent	surface and margin	2.0 – 2.1	5.4 – 5.8	pink	filiform	2.75 – 2.83	0.50 – 0.55	top	erect	yellow
AL 1113	green	present	flat	absent	surface only	2.1 – 2.6	5.6 – 6.8	pink	filiform	2.5 – 2.8	0.75	top	erect	yellow
AL 1162	green	present	flat	absent	surface and margin	4.4 – 5.4	10.6 – 12.3	cerise	filiform	3.5 – 3.7	2.1 – 2.4	top	erect	yellow
AL 1164	red	absent	flat	absent	surface and margin	2.5	5.0 – 5.8	pale pink	filiform	2.4 – 2.8	0.53 – 0.60	top	erect	yellow
AL 1172	red	absent	flat	absent	surface and margin	3.0 – 3.4	5.9 – 6.9	pale pink	filiform	2.8 – 3.3	0.63 – 0.70	top	erect	yellow
AL 1173	green	present	flat	absent	surface only	4.6 – 5.0	8.5 – 9.6	pink	filiform	3.9 – 4.3	1.0 – 1.1	top	erect	yellow
AL 1193	orange-red	absent	flat	absent	surface and margin	4.1 – 4.8	9.5 – 9.9	apricot	deltoid	3.4 – 3.8	1.7 – 1.8	margin	erect	yellow
AL 1195	green	absent	flat	absent	surface only	3.6 – 3.8	9.3 – 10.3	mauve	deltoid	2.4 – 2.8	2.3 – 2.5	margin	erect	yellow
AL 1200	green	present	flat	present	surface only	4.6 – 4.9	7.3 – 7.5	white	cobra-like	3.8 – 4.0	1.1 – 1.3	margin	hooded	red
AL 1208	orange-red	absent	flat	absent	surface only	3.5 – 3.8	8.0 – 8.8	orange	deltoid	3.3 – 3.4	1.45 – 1.55	margin	erect	yellow
AL 1215	green	present	flat	present	surface only	4.6 – 5.3	6.8 – 7.4	white	cobra-like	3.6 – 3.9	0.8 – 1.1	margin	hooded	red
AL 1283	green	present	flat	absent	surface and margin	3.9 – 5.6	10.8 – 11.3	cerise	deltoid	2.2 – 2.7	1.7 – 1.9	margin	erect	yellow

Appendix IV. (continued)

