Identification of molecular markers linked to quantitative traits and disease resistance genes in wheat (*Triticum aestivum* L.)

by

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Thesis submitted to The University of Adelaide for the degree of Doctor of Philosophy

July 1998

Errata

Page	Para/line/section	Correction
v	L6	amplified
vi	P2, L10	7A
7	P3, L2	… cereal crops.
13	P2, L11	Sourdille *et al.* identified…
19	L6	… 124 different wheat…
24	2.5.1.1	4mM spemidine
28	P2, L5	… which an equal volume…
30	P2, L2&3	National Diagnostics
30	P2, L6	… was performed using…
36	P3, L4	… segregation at some loci.
52	5.2.2	… aliquots of DNA…
52	5.2.3	… heritability for milling yield,
63	AFLP, L5	0.1X TE
72-73	Table 1	*tauschii*
75	Sr22, L10	… expected number of heterozygous…
86	L1	… unstable locus (Jeffreys *et al.* 1988).

Chapter III

Statistical tests as described in Holm 1979, (Scand J Statist, 6:65-70) could be used to assess marker data showing distorted segregation ratios. A more appropriate measure of the level of AFLP polymorphism could also have been adopted.

Chapters IV and V

Other statistical methods (Jiang and Zeng, 1995. Genetics 140:1111-1127) could be used to further analyse the protein and milling yield field trial data.

Chapter VII

Chi-squares in Table 2 were calculated based on the expected segregation ratios of the resistance genes in the population at the F_4 stage.

Chapter VIII

Coefficients of parentage could be calculated and the results compared to the relationships obtained using both the RFLP and SSR data.

Declaration

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

I give my consent to the librarian of the Barr-Smith Library, The University of Adelaide, or his/her appointed agent to make this thesis freely available for photocopying or loan.

Garry Parker . Date: 9/7/98

Acknowledgments

I would like to sincerely thank my supervisors, Dr. Peter Langridge and Dr. Tony Rathjen who have guided and supported me throughout the course of my research. I have appreciated their help, friendship, and advice and I am indebted to them. I would also like to offer thanks to Dr. Ken Chalmers for his support, supervision and patience during my studies.

Special thanks to Dr. Harbans Bariana for his help in screening the population for rust resistance.

I have also appreciated the support of Dr. Miguel Lopes, Angelo Karakousis, Jodie Kretschmer, and the other members of the Langridge laboratory whom I have had the good fortune of working with. Their input and guidance has been invaluable at times.

Thanks to Chris Stone, Stewart Milde, and other members of the wheat breeding unit at the Waite Institute for their technical support in sowing and harvesting field trials. I would also like to thank Geoff Palmer and the members of the SARDI grain quality laboratory for their help and technical support.

The financial support of Arnott's Biscuits throughout my studies is gratefully acknowledged. Thanks also to the Australian Research Council and the Department of Employment, Education and Training for the provision of an Australian Postgraduate Research Award.

To Nick Paltridge (Rabbit) and Juan Juttner (Piglet), thanks for the many discussed fishing expeditions, as well as those that came to fruition. The light lab entertainment was also appreciated.

I would like to thank my family for their prayers and support over the years. Their love and encouragement has been, and continues to be a source of strength.

Finally to my beautiful wife Andrea, thankyou for the loving support and patience which you have given me throughout my studies. Your encouragement has helped me through many of the difficult and frustrating times.

"Unless a kernel of wheat falls to the ground and dies, it remains only a single seed. But if it dies, it produces many seeds. The man who loves his life will lose it...."

Jesus Christ, A.D. 33

Publications

Parker, G.D., Chalmers, K.J., Rathjen, A.J. and Langridge, P. (1998). Mapping of loci associated with flour colour in wheat (*Triticum aestivum* L.). *Theoretical and Applied Genetics*, (In press).

Parker, G.D., Chalmers, K.J., Rathjen, A.J., Bariana, H. and Langridge, P. (1998). Molecular mapping of rust resistance in wheat (*Triticum aestivum* L.). (in preparation)

Conferences

(Invited speaker)

Eighth Assembly Wheat Breeding Society of Australia. Canberra (September 1996) "Identification of molecular markers linked to protein, flour colour and milling yield in wheat."

5th International Congress of Plant Molecular Biology. Singapore (September 1997) "Identification of molecular markers linked to flour colour and milling yield in wheat."

Abbreviations

°C	:	degrees celcius
µCi	:	microcurie
µg	:	microgram
µl	:	microlitre
µM	:	micromolar
AFLP	:	amlified fragment length polymorphism
BAC	:	bacterial artificial chromosome
g	:	grams
GS	:	genetic similarity
LOD	:	log likelihood
M	:	molar
MAS	:	marker assisted selection
ml	:	millilitre
mM	:	millimolar
ng	:	nanogram
NIL	:	near isogenic line
QTL	:	quantitative trait loci
RAPD	:	randomly amplified fragment length polymorphism
RFLP	:	restriction fragment length polymorphism
SSM	:	slip strand mispairing
SSR	:	simple sequence repeat
STS	:	sequence tagged site
UCO	:	unequal cross over

Summary

The work presented in this thesis was conducted with the broad aim of identifying molecular markers linked to quantitative trait loci (QTLs) associated with traits affecting wheat quality, as well as rust resistance genes. To this end, a 'partial linkage map' comprising 70 RFLP, 11 SSR and 43 AFLP markers was constructed using a population of 150 F_4 derived single seed descent (SSD) lines generated from a cross between two hexaploid wheat cultivars, 'Schomburgk' and 'Yarralinka'. The RFLP markers detected the highest level of polymorphism between the parental lines (49.8%) followed by the SSR (36%) and AFLP (11.1%) markers. While the markers were poorly dispersed across all linkage groups, within individual groups they appeared to be evenly distributed, with the exception of a few clusters of AFLP markers. The linkage map was used to identify loci associated with important traits segregating in the mapping population, and major QTLs associated with the segregating traits were successfully identified and mapped.

The mapping population segregated for three important quality traits: grain protein content, milling yield and flour colour. Data for each of these traits were obtained by assaying seed collected from field trials conducted at different sites over two seasons. A total of seven markers were found to be associated with protein content. However, each of the associated markers were linked to protein in only one of the data sets. The heritability of protein in this population was found to be extremely low ($H^2 = 0.11$), indicating that a large proportion of the observed variation had arisen as a result of environmental influences, although there appeared to be a real genetic difference between the parental lines. The estimated heritability of milling yield in the population was calculated to be 0.48. In the preliminary analysis using RFLP and SSR markers, two regions were identified on chromosomes 3A and 7 which were significantly associated with milling yield, accounting for 22% and 19% of the genetic variation, respectively. Bulked segregant and AFLP analysis identified other markers linked to these loci, as well as an additional region on chromosome 5 which accounted for 19% of the genetic variation. The heritability of flour colour in the population was found to be high (0.67). Two QTLs were identified in the preliminary analysis on chromosomes

3A and 7A, which accounted for 13% and 60% of the genetic variation, respectively. A detailed analysis of the major locus on 7A was conducted through fine mapping of AFLP markers identified using bulked segregant analysis (BSA). Seven additional linked markers were identified by BSA and mapped close to the locus on chromosome 7A.