Collection	PLC	LPT	LFG	LRS	LTD	FSL	FPL	FPC	FFF	FFL	FAL	FAP	FAE	FAC
AL 1284	green	present	flat	absent	surface only	4.6 – 5.4	10.0 – 11.5	pink	filiform	3.9 – 4.9	0.8 – 1.2	top	erect	yellow
AL 1289	green	present	flat	absent	surface and margin	4.5 –5.1	8.6 – 9.1	mauve	filiform	3.5 – 3.8	0.9 – 1.3	top	erect	yellow
AL 1318	green	present	flat	present	surface only	5.4 – 5.9	7.0 – 7.8	white	cobra-like	3.7 – 3.8	0.83 – 0.95	margin	hooded	red
AL 1322	maroon-red	absent	grooved	absent	surface only	4.5 – 5.0	14.0 – 14.5	purple	deltoid	2.0 – 2.3	2.3 – 2.5	margin	erect	yellow
AL 1325	green	present	grooved	absent	surface only	3.6 – 4.0	9.5 – 10.8	magenta	deltoid	3.9 – 4.0	1.45 – 1.65	margin	erect	red
AL 1352	green	absent	flat	absent	surface and margin	3.9 – 4.1	5.5 – 6.5	white	filiform	2.3 – 2.5	1.0 – 1.2	top	erect	yellow
AL 1697	green	absent	grooved	absent	surface and margin	3.0 – 3.4	8.6 – 9.0	pink	filiform	1.6 – 2.1	1.8 – 2.0	top	erect	yellow
AL 1716	green	present	flat	absent	surface and margin	4.1 – 4.4	11.0 – 11.5	cerise	filiform	3.0 – 3.6	1.8 – 2.0	top	erect	yellow
AL 1730	green	present	flat	present	surface only	4.8 – 5.3	6.6 – 6.8	pale pink	cobra-like	3.5 – 3.8	0.75 – 0.95	margin	hooded	red
AL 1734	red, green leaves	absent	flat	absent	surface and margin	3.0 – 3.9	6.6 – 8.4	pink	deltoid	1.5 – 2.5	1.2 – 1.4	margin	erect	yellow
AL 1749	green	present	flat	absent	surface only	4.1 – 4.8	10.4 – 11.3	pink	filiform	2.6 – 3.0	1.4 – 1.9	top	erect	yellow
AL 2206	green	present	flat	absent	surface and margin	4.6 – 5.0	12.5 – 13.9	cerise	deltoid	2.4 – 3.0	1.8 – 2.3	margin	erect	red
AL 2216	green, red glands	absent	flat	absent	surface and margin	3.4 – 3.6	9.3 – 11.5	pink	filiform	2.1 – 3.4	1.8 – 2.5	top	erect	yellow
AL 2219	green	absent	grooved	absent	surface only	3.4 – 3.9	8.3 – 8.6	pink	filiform	2.3 – 2.5	2.05 – 2.25	top	erect	yellow
AL 2220	green	present	flat	absent	surface only	4.1 – 4.6	11.8 – 12.3	cerise	deltoid	2.8 – 3.8	1.4 – 1.8	margin	erect	yellow
AL 2225	reddish	absent	flat	absent	surface and margin	2.5 – 3.0	5.4 – 6.0	pink	filiform	2.6 – 2.8	0.63 – 0.70	top	erect	yellow
AL 2226	green	present	flat	absent	surface only	3.1 – 4.0	5.6 – 6.5	pink	filiform	3.8 – 4.4	0.75 – 0.88	top	erect	yellow
AL 2227	green	present	grooved	absent	surface only	4.9 – 5.8	10.8 – 13.8	cerise	deltoid	3.1 – 3.9	1.8 – 2.0	margin	erect	yellow
AL 2242	green	present	grooved	absent	surface only	4.6 – 5.5	13.1 – 14.0	cerise	deltoid	2.6 – 3.3	2.3 – 2.5	margin	erect	yellow
AL 2243	green	present	flat	absent	surface only	4.3 – 5.0	11.5 – 12.0	cerise	filiform	2.4 – 3.1	2.4 – 2.8	top	erect	yellow
AL 2255	green	present	flat	absent	surface only	3.9 – 4.5	6.1 – 7.2	pink	filiform	3.9 – 4.8	0.75 – 1.1	top	erect	yellow

Appendix IV. (continued)