Molecular markers linked to four stem-rust and two leaf-rust resistance genes currently of significance to the Australian wheat breeding program were also identified. These resistance genes included *Sr5*, *Sr9e*, *Sr22*, *Sr36*, *Lr3*, and *Lr20*. The locations of each of the six genes based on linkage data were in agreement with the results of previous genetic, cytological and molecular studies. The identification of markers linked to these resistance genes should enable breeders to monitor and pyramid the resistance alleles effectively and accurately. Five of the 150 lines in the mapping population were found to contain resistance alleles for five of the six resistance genes and should provide a valuable breeding resource.

Finally, two of the molecular marker systems used in mapping, SSRs and RFLPs, were compared to determine whether they detect the same patterns of genetic relationships between 124 Australian wheat cultivars. The RFLP data consisted of 1955 polymorphic bands generated by Paull *et al.* (1998), while the SSR data consisted of 165 scorable bands derived from 18 SSRs. The results of the dendrograms produced from the genetic similarity (GS) estimates showed that the two marker systems generated relatively independent estimates of GS between the 124 cultivars. The average level of GS between the 124 cultivars based on the SSR data (0.33) was much lower compared with the RFLP data (0.75), reflecting the hypervariability of the SSR loci. The differences between the two marker systems in determining GS reflect the different mechanisms by which allelic variation is generated.

Contents

Chapter I: General Introduction

Chapter II: General Materials and Methods

Chapter III: General Mapping

Chapter IV: Wheat Protein

Chapter V: Milling yield

Chapter VI: Mapping loci associated with flour colour in wheat (*Triticum aestivum* L.)
(In press in Theoretical and Applied Genetics)

Chapter VII: Molecular mapping of Rust Resistance in Wheat (*Triticum aestivum* L.)
(To be submitted to Theoretical and Applied Genetics)

Chapter I

General Introduction

1.1 Objectives

The objective of this introduction is to present an overview of the development of molecular markers, to assess their strengths and weaknesses, and to discuss their application in plant breeding using examples from various species. To date, a significant amount of research has been carried out in this area, making it difficult to review comprehensively (for review see Rafalski *et al.* 1996). The material presented in this chapter is by no means an exhaustive summary of all the research published in this field. Rather, it provides an overview of the literature, and presents background information relevant to the experiments described in the subsequent chapters.

1.2 Genetic linkage maps and markers: A general overview

1.2.1 Isozymes and morphological markers

The development of genetic linkage maps originated with the use of naturally occurring variation between enzymes and morphological traits, which could be used to detect and map single trait loci in segregating populations (Tanksley *et al.* 1982). Isozyme markers are generally co-dominant and provided a useful source of markers for the development of linkage maps prior to the establishment of alternative molecular marker approaches. Once map positions of isozyme genes had been established, they could be used to locate other genes which may be linked (Tanksley and Rick 1980). Large numbers of isozyme markers could be assayed simultaneously in segregating populations while retaining the ability to determine the genotype at each locus for every individual. Similarly, morphological markers were used to generate early linkage maps, but their application in mapping has been limited due to the low

number of markers that can be scored in any one population. Overall, isozyme and morphological markers only offered limited genome coverage, were time consuming to assay, and detected relatively low levels of polymorphisms compared with more recently developed marker systems.

1.2.2 Restriction Fragment Length Polymorphism

The development of restriction fragment length polymorphism (RFLP) markers revolutionised plant genome mapping, offering a new source of virtually unlimited co-dominant markers with extensive genome coverage (Beckmann and Soller 1983). RFLPs are polymorphisms that are detected as differences in the length of defined DNA fragments produced by digesting genomic DNA with restriction endonucleases. The existence of genetic variation in the DNA nucleotide sequence between individual plants results in changes in the distribution of cleavage sites along respective DNA molecules. As a result, the digestion of genomic DNA with restriction enzymes produces a mix of restriction products varying in size (Beckman and Soller 1986). In the majority of species, the genome is so large that many restriction fragments are formed, resulting in a continuous smear when separated by gel electrophoresis. For this reason, the DNA is transferred to the solid support of a nylon membrane allowing specific fragments to be detected from within the smeared background through hybridisation using an appropriately labelled probe.

Probes are obtained by either synthesising specific regions of the genome using cloned DNA, or by testing clones from a random DNA library and selecting those which are single or low copy (Beckman and Soller 1986). If two individuals differ for a particular restriction site that affects the size of the DNA fragment homologous to the probe, then a band will appear at a different location in their respective autoradiographs. Variation in restriction sites can therefore be detected as restriction fragment length polymorphisms. Similarly, insertions or deletions between restriction sites will also generate fragment length polymorphisms. The mode of inheritance of these markers is predominantly co-dominant so that heterozygotes will show two discrete allelic bands, provided the probe can bind to both alleles. Additionally the stability and

unlimited availability of RFLP markers make them ideal 'scaffold' markers for developing base maps in many crop species (Graner *et al.* 1991). Other applications of RFLP markers include varietal identification and the introgression of alleles from wild or related species into breeding programs. Although RFLPs have played an important role in developing linkage maps, they are not ideally suited to large scale diagnostic applications (Rafalski and Tingey 1993). This is primarily because they are labour intensive, time consuming, and require a relatively large amount of DNA to perform the assay.

1.2.3 Randomly Amplified Polymorphic DNA

The development of the polymerase chain reaction (PCR) (Saiki *et al.* 1985) resulted in new types of DNA markers being established, including randomly amplified polymorphic DNA (RAPD) (Williams *et al.* 1990). The development of RAPDs was based on the use of short primers of arbitrary sequence to amplify random segments of genomic DNA. Polymorphisms detected among the amplified products arise as a result of changes in specific nucleotide sequence information in one or both of the priming sites. These changes are visible as the presence or absence of a particular RAPD band, or variation in size of the amplified fragment when the products are separated by gel electrophoresis (Rafalski and Tingey 1993). Many single nucleotide changes in the primer sequence cause a complete change in the pattern of amplified DNA fragments, with small differences in the number of base pairs amplified able to be detected following electrophoresis (Williams *et al.* 1990).

When they were initially developed, it was thought that RAPDs had the potential to provide a new source of unlimited genetic markers for use in the construction of genetic maps. However, studies have since shown that RAPDs have several drawbacks, including their dominant mode of inheritance (Rafalski and Tingey 1993), as well as problems associated with their reproducibiliy (Talbert *et al.* 1994; van Eck *et al.* 1995; Nagaoka and Ogihara 1997). Other limitations relate to the extent to which products can be considered to be allelic when derived from different varieties and species. Furthermore, it has been noted that the short arbitrary primers may amplify sequences homologous to repetitive DNA in complex genomes (Powell *et*

al. 1995). However, once a marker is found linked to a trait of interest, it is possible to convert the RAPD assay into a more reproducible PCR-type assay using techniques such as allele specific PCR, allele specific ligation, or sequence characterised amplified region (SCAR) assays (Rafalski and Tingey 1993). This approach has been used successfully in wheat (Schachermayr *et al.* 1994; Dedryver *et al.* 1996).

1.2.4 Microsatellites

The discovery of microsatellites or simple sequence repeats (SSRs) provided researchers with an alternative source of PCR based markers for the construction of genetic maps. SSRs are regions of di-, tri-, or tetranucleotide repeats interspersed throughout eukaryotic genomes (Levison and Gutman 1987; Röder *et al.* 1995). A large proportion of genomes consist of these tandem arrays of nucleotide repeats of various lengths (Devos *et al.* 1995). These regions are generally highly polymorphic due to the large amount of variation in the number of repeats. SSRs are not confined to untranslated regions only, but can be found within coding regions (Tautz *et al.* 1986). Allelic variation of SSRs is thought to arise as a result of slipped-strand mis-pairing (SSM) (Levison and Gutman 1987; Weber and Wong 1993). SSM involves denaturing and displacement of strands of a DNA duplex followed by mis-pairing of complementary bases at the site of an existing tandem repeat. When followed by replication or repair, this can lead to insertions or deletions of one or several of the short repeat units (Levison and Gutman 1987). Unequal crossing-over can also generate tandem duplications in DNA, as well as insertion or deletion events in the sequences that flank the SSR region. All of these mechanisms can potentially generate allelic variation, providing an important source of highly polymorphic markers.

These polymorphisms are detectable by PCR amplification using primers specific for the regions flanking the repeat array. The high level of polymorphism, interspersion rate and co-dominant nature of SSRs makes them an abundant source of markers. Their application may be especially useful for mapping in species with little intra-specific polymorphism, including inbreeding species such as wheat (Röder *et al.* 1995). However, there are several drawbacks

associated with SSRs, including the high cost and length of time required for their development. Although the initial costs may be significant, once developed the cost of implementing these markers is greatly reduced. In addition, they are easily transferable between laboratories as the sequence information can be distributed, allowing other research groups to generate their own primers.

1.2.5 Amplified Fragment Length Polymorphism

The development of amplified fragment length polymorphisms (AFLPs) (Vos *et al.* 1995) has produced another source of PCR based markers with the potential to rapidly saturate genetic maps. The technique is based on the detection of genomic restriction fragments by PCR amplification and can be used for all types of DNA, irrespective of its origin or complexity. Furthermore, fingerprints can be produced without prior sequence knowledge using a range of generic primers. Initially the DNA is cut with restriction enzymes, after which double stranded adaptors are ligated to the ends of the fragments to generate templates for DNA amplification. The sequences of the adaptors and the adjacent restriction sites serve as primer binding sites for amplification of the restriction fragments. Selective nucleotides are included at the 3' ends of the PCR primers restricting DNA synthesis to a subset of the restriction sites. In plant species, amplification is generally carried out in two steps. An initial pre-amplification is performed using primers containing only one selective base, followed by a selective amplification step using primers with up to three or more selective nucleotides attached at the 3' end. This provides a control for the number of polymorphisms and bands generated in each reaction.

AFLP is similar to RFLP except that it uses PCR amplification instead of Southern hybridisation for the detection of restriction fragments, requiring much less template DNA. AFLPs also have a much higher multiplex ratio than RFLPs. DNA of any origin or complexity can be used with the number of restriction fragments detected in complex genomes being virtually unlimited. In contrast to RAPDs, almost every AFLP reaction is useful, with large numbers of polymorphic bands common (Mackill *et al.* 1996). Additionally, the majority of AFLP fragments correspond to unique positions on the genome, and can be used as markers in

genetic and physical maps. AFLPs have the capacity to identify large numbers of loci, increasing their ability to detect polymorphism compared to other PCR based methods such as RAPDs and SSRs (Thomas *et al.* 1995). Initial work has shown that AFLP markers are relatively well dispersed and can be used to fill gaps in established maps without interrupting RFLP clusters (Becker *et al.* 1995). Apart from generating maps, the technique can also be used to generate fingerprints of cloned DNA segments, bacterial artificial chromosomes (BACs), or yeast artificial chromosomes (YACs), allowing individual clones to be compared (Vos *et al.* 1995). Alternative mapping approaches involving the identification of large numbers of markers from small numbers of samples, such as bulked segregant analysis, will also be improved with AFLP analysis. These techniques rely on the rapid detection of large numbers of polymorphisms, a requirement that can be readily achieved using AFLPs (Mackill *et al.* 1996). However, one draw back of AFLP marker data is that it is difficult to verify allelism at present, so that alleles are mapped rather than specific loci (van Eck *et al.* 1995). It has also been unclear whether AFLP markers can be considered to be chromosome specific and transferable between populations. However, recent work by Waugh *et al.* (1997) has indicated that AFLP markers are chromosome specific, allowing specific loci to be mapped in different populations.

1.3 Applications of marker technology in plant breeding

1.3.1 Development of linkage maps

With the development of different molecular marker technologies in the past two decades, researchers have been able to use these tools to construct detailed genetic linkage maps. The combination of different marker assays has enabled a large number of maps to be generated in many different plant species. These maps have helped in the localisation of genes of interest, and have provided the basis for the development of alternative strategies for plant breeding. In addition, they have played a critical role in the characterisation and isolation of specific genes

(Marino *et al.* 1996; Baker *et al.* 1997). The development of maps in related species has allowed the comparison of maps and gene order resulting in the identification of regions of synteny.

Some examples of genetic maps that have been generated in different plant species are listed in Table 1.1. As expected from cytogenetic analyses, the number of linkage groups varies considerably between species, with the sizes of the linkage maps also showing significant variation. For species with many chromosomes or large genomes, a large number of markers are required to give good genome coverage. One of the main strategies for increasing the number of markers has been the use of multiple marker systems. A number of studies have shown that the merging of different marker data for a specific mapping population generally results in an increase in map length, with very little change in marker order (Becker *et al.* 1995; van Eck *et al.* 1995; Schondelmaier *et al.* 1996).

The use of multiple marker systems also increases the probability of obtaining a more uniform coverage as different genomic regions are detected by the different marker systems. Although the different types of markers are generally well distributed over linkage groups, they often show a tendency to map to specific regions of the chromosome arms (McCouch *et al.* 1988; Mackill *et al.* 1996; Taramino and Tingey 1996). Both RFLP and AFLP markers have been found to cluster in some species (Graner *et al.* 1991; Tanksley *et al.* 1992; Barzen *et al.* 1995; Schondelmaier *et al.* 1996) with RFLPs generally targeting low copy or expressed genes, while AFLPs target regions with a high density of particular restriction sites. AFLPs generated with *Mse1* and *Pst1* have been found to cluster in centromeric regions or at the edges of genomic segments heavily mapped with RFLPs (Waugh *et al.* 1996). However they rarely map within clusters of other markers (Becker *et al.* 1995). The cause of this clustering phenomenon may be related to the occurrence of multiple specific genomic sequences that are targeted by AFLP, or the occurrence of centromeric or telomeric suppression of recombination.

Although a large number of maps have been developed in a wide range of species, the focus of this review is on cereals crops. A significant number of genetic maps have been generated in

Table 1.1 Examples of genetic maps developed in a broad range of species using molecular markers.