Collection	PLC	LPT	LFG	LRS	LTD	FSL	FPL	FPC	FFF	FFL	FAL	FAP	FAE	FAC
AL 2273	green	present	flat	absent	surface only	3.3 – 3.6	8.4 – 9.1	cerise	filiform	4.1 – 4.6	0.75 – 1.0	top	erect	yellow
AL 2299	green, red glands	absent	flat	absent	surface and margin	3.3 – 3.5	6.5 – 7.1	pink	filiform	3.4 – 4.0	0.65 – 0.75	top	erect	yellow
AL 2302	red	absent	flat	absent	surface and margin	2.3 – 2.6	4.3 – 5.0	white	filiform	3.1 – 3.3	0.38 – 0.45	top	erect	yellow
AL 2304	green	present	flat	absent	surface only	4.0 – 5.8	8.8 – 10.6	cerise	filiform	3.1 – 4.4	1.4 – 1.7	top	erect	yellow
AL 2461	green	absent	grooved	absent	surface only	3.4 – 3.7	7.8 – 8.6	pink	filiform	1.1 – 1.5	2.1 – 2.5	top	erect	yellow
AL 2462	red	absent	flat	absent	surface and margin	2.8 – 3.8	5.5 – 6.3	pink	filiform	3.9 – 4.1	0.83 – 0.90	top	erect	yellow
AL 2508	green	present	grooved	absent	surface only	3.8 – 4.3	9.9 – 10.6	pink	filiform	3.3 – 3.4	1.6 – 2.0	top	erect	yellow
AL 2523	green	present	flat	present	surface only	7.1 – 7.9	8.6 – 9.8	white	cobra-like	5.3 – 5.5	1.0 – 1.2	margin	hooded	red
AL 2525	red	absent	flat	absent	surface and margin	3.6 – 4.1	7.7 – 8.4	orange	filiform	2.5 – 3.6	1.3 – 1.5	top	erect	yellow
AL 2526	green	present	flat	present	surface only	4.9 – 5.1	6.1 – 6.8	white	cobra-like	3.5 – 3.8	0.9 – 1.0	margin	hooded	red
AL 2542	green	present	grooved	absent	surface and margin	5.6 – 6.0	10.3 – 10.8	cerise	filiform	3.9 – 4.5	1.2 – 1.4	top	erect	yellow
CR	green	absent	flat	absent	surface and margin	3.1 – 3.5	5.1 – 5.6	pink	filiform	2.4 – 2.6	0.73 – 0.80	top	erect	yellow
DEM 1812	green	absent	flat	absent	surface only	4.4 – 4.9	8.1 – 9.5	pink	filiform	3.8 – 4.0	1.3 – 1.6	top	erect	yellow
FR s.n. 'A'	green	present	flat	absent	surface and margin	3.0 – 3.5	3.8 – 4.0	white	filiform	2.8 – 3.0	0.40 – 0.43	top	erect	yellow
FR s.n. 'B'	green	present	flat	absent	surface and margin	2.6 – 2.9	4.5 – 5.3	pink	filiform	2.8 – 3.1	0.55 – 0.65	top	erect	yellow
JGC 738A	green	present	grooved	absent	surface only	6.0 – 6.8	11.0 – 11.4	pink/mauve	filiform	4.5 – 4.6	1.0 – 1.3	top	erect	yellow
JGC 738B	green	absent	flat	absent	surface only	3.8 – 4.0	9.3 – 9.5	pink/ mauve	filiform	4.0 – 4.6	0.75 – 0.88	top	erect	yellow
JGC 739	green	present	grooved	absent	surface only	4.3 – 4.9	9.3 – 9.5	orange	deltoid	4.6 – 4.8	0.75 – 1.2	margin	erect	red
Dro 23	green	present	flat	present	surface only	5.3 – 5.8	6.3 – 6.7	pale pink	cobra-like	2.6 – 3.3	0.58 – 0.90	margin	hooded	red
Dro 42	green	absent	flat	absent	surface and margin	3.5 – 3.8	6.5 – 7.1	pale pink	filiform	3.4 – 3.5	0.88 – 0.95	top	erect	yellow
Dro 53	green	absent	flat	absent	surface and margin	3.6 – 3.8	6.3 – 6.9	pale pink	filiform	3.0 – 3.4	0.85 – 1.0	top	erect	yellow

Appendix IV. (continued)