Species	Common name	Linkage groups	Map size (cM)	Number of loci	Reference
Arachis hypogeae	peanut	11	1063	117	Halward *et al.* 1993
Beta vulgaris	sugar beet	9	621	413	Hallden *et al.* 1996
Brassica rapa	oilseed	10	1785	139	Teutonico and Osborn 1995
Brassica napus	rapeseed	19	1441	207	Uzunova *et al.* 1995
Capsicum annum	capsicum	14	820	85	Lefebvre *et al.* 1995
Glycine max	soybean	26	3441	840	Keim *et al.* 1997
Gossypium hirsutum	cotton	41	4675	705	Reinisch *et al.* 1994
Helianthus annuus	sunflower	17	1380	234	Berry *et al.* 1995
Lactuca sativa	lettuce	17	1950	319	Kesseli *et al.* 1994
Lycopersicum esculentum	tomato	12	1276	1030	Tanksley *et al.* 1992
Malus domestica	apple	24	950	360	Hemmat *et al.* 1994
Medicago sativa	alfalfa	8	659	89	Kiss *et al.* 1993
Phaseolus vulgaris	bean	15	827	152	Nodari *et al.* 1993
Pinus radiata	pine	16	1382	208	Devey *et al.* 1996
Pisum sativum	garden pea	7	1700	151	Ellis *et al.* 1992
Prunus	peach/almond	9	800	118	Foolad *et al.* 1995
Saccharum spontaneum	wild sugarcane	42	1812	216	Dasilva *et al.* 1993
Secale cereale	rye	7	760	127	Senft and Wricke *et al.* 1996
Solanum tuberosum	potato	12	1120	193	Jacobs *et al.* 1995
Vigna unguicalata	cowpea	10	684	97	Menancio-Haytea *et al.* 1993
Vigna radiata	mungbean	14	1570	145	Young *et al.* 1993
Vitis ssp	grapevine	19	1477	225	Lodhi *et al.* 1995
Zea mays	Maize	10	1859	215	Gardiner *et al.* 1993

cereal crops in the past decade, with examples listed in Table 1.2. The mapping of diploid species has progressed more rapidly than polyploid species, with high density maps available in both barley and rice (Kleinhofs *et al.* 1993; Langridge *et al.* 1995; Causse *et al.* 1994; Yang *et al.* 1997). With rice in particular, the construction of high density maps has progressed to a stage where BAC libraries have been constructed (Yang *et al.* 1997). The high density maps enable contigs to be constructed as a first step in map-based cloning in these regions. Several disease resistance genes have been cloned in barley and rice using map based cloning (for review see Baker *et al.* 1997). The development of genetic maps in wheat has been slower compared to diploid crops. One of the difficulties in wheat has been the low levels of intraspecific polymorphism and the large number of linkage groups (Nelson *et al.* 1995a, 1995b; Marino *et al.* 1996). The majority of mapping in wheat has been performed using RFLP markers. However, the development of PCR based markers, such as SSRs (Röder *et al.* 1995) and AFLPs (Vos *et al.* 1995) has provided an additional source of markers to accelerate map construction.

1.3.2 Analysing Quantitative trait loci (QTLs)

1.3.2.1 The nature of QTLs

Many traits are influenced by multiple genes as well being influence by environmental effects. These traits are known as quantitative traits. Quantitative characters have been an important component of genetic analysis for over a century as they are expressed in populations of all eukaryotes. For many of these traits, very little is known about the number of genes involved or their chromosomal location. The concept of multiple loci controlling traits was developed after early research indicated that the inheritance patterns of some traits were more complex than others. Sax (1923) carried out early studies in beans looking at characters such as bean size and seed coat pigmentation and found evidence for control by several loci. Thoday (1961) also noted that the variation of quantitative traits arose as a result of genetic variation at a number of loci, with the contribution of each locus varying, making it difficult to handle loci individually.

Table 1.2 Examples of important genetic maps developed in different cereal crop species using a range of molecular markers. A number of traits have been identified from these maps and these are listed in the table.

Species	Common name	Number of loci	Markers used	Traits mapped	Reference
Oryza sativa	Rice	135	RFLP	-	McCouch *et al.* 1988
		726	RFLP, SSR, RAPD, isozyme	Over 80 genes of agronomic importance	Causse *et al.* 1994
		115	RFLP	QTLs for heading date & plant height	Li *et al.* 1995
		127	RFLP	Root morphology & drought resistance loci	Champoux *et al.* 1995
		137	RFLP	Six agronomic traits	Lu *et al.* 1996b
		176	AFLP, RFLP, RAPD	-	Mackill *et al.* 1996
Hordeum vulgare	Barley	88	RFLP	-	Graner *et al.* 1991
		117	RFLP & RAPD	Height, heading date and growth habit	Baura *et al.* 1993
		94	RFLP & protein loci	Vernalization & photoperiod	Laurie *et al.* 1995
		282	AFLP, RFLP, SSR & protein loci	-	Becker *et al.* 1995
		587	RFLP	-	Langridge *et al.* 1995
		85	Isozyme, RFLP & RAPD	Grain yield and yield components	Kjaer & Jensen 1996
Triticum aestivum	Wheat	29	1A - RFLP and known function	-	Dubcovsky *et al.* 1995
		39, 58, 28	Group1 - RFLP & known function	-	Van Deynze *et al.* 1995
		57, 60, 56	Group2 - RFLP	*Lr13, Sr16*	Nelson *et al.* 1995a
		58, 62, 40	Group3 - RFLP & known function	Red grain colour	Nelson *et al.* 1995b
		45, 35, 18	Group4 - RFLP & known function	*Ha, Lr34, Vrn1, Vrn3, Rc1*	Nelson *et al.* 1995c
		40, 49, 30	Group5 - RFLP & known function	*Vrn1*, *Vrn3*, *Pm2*, *Ha*	Nelson *et al.* 1995c
		9, 21, 20	Group5 - RFLP & known function	-	Xie *et al.* 1993
		29, 24, 21	Group6 - RFLP	-	Marino *et al.* 1996
		51, 38, 19	Group7 - RFLP & known function	*Lr34*, *Pm1*, Rc1, *Rc3*	Nelson *et al.* 1995c
		95	RFLP	-	Lui and Tsunewaki 1991
		139	RFLP, morpholgical & known function	Grain protein	Blanco *et al.* 1996
		1014	Consensus - RFLP and known function	-	Gale *et al.* 1995

Thoday (1961) realised that the understanding of quantitative variation would have greater precision if individual relevant genes could be mapped more accurately on linkage maps. These early observations regarding the nature of quantitative traits were supported by further studies in species such as tomato (Tanksley *et al.* 1982) which identified many traits which showed continuous variation, seemingly controlled by multiple genes with varying effects.

With some quantitative traits, the phenotypic variation is determined largely by the genotype at several loci while the environment has little impact. However, with other traits the environmental effects have a significant impact on the phenotype while genetic factors play a minor role (Hartl *et al.* 1988). The majority of quantitative traits are difficult to dissect using pedigree based methods, as the effects of segregating alleles at one locus may be masked by the effects of other loci and environmental factors. However these traits can be effectively dissected and mapped using genetic linkage maps of populations of inbred or double haploid lines segregating for the trait of interest.

1.3.2.2 Dissecting quantitative traits and interval mapping

Mapping monogenic traits is simple provided the trait can be assayed reliably. However, the identification of molecular markers linked to genes that control quantitative traits is difficult due to their complex mode of inheritance (For review see Kearsey and Farquhar 1998). The basic problem associated with QTL mapping is that the trait phenotype is a single value, which is the product of the combined allelic effects of many genes as well as the environment. However, QTLs can be detected based on the identification of associations between genetically determined phenotypes and specific genetic markers (McCouch and Doerge, 1995). QTL mapping requires a high density genetic map to increase its precision and to ensure coverage of the entire genome (Thoday 1961; Rafalski and Tingey 1993). The initial limitation of incomplete genetic maps was overcome by the development of molecular markers in both plant and animal species. The development of markers which showed high levels of variability provided a means for developing the framework in which QTLs could be located. These markers could be established in any population allowing QTLs to be mapped in many species (Kearsey and

Farquhar 1998). The establishment of markers on all chromosomes allows researchers to obtain indirect information about the genotype of a target gene based on the genotypes of markers located close to the gene of interest (Jansen 1996).

The majority of early studies on quantitative traits involved statistical techniques based on means, variances and covariances, with little knowledge of the number or exact location of the genes involved. Sax (1923) originally assessed markers on an individual basis and related the genotypic mean for individuals with a particular marker to the mean of the values for the trait of interest. Using this approach, when markers are identified linked to the target gene, the marker and QTL loci will be associated. Single marker analysis does not use information from the order of markers on the map. Rather it divides the population into classes based on the genotype at each of the marker loci (McCouch and Doerge 1995). Associations can be statistically tested (*t*-test analysis of variance and regression) and likelihood values for the presence of a QTL plotted for each marker position. A QTL is declared if there is a significant difference in the mean phenotypic score for each of the groups. However, using this method it is not possible to calculate genotypic means for a target locus in an interval between markers, because there are no observable genotypes. Weller (1986) adopted the technique of maximum likelihood for mapping and analysing QTLs using genetic markers in tomato. The principle of maximum likelihood is to maximise the value of the likelihood of obtaining an observed set of phenotypes by maximising the value of the likelihood of expression with respect to p (recombination fraction). The log value of the expression is used as it is easier to differentiate and will have its maximum at the same value of p as the original expression. This method was more powerful than the first approach, as it enabled the estimation of QTLs under partial linkage. It was effective for large QTL effects and particularly useful for analysing data from field trials.

Paterson *et al.* (1988) used interval mapping, which allowed inferences to be made about points throughout the entire genome and helped to overcome the problems associated with QTL mapping based on single markers. These problems included underestimating phenotypic effects of QTLs, the large numbers of progeny required for mapping, and the inability to clearly

resolve genetic locations of QTLs. The method of LOD scores used in human genetics was adopted to overcome some of these problems. With interval mapping the 'most likely' phenotypic effect of a QTL affecting a trait and the odds ratio (probability of data arising from a QTL with this effect divided by the probability that it would have arisen given there was no QTL) can be computed for each marker position in the genome (Paterson *et al.* 1988). The LOD score is defined as the $\log_{10}$ of the odds ratio and summarises the strength of the evidence for a QTL at any particular position. Therefore, if the LOD score exceeds a predetermined threshold, then a QTL is deemed to be present. Lander and Botstein (1989) also adopted the approach of interval mapping and recommended that a LOD score between 2 and 3 should be used to ensure a false positive rate of less than 5%. Interval mapping using maximum likelihood can provide accurate estimates of linkage. However, the numerical methods required to maximise the likelihood of the expression become increasingly difficult as the model becomes more complex. An example of this is when data are analysed for the presence of two or more linked or interacting QTL.

Although interval mapping has had a significant impact on the identification of single QTLs in backcross and F_2 populations, the approach was limited in early computer packages such as MapMaker/QTL (Lander *et al.* 1987), as recombinant inbred lines and other population designs were not addressed. Haley and Knott (1992) developed regression methods which could easily be implemented in standard computer packages to perform flanking marker analyses for the detection of QTL. These methods could be implemented in the same way as maximum likelihood methods and generated similar estimates of linkage. The regression method could also be used to analyse data in which two linked or interacting QTL are present. The use of nonparametric statistics for mapping QTLs and determining threshold values for significance has improved the application of interval mapping. These have been implemented in custom written software packages such as MapManager QT (Manly and Elliott 1993) and Q-gene (Nelson 1997).

With Q-gene (Nelson 1997), single marker analysis is performed by obtaining a representative statistic (F) as a measure of the fit of a linear regression of phenotype on marker genotype. The

plotting of each marker in map order allows significant genomic regions to be identified. For interval mapping, the regression method proposed by Haley and Knott (1992) fits a model which expresses phenotype as a linear combination of the expected genetic effects of a modelled QTL and is conditional on the distance and genotypes of flanking markers. The location of the QTL is modelled at different points along the chromosome and a regression statistic is plotted to indicate positions of higher likelihood. Using this method, marker data can even be derived for plants with missing data (Nelson 1997).

1.3.2.3 Examples of QTLs mapped in different crops

A large number of studies have been carried out in the last decade, aimed at locating QTLs in a variety of crop species. Of these, many studies have targeted QTLs in horticultural crops such as tomato, where markers have been identified linked to economically important traits such as fruit mass, pH of juice and concentration of soluble solids (Paterson *et al.* 1988). Similarly, extensive molecular studies have been performed in maize. Edwards *et al.* (1992) identified significant trait-locus associations for 20 quantitative traits in a population of 187 F_2 maize lines, while Austin and Lee (1996) identified QTLs associated with flowering date and plant height. Of all the cereal crops, rice has been the most extensively mapped, with a significant number of studies aimed at identifying QTLs associated with different traits. Causse *et al.* (1994) generated a detailed map containing a total of 726 markers which was used to locate over 80 genes associated with agronomic traits. Other loci that have been mapped in rice include QTLs controlling heading date and plant height, which were identified by Li *et al.* (1995) using an RFLP based map. Additionally, Champoux *et al.* (1995) mapped putative QTLs associated with five parameters related to root morphology using an RFLP map generated from 203 recombinant inbred lines.

Similarly, a large number of studies have been carried out aimed at identifying QTLs in barley. Early mapping studies were performed using biochemical markers with some success in identifying QTLs controlling plant height and ear emergence (*vrn 1*) (Hackett *et al.* 1992). However, the development of improved, alternative marker approaches enabled more detailed

maps to be generated. Barua *et al.* (1993) used a number of marker systems to generate a map from a double haploid (DH) population of barley. Using this map they were able to identify seven markers linked to the *denso* locus on chromosome arm 3HL, and a QTL associated with days to heading on chromosome 6H. The *denso* locus accounted for more than 80% of the phenotypic variation in plant height. Laurie *et al.* (1995) also mapped QTLs associated with days to heading and photoperiod response in barley using a double haploid population. Two major photoperiod genes were identified on chromosome arms 2HS and 1HL. A QTL controlling days to heading was also identified on chromosome 3 and was believed to be the *denso* locus previously identified by Barua *et al.* (1993). Other QTLs associated with flowering time were also identified on chromosomes 4HL, 5HL, 6HL and 7HL.