Collection	SES	SEO	SEL	SEW	PSH	NPL	NPW	PCU	PFS	SLR	POL	POW
AL 84	ovoid - ellipsoid	foveolate	572	310	elliptic	20	7	deeply concave	undulate	present	20.5	44.7
AL 261	ovoid	reticulate	510	334	tetragonal	18	5	deeply concave	smooth	absent	22.1	66.3
AL 962	ovoid - ellipsoid	reticulate	429	264	transversely hexagonal	10	5	concave	undulate	absent	35.5	51.3
AL 965	ellipsoid	furrowed	736	390	rectangular	12	5	deeply concave	wavy	present	93.9	59.0
AL 976	ovoid - ellipsoid	reticulate	361	238	longitudinally hexagonal	8	6	flat	undulate	absent	49.5	33.4
AL 1031	ovoid	reticulate	446	267	transversely hexagonal	9	5	concave	smooth	absent	35.9	58.6
AL 1036	ovoid	foveolate	558	411	elliptic	24	5	deeply concave	smooth	present	23.8	51.8
AL 1103	ovoid	reticulate	262	187	longitudinally hexagonal	7	5	flat	undulate	absent	48.9	31.7
AL 1108	ovoid - ellipsoid	reticulate	273	170	tetragonal	7	5	flat	undulate	absent	44.1	25.1
AL 1113	ovoid - ellipsoid	foveolate	433	273	elliptic	10	6	deeply concave	undulate	present	18.6	48.1
AL 1162	ovoid	foveolate	463	349	elliptic	20	5	deeply concave	smooth	present	20.8	43.4
AL 1164	ovoid	reticulate	266	157	tetragonal	6	5	flat	undulate	absent	45.6	24.7
AL 1172	ovoid - ellipsoid	reticulate	308	175	longitudinally hexagonal	6	4	flat	undulate	absent	58.8	33.4
AL 1173	ellipsoid	foveolate	467	282	elliptic	18	5	deeply concave	undulate	present	22.5	41.2
AL 1193	ovoid	reticulate	393	250	longitudinally hexagonal	8	5	flat	undulate	absent	56.1	47.3
AL 1195	ovoid - ellipsoid	reticulate	349	199	longitudinally hexagonal	9	5	concave	undulate	absent	51.2	30.0
AL 1200	ellipsoid	furrowed	738	427	rectangular	10	5	deeply concave	wavy	present	101.9	60.5
AL 1208	ovoid - ellipsoid	reticulate	333	228	longitudinally hexagonal	7	5	flat	undulate	absent	44.4	53.1
AL 1215	ellipsoid	furrowed	694	357	rectangular	9	5	deeply concave	wavy	present	80.7	49.5
AL 1283	ovoid - ellipsoid	foveolate	607	380	elliptic	24	6	deeply concave	smooth	present	22.6	45.5
AL 1284	ovoid - ellipsoid	foveolate	534	297	elliptic	16	5	deeply concave	smooth	present	27.4	52.0

Appendix IV. (continued)