Despite the availability of suitable marker technology, the identification of markers linked to important QTLs in wheat has been slow due to the difficulty associated with the construction of detailed linkage maps. The development of detailed maps has been hindered by the large number of chromosomes, and low levels of intraspecific polymorphism (Nelson *et al.* 1995a, 1995b). Anderson *et al.* (1993) carried out a preliminary study in wheat aimed at identifying loci associated with resistance to pre-harvest sprouting. RFLP markers were used to create a map using two recombinant inbred (RI) populations in which four QTLs were identified which showed significant associations with resistance to pre-harvest sprouting. Additionally, Sourdille *et al.* (1996) conducted linkage studies to identify markers linked to genes affecting kernel hardness in wheat using a population of single seed descent lines (SSD) previously mapped with over 1100 loci (Nelson *et al.* 1995a, 1995b, 1995c). They identified a major QTL associated with kernel hardness on chromosome arm 5DS which was designated *ha*. The QTL accounted for 63% of the observed phenotypic variation in the population. Four other minor QTLs were also identified on chromosomes 2A, 2D, 5B, and 6D. Another important quantitative trait in wheat that researchers have tried to dissect is the level of protein concentration in the grain. Joppa and Cantrell (1990) carried out preliminary work by substituting each of the *T. dicoccoides* chromosomes into a durum line and assessing the effects on total protein concentration. Chromosomes 6B, 2A, 5B, 3A and 6A were found to significantly increase grain protein-content. Blanco *et al.* (1996) carried out additional work on

grain protein content in tetraploid wheat by characterising a set of 65 recombinant inbred lines using RFLP and morphological markers. Using data generated from field trials, a total of six putative QTLs associated with protein were identified on chromosome arms 4BS, 5AL, 6AS, 6Bs and 7BS. Only two of the QTLs, on chromosome arms 4BS and 6AS were significantly associated with protein concentration in all three field trials. These results highlight the large environmental effect on traits such as protein, making it difficult to find significant associations between markers and QTLs.

1.3.3 Mapping qualitative traits: disease resistance genes

Qualitative traits such as disease resistance loci are easier to map in segregating populations than most QTLs, because their phenotypic expression is generally not significantly influenced by environmental factors or interactions. Studies aimed at identifying markers linked to important disease resistance genes have been performed in a number of horticultural species including tomato, potato, lettuce and peanut (Thomas *et al.* 1995; Brigneti *et al.* 1997; Paran *et al.* 1991; Garcia *et al.* 1996). In addition, a significant number of resistance genes have been cloned including genes from Arabidopsis, tomato, tobacco, flax, sugar beet, rice and maize (for review see Baker *et al.* 1997). Similarly, a large number of studies have identified markers linked to disease resistance genes in the major cereal crops, including wheat and barley (Table 1.3). Although many resistance genes have been identified and characterised phenotypically, including more than ninety in wheat (McIntosh 1995; Knott 1989), a large proportion of these resistance genes have not been localised on genetic linkage maps.

In order to prevent the rapid breakdown of resistance genes in the field, multiple genes can be combined in new varieties to ensure more durable resistance (Roelfs, 1988; Long *et al.* 1993; Long *et al.* 1994). This can be difficult and time consuming due to the lack of pathogen isolates with appropriate virulences (Schachermayr *et al.* 1995). Using glasshouse based screening procedures, it is difficult and often impossible to determine whether a resistant line carries more than one resistance gene for a particular pathogen isolate. Therefore, the implementation of pyramiding strategies for the development of durable resistance would be considerably easier

Table 1.3 Examples of resistance genes mapped in wheat and other cereals using molecular markers.

Crop	Resistance genes	Chromosomal Location	Closest markers	Reference
Oat	*Pg3* (Stem rust)	-	*ACOpR-2*	Penner *et al.* 1993
Rice	Xa-4, Xa-5, Xa-13, Xa-21 (bacterial blight)	11, 5, 8, 11	*XNpb181, XRZ207, XRZ28, XpTA818*	Huang *et al.* 1997
Rice	tXa-5 (bacterial blight)	5	*XRZ390, XRG556*	Blair and McCouch 1997
Rice	tXa21 (bacterial blight)	11	Cloned	Song *et al.* 1995
Rice	tMlo	4	Cloned	Büschges *et al.* 1997
Barley	*Rh* (scald resistsnce)	3HL	*Xcdo1174, XOPR3-H550*	Baura *et al.* 1993
Barley	Rh2 (scald resistance)	1S	*Xcdo545*	Schweizer *et al.* 1995
Barley	stripe mosaic virus	1S	*Xabc455, Xabg011*	Edwards and Steffenson 1996
Barley	*Ha2* (Cereal cyst nematode)	2H	*Xpsr901, Xawbma21*	Kretschmer *et al.* 1997
Barley	bacterial leaf streak	3L	*Xabc171, Xabg377*	El Attari *et al.* 1998
Barley	stripe rust QTLs	3, 4, 7	*Xe36m36a, Bmy1, Xcdo57*	Toojinda *et al.* 1998
Barley	*Yd2* (BYDV)	3	*YLM*	Paltridge *et al.* 1998
Wheat	*Wwm1* (wheat strek mosaic)	4	*Xwg232,STSJ15*	Talbert *et al.* 1996
Wheat	*Yr15* (stripe rust)	1B	$OPB13_{1420}$, *Nor1*	Sun *et al.* 1997
Wheat	*Lr1* (leaf rust)	5DL	*XpTAG621*	Feuillet *et al.* 1995
Wheat	*Lr9* (leaf rust)	6BL	*Xmwg684*, OPR-15_{950}	Schachermayr *et al.* 1994
Wheat	*Lr9* (leaf rust)	6BL	*XksuD27*	Autrique *et al.* 1995
Wheat	*Lr10* (leaf rust)	1AS	*STSLrk 10-6*	Schachermayr *et al.* 1997
Wheat	*Lr19* (leaf rust)	7DL	*XksuG39*	Autrique *et al.* 1995
Wheat	*Lr19* (leaf rust)	7DL	*Ep-D1*	Winzeler *et al.* 1995
Wheat	*Lr22* (leaf rust)	2DS	*Xpsr150, Xpsr666*	Bonhomme *et al.* 1995
Wheat	*Lr24* (leaf rust)	3DL	*J9-STS, Xpsr1205*	Schachermayr *et al.* 1995
Wheat	*Lr24* (leaf rust)	3DL	*Xbcd147, Xbcd372*	Autrique *et al.* 1995
Wheat	*Lr24* (leaf rust)	3DL	$SC\text{-}H5_{700}$	Dedryver *et al.* 1996
Wheat	*Lr32* (leaf rust)	3DS	*Xbcd1278, Xcdo395*	Autrique *et al.* 1995
Wheat	*Sr22* (Stem rust)	7AL	*Xpsr165, Xpsr129*	Paull *et al.* 1994
Wheat	*Cre* (Cereal cyst nematode)	2L	*Xglk605, Xabc451*	Williams *et al.* 1994

with the identification of linked markers for each resistance gene involved (Feuillet *et al.* 1995). Linked markers could then be applied in marker assisted selection to select for lines that contain multiple resistance loci. In addition, detailed linkage maps of areas in the genome which contain resistance genes are a pre-requisite for map based cloning.