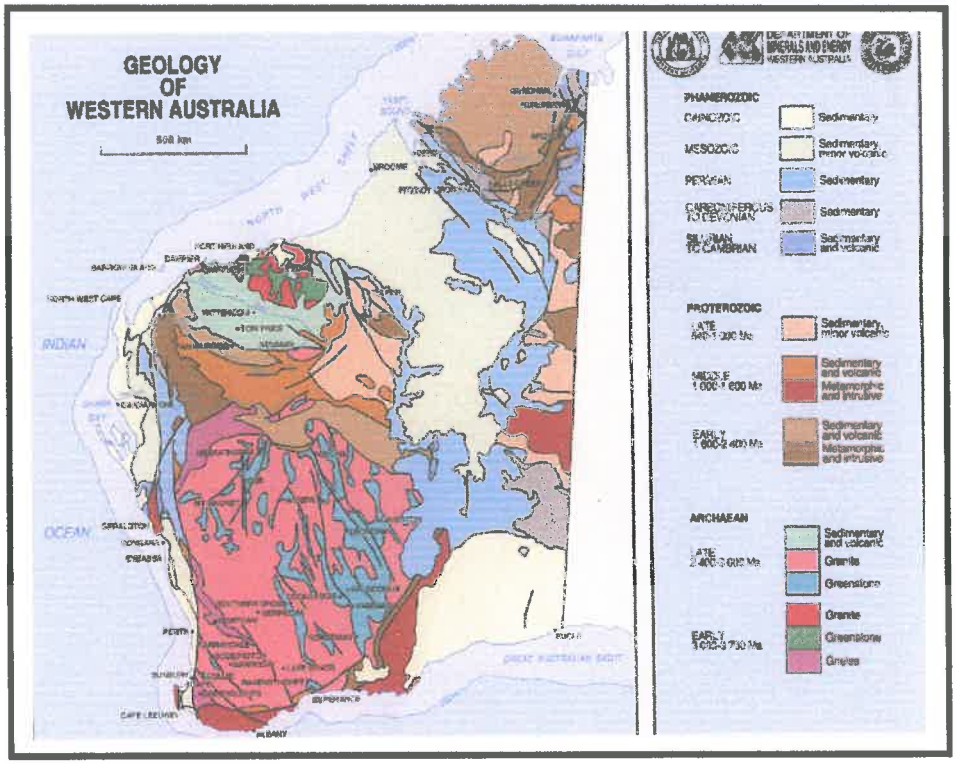
Collection	SES	SEO	SEL	SEW	PSH	NPL	NPW	PCU	PFS	SLR	POL	POW
AL 1289	ovoid – ellipsoid	foveolate	473	273	elliptic	16	5	deeply concave	smooth	present	24	47.1
AL 1318	ellipsoid	furrowed	912	432	rectangular	11	5	deeply concave	wavy	present	96.4	44.0
AL 1322	ovoid	reticulate	354	257	longitudinally hexagonal	8	5	flat	undulate	absent	49.5	64.6
AL 1325	ovoid – ellipsoid	foveolate	532	330	elliptic	24	7	deeply concave	smooth	present	16.9	34.6
AL 1352	ovoid – ellipsoid	reticulate	466	277	transversely hexagonal	12	6	concave	undulate	absent	27.6	53.0
AL 1697	ovoid – ellipsoid	reticulate	375	232	transversely hexagonal	9	5	flat	undulate	absent	30.7	46.7
AL 1716	ovoid	foveolate	443	293	elliptic	16	5	deeply concave	undulate	present	30.4	62.5
AL 1730	ellipsoid	furrowed	755	402	rectangular	10	6	deeply concave	wavy	present	99.6	62.5
AL 1734	ovoid – ellipsoid	reticulate	314	175	longitudinally hexagonal	7	5	flat	undulate	absent	50.9	31.9
AL 1749	ovoid – ellipsoid	foveolate	415	279	elliptic	20	5	concave	undulate	present	25.1	45.9
AL 2206	ovoid	foveolate	540	350	elliptic	16	6	deeply concave	undulate	present	24.4	41.6
AL 2216	ellipsoid	reticulate	470	270	transversely hexagonal	13	7	flat	undulate	absent	30.1	40.5
AL 2219	ovoid – ellipsoid	reticulate	406	255	transversely hexagonal	10	5	concave	undulate	absent	35.6	53.7
AL 2220	ellipsoid	foveolate	699	389	elliptic	20	6	deeply concave	undulate	present	22.6	47.5
AL 2225	ovoid	reticulate	290	183	tetragonal	6	5	flat	undulate	absent	53.3	31.3
AL 2226	ovoid	foveolate	476	323	elliptic	12	5	deeply concave	smooth	present	19.5	45.7
AL 2227	ovoid – ellipsoid	foveolate	538	365	elliptic	24	7	concave	smooth	present	19.3	54.0
AL 2242	ovoid	reticulate	508	316	tetragonal	7	5	deeply concave	undulate	absent	23.9	46.5
AL 2243	ovoid – ellipsoid	foveolate	478	267	elliptic	20	5	concave	undulate	present	22.1	49.0
AL 2255	ovoid	foveolate	585	372	elliptic	20	6	deeply concave	smooth	present	24.1	54.0
AL 2273	ellipsoid	foveolate	639	405	elliptic	24	8	deeply concave	undulate	present	14.3	32.4

Appendix IV. (continued)

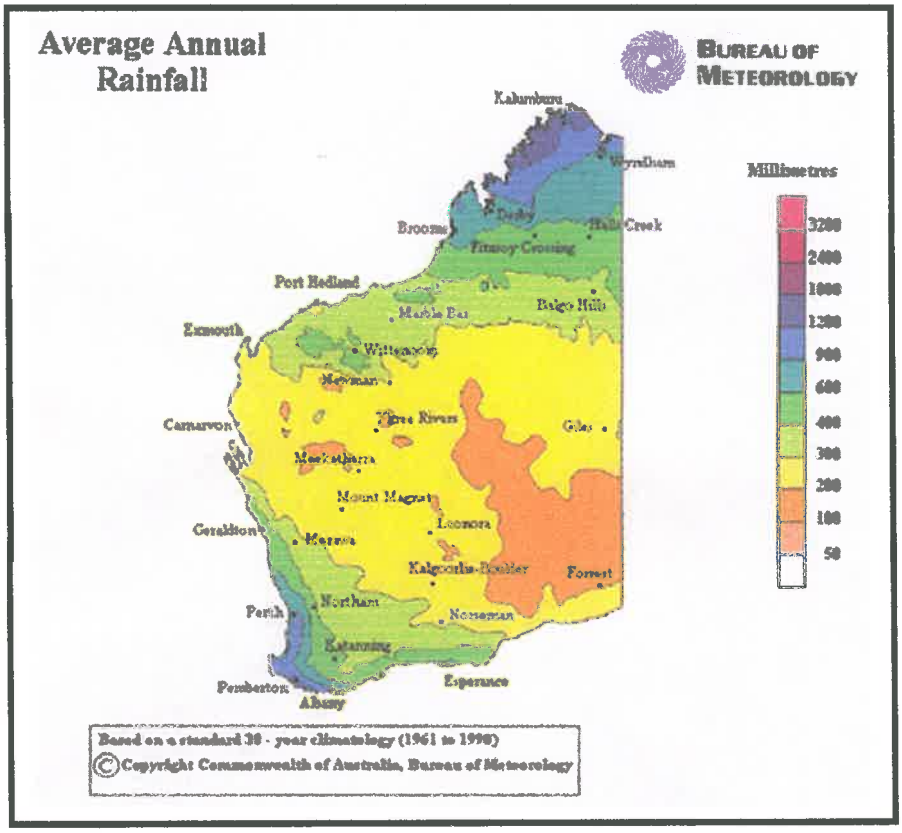
Collection	SES	SEO	SEL	SEW	PSH	NPL	NPW	PCU	PFS	SLR	POL	POW
AL 2299	ovoid – ellipsoid	reticulate	466	320	tetragonal	9	6	flat	undulate	absent	40.5	68.8
AL 2302	ovoid	reticulate	312	213	tetragonal	8	5	flat	undulate	absent	39.9	38.6
AL 2304	ellipsoid	foveolate	853	482	elliptic	26	7	deeply concave	undulate	present	21.5	40.4
AL 2461	ovoid	reticulate	396	234	transversely hexagonal	10	5	flat	undulate	absent	30.1	48.5
AL 2462	ovoid	reticulate	349	213	longitudinally hexagonal	7	5	flat	undulate	absent	54	34.3
AL 2508	ovoid	foveolate	441	307	elliptic	16	6	deeply concave	undulate	present	22.8	41.9
AL 2523	ellipsoid	furrowed	805	404	rectangular	9	5	deeply concave	wavy	present	92.5	78.9
AL 2525	ovoid - ellipsoid	reticulate	391	238	tetragonal	9	5	flat	undulate	absent	50.8	39.8
AL 2526	ellipsoid	furrowed	806	399	rectangular	9	5	deeply concave	wavy	present	109.2	50.2
AL 2542	ellipsoid	foveolate	573	346	elliptic	24	6	deeply concave	undulate	present	19.8	41.7
CR	ovoid	reticulate	346	234	transversely hexagonal	10	6	concave	undulate	absent	27.7	44.5
DEM 1812	ovoid	reticulate	433	295	transversely hexagonal	9	6	deeply concave	undulate	absent	31.8	54.3
FR s.n. 'A'	ovoid	reticulate	527	353	tetragonal	14	8	flat	smooth	absent	34.8	52.0
FR s.n. 'B'	ovoid - ellipsoid	reticulate	408	271	transversely hexagonal	11	6	deeply concave	undulate	absent	28.4	51.7
JGC 738A	ovoid	foveolate	477	326	elliptic	16	6	deeply concave	smooth	present	25.9	49.4
JGC 738B	ovoid	reticulate	406	274	transversely hexagonal	8	5	concave	smooth	absent	43.6	59.7
JGC 739	ovoid - ellipsoid	foveolate	709	448	elliptic	24	6	deeply concave	smooth	present	24	58.3
Dro 23	ellipsoid	furrowed	912	399	rectangular	10	5	deeply concave	wavy	present	136.9	55.6
Dro 42	ovoid - ellipsoid	reticulate	405	273	transversely hexagonal	9	5	concave	undulate	absent	36.8	61.6
Dro 53	ovoid	reticulate	404	297	transversely hexagonal	9	5	concave	undulate	absent	28.3	61.5