1.3.4 Use of bulked segregant analysis (BSA) in mapping

The chromosome number and low levels of intraspecific variation in species such as wheat make it difficult to generate high density linkage maps. However, the detection of QTLs requires maps with a high level of genome coverage, to maximise the probability of identifying as many major loci as possible. An alternative option to increasing the map density, is the use of techniques such as bulked segregant analysis (BSA) (Michelmore *et al.* 1991). BSA involves the screening of two bulked DNA samples derived from contrasting segregants in a single population, with the aim of identifying polymorphic markers between the bulks. The bulks are constructed by pooling DNA from individuals that have identical or similar phenotypes for a trait of interest from each tail of the distribution. Each of the pools will have distinct alleles in the region of interest while the remainder of the genome will have equal contributions of alleles from both parents. Linkage between polymorphic markers and the targeted locus or trait can be confirmed by screening the markers on the whole population from which the bulks were constructed.

A number of studies have been reported where BSA has been successfully implemented in identifying markers linked to traits of interest. Michelmore *et al.* (1991) used BSA to identify markers linked to disease resistance genes for downy mildew in lettuce. They demonstrated that markers could be reliably identified in a 25 cM window on either side of the target locus. Chalmers *et al.* (1993) also used BSA to identify RAPD markers linked to QTLs associated with milling energy in barley, while Barua *et al.* (1993) applied BSA to identify RAPD markers linked to a *Rhynchosporium* resistance locus in barley. Similarly, Garcia *et al.* (1996) identified markers linked to root-knot nematode (*Meloidogyne arenaria*) resistance genes in peanut using BSA. The results of these and other studies show that the use of BSA in

combination with rapid PCR based marker techniques is an effective way of rapidly identifying markers linked to specific regions or traits of interest.

1.3.5 Application of markers through marker assisted selection (MAS)

One of the most important applications of molecular markers is their use as a selection tool in breeding programs. The manipulation of desirable traits is an important objective in both genetic research and plant breeding. However, many important quality related traits cannot be measured early in plant development and require either seed or flour samples for testing. Therefore, the identification of markers linked to important traits could result in the development of more efficient breeding strategies, enabling breeders to discard unwanted genotypes early in the program. The use of MAS will also overcome difficulties caused by limited seed supplies and large environmental influences (Hackett *et al.* 1992; Blanco *et al.* 1996). Additionally, linked markers will be useful in the transfer of genes from wild species into breeding lines by enabling more accurate and effective accelerated backcrossing (Paterson 1988; Stuber 1995; Blanco *et al.* 1996). This is done by selecting for the appropriate region and selecting against the rest of the donor genome (Paterson 1988; Lander and Botstein 1989).

Although many studies have identified markers linked to traits of interest in a variety of crops, the application of these markers as selection tools by breeders is still in its infancy. MAS requires assays that are fast, reliable and easy to implement in order to maximise its potential. PCR based markers, such as sequence tagged site (STS) (Olson *et al.* 1989) or sequence characterised amplified region (SCAR) markers (Paran and Michelmore 1993) are particularly suited to MAS. The conversion of linked markers to STSs overcomes problems such as the lengthy assay times associated with RFLPs and AFLPs, and the lack of reliability with RAPD markers. There are several examples where RAPD, RFLP and AFLP markers have been converted to STS markers for use in MAS (Williams *et al.* 1990; Schachermayr *et al.* 1995; Feuillet *et al.* 1995; Dedryver *et al.* 1996; Talbert *et al.* 1996; Blair and McCouch 1997; Huang *et al.* 1997; Paltridge *et al.* 1998; Toojinda *et al.* 1998).

One of the most powerful applications of QTL mapping in plant breeding is the ability to determine the allelic composition of individual segregants which out-perform both parents (McCouch and Doerge, 1995). Transgressive segregation occurs when alleles from different loci recombine to give progeny with a phenotype greater or less than the observed phenotype of either parent. By identifying QTLs associated with a trait through linked markers and knowing the parental origins of those alleles, transgressive segregation can be explained and manipulated. Maintaining set breeding methods and detailed records of crosses in breeding programs also makes it possible to trace the ancestry of genes using molecular markers.

The question of whether markers linked to a QTL in one cross are applicable in other populations is an important consideration relating to the wider utility of molecular markers in breeding programs. If closely flanking markers can be applied in other crosses, they will greatly increase the efficiency of marker assisted selection. For example, several genomic regions associated with plant height, ear height, anthesis and silk emergence in maize have been identified in more than one population as well as in populations of sorghum (Austin and Lee 1996).

1.4 Application of markers in determining genetic similarity

Molecular marker technology is also playing an important role in the identification of genetic relationships between different cultivars. Relationships between genotypes have been developed in the past using three main sources of information: geographic location, pedigree data, and plant characteristics. Plant characteristics include agronomic traits, morphological markers, biochemical markers, and recently molecular markers (Schut *et al.* 1997). A number of molecular marker techniques have been successfully used to identify genetic relationships in different species. Examples of these include the use of RAPDs in Brassica, RFLPs in maize and wheat, SSRs in barley and soybean, and AFLPs in barley, pea and lettuce (dos Santos *et al.* 1994; Messmer *et al.* 1993; Paull *et al.* 1998; Melchinger *et al.* 1994; Russell *et al.* 1997;

Powell *et al.* 1997; Lu *et al.* 1996; Hill *et al.* 1996). The determination of genetic relationships based on genetic similarity estimates, and the assessment of genetic diversity provides plant breeders with a useful source of information for the selection of new parental material.

In the past, breeders have relied on pedigree records when selecting parental material for new crosses. However, accurate co-ancestry estimates are dependent on the availability of reliable and detailed pedigree records. In contrast, DNA based polymorphisms allow more direct comparisons of the variation in nucleotide sequences for estimating genetic relationships. Information about the genetic similarity of different cultivars allows breeders to predict more accurately different aspects of breeding behaviour including heterosis, transgressive segregation and genetic variance (Messmer *et al.* 1993; dos Santos *et al.* 1994). It also provides important information regarding the population size required, and the level of selection intensity required to select for superiour segregants. Crosses are generally made between genotypes that have been highly selected with proven performance in the field. As a result, the number of genotypes being recycled is small, narrowing the base of elite germplasm. An accurate understanding of the genetic similarity between cultivars should help maintain a higher level of genetic diversity, and sustain long term selection (Graner *et al.* 1994).

1.5 Summary and research objectives

The development of various molecular marker techniques in the past 15 years has enabled detailed genetic linkage maps to be constructed in many crop species. These maps have been used to identify many important loci and QTLs in a range of mapping populations. Molecular markers linked to important traits have also been applied in plant breeding programs through MAS. This technology has provided breeders with an additional selection tool, enabling them to begin selecting at the genetic level much earlier in the breeding program. Although a large number of traits have been mapped in the cereals, very little work has been carried out aimed at mapping QTLs associated with traits affecting wheat quality.

The aim of this research was to map and identify markers closely linked to loci associated with important traits in wheat. These included traits related to wheat quality, such as grain protein content, milling yield and flour colour, as well as six important stem rust and leaf rust resistance genes (Sr5, Sr9e, Sr22, Sr36, Lr3a and Lr20). In addition, two different marker systems (RFLPs and SSRs) were compared to assess their ability to detect genetic relationships between different 124 wheat cultivars.