Appendix V. Glasshouse-grown plant of *Drosera indica* showing a red stripe on the abaxial surface of the leaf.

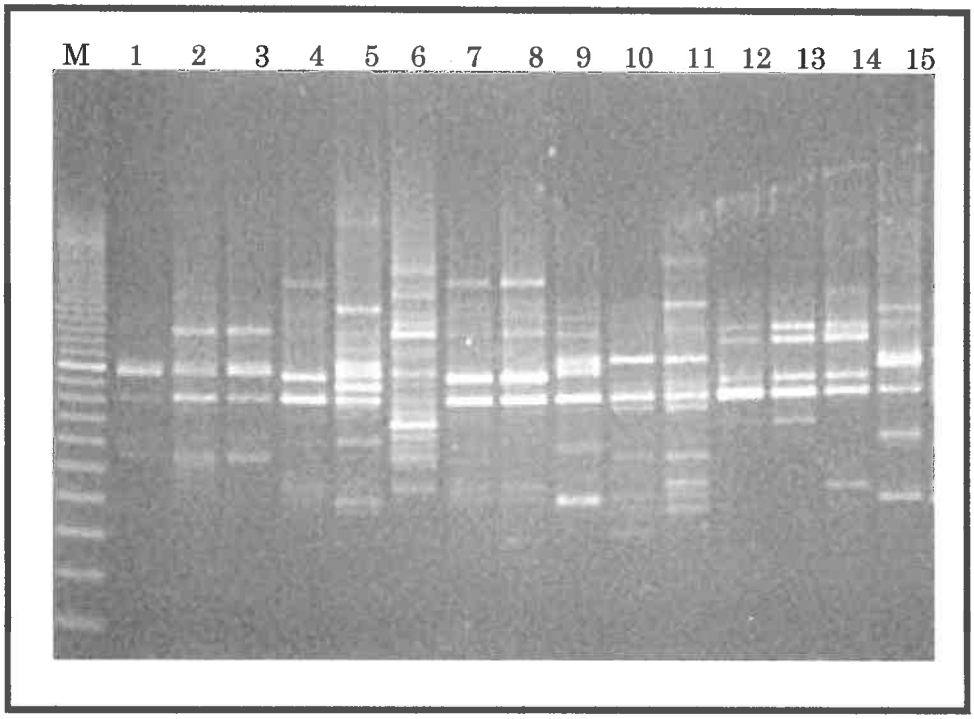
Appendix VI. The Geology of Western Australia (Source: Department of Minerals and Energy, Western Australia)



Appendix VII. Map of the Average Annual Rainfall of Western Australia
(Source: Bureau of Meteorology, Australia)



Appendix VIII. Gel electrophoresis of RAPD fragments of *Drosera indica* amplified using primer OPA03.



M : 100-bp ladder
1 : dro14
2 : dro17
3 : dro21
4 : dro22
5 : dro23
6 : dro24
7 : dro29
8 : dro34
9 : dro36
10 : dro38
11 : dro39
12 : dro42
13 : dro53
14 : JGC738A
15 : JGC738B