Chapter II

General Materials and Methods

2.1 Plant Material

2.1.1 Mapping population

A recombinant inbred population derived from hexaploid wheat segregating for flour colour, milling yield, protein percentage and several disease resistance genes was used for mapping. The population consisted of 150 F_4 and F_5 derived lines developed through single seed descent (SSD) by Dr. A.J. Rathjen, Waite Agricultural Research Institute, from a cross between the Australian cultivars 'Schomburgk' and 'Yarralinka'. These two varieties segregated for a number of traits including quality traits (protein, milling yield and flour colour), maturity and a number of rust resistance genes. Based on the results of the genetic similarity study oulined in Chapter 8, 'Schomburgk' and 'Yarralinka' are relatively closely related to (genetic distance = 0.197), a result of the common genetic material in their pedigrees. However there is enough variation between them to expect some level of heterozygosity for mapping.

Pedigree 'Schomburgk': - (W3589*Oxley) * Warigal **2) * Aroona **2)

Pedigree 'Yarralinka': -

((Mengavi/Siete Cerros/3/Mengavi/Siete Cerros//Crim/4/Combination III*Warigal#))/13/9

Four seeds of each of the SSD lines were grown in large pots in the glasshouse at 25°C for six weeks. The leaves were then harvested, frozen in liquid nitrogen and stored in preparation for DNA extraction.

2.1.2 Nullisomic-tetrasomic lines

Wheat nullisomic-tetrasomic lines were obtained from Mr Terry Miller (John Innes Centre, Norwich). Each nullisomic-tetrasomic line is deficient for one pair of the 21 pairs of wheat chromosomes, with the missing chromosome pair compensated for by the addition of an extra pair of one of its homologous chromosomes from a different genome. Four seeds of each line were grown in small pots in the glass house at 25°C for several weeks before leaf material was collected for DNA extraction. The DNA extracted from these lines were used to assign markers to specific chromosomes.

2.2 Nucleic acid extraction

2.2.1 Small scale genomic DNA preparation

The method used for small scale extraction of DNA from leaves was modified from Rogowsky *et al.* (1991). A 10 cm long piece of healthy leaf was placed in a 2 ml Eppendorf tube and frozen in liquid nitrogen. The sample was then crushed with a small pestle to a fine powder after which 600 µl DNA extraction buffer (1% sarkosyl, 100 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA, pH 8.5) was added and homogenised with the leaf powder to form a slurry. Extraction was performed by adding 600 µl of cold phenol solution [phenol:chloroform:iso-amylalcohol (25:24:1) equilibrated with 1M Tris-HCl pH 8.0] and mixing on an orbital rotor for 10 minutes. The sample was centrifuged for 10 minutes at 20,160 g and the supernatant transferred to a fresh tube to repeat the phenol extraction step. After the supernatant was collected, 60 µl 3M NaAc (pH 4.8) and 600 µl isopropanol was added and mixed gently at room temperature to allow the DNA to precipitate. The DNA was then pelleted by centrifugation for 5 minutes at 20,160 g and the supernatant discarded. After washing the pellet

with 1 ml 70% ethanol, the DNA was air dried and resuspended overnight at 4°C in 50 µl R40 (40 µg/ml RNAse A in TE buffer).

2.2.2 Medium scale genomic DNA preparation (Guidet *et al.* 1991)

Approximately 2g of harvested leaf material was crushed to a fine powder in liquid nitrogen using a mortar and pestle. When the powder had partially thawed, 4ml of extraction buffer was added and mixed forming a slurry. The slurry was transferred to a 10 ml plastic tube together with 4ml of cold phenol solution and mixed on an orbital rotor for 10 minutes.

After centrifugation for 10 minutes at 12,900 g the supernatant was dispensed into a silica matrix tube and re-extracted with 4ml phenol/chloroform/iso-amylalcohol (25:24:1). The tubes were centrifuged again for 10 minutes at 2060 g and the extraction step repeated. After the third extraction the supernatant was dispensed into fresh 10 ml plastic tubes to which 400 µl 3M NaAc (pH 4.8) and 4ml isopropanol were added. The tubes were mixed gently to precipitate the DNA and centrifuged for 10 minutes at 12,900 g to pellet the DNA. The supernatant was poured off and the pellet washed with 4ml 70% ethanol, air dried and resuspended overnight at 4°C in 350 µl R40.

To calculate the concentration of the DNA, 10 µl of the sample was added to 990 µl TE buffer and vortexed before reading the absorption in a spectrophotometer at A260 (concentration µg/ µl = A260x100x50/1000).

2.3 Plasmid DNA extraction

Plasmid DNA extraction was based on the method outlined by Maniatis *et al.* (1989).

2.4 PCR amplification of cloned insert DNA

The cloned insert DNA was amplified from various vectors (including PUC18, PUC19 and PTZ19U) using the polymerase chain reaction with M13 forward and reverse primers. The primers were synthesised using an Applied Biosystems 392 oligonucleotide synthesiser according to the manufacturers instructions. The reaction mix contained 50mM Tris HCl, 6.5 mM $MgCl_2$ (25 mM), 48μM each dNTP, 0.3 μg each primer, 1 μg plasmid DNA and 1 unit Taq polymerase in a total volume of 50 μl. Temperature conditions for PCR were 95°C for 3 minutes, followed by 35 cycles of 96°C for 1 minute, 55°C or 58°C for 2 minutes, 72°C for 2 minutes with a final step of 72°C for 5 minutes and 25°C for 5 minutes.

2.4.1 Recovery of insert DNA from agarose gels using glass-milk

Following gel electrophoresis of the PCR product, the required band was excised from the gel after staining with ethidium bromide and visualising on a long wave ultraviolet transilluminator (340 nm). The excised agarose block was transferred to a 1.5 ml eppendorf tube and weighed. The DNA was recovered according to the standard protocol supplied with the Geneclean kit (Bio 101) [Bresatec, Australia].

2.5 RFLP analysis

A total of 217 RFLP markers were screened for their ability to identify polymorphisms between the parents. These DNA clones were obtained through the Australian Triticeae Mapping Initiative. The majority of these markers have been mapped in wheat and their chromosomal locations identified (Gale *et al.* 1995; Van Deynze *et al.* 1995; Nelson *et al.* 1995a, 1995b, 1995c; Marino *et al.* 1996). The clones were selected on the basis of their availability and map location, in order to obtaining maximum genome coverage.

2.5.1 Southern blot analysis

2.5.1.1 Digestion of DNA

Digestion of genomic DNA was performed in 1.5 ml Eppendorf tubes containing 3 μg DNA, 4mM spermidine, 0.1 μg BSA (acetylated), 100 mM Tris-HCl, 0.5 M potassium acetate, 100mM magnesium acetate, 10 mM spermidine, 10 mM DTT and 20 units of restriction enzyme in a total volume 10 μl with water. The samples were incubated at 37°C for 5 hours.

2.5.1.2 Gel electrophoresis and transfer to nylon membrane

After digestion, 2 μl 6X Ficoll loading buffer (15% [w/v] ficoll 400, 0.25% [w/v] bromophenol blue, 0.25% [w/v] xylene cyanol FF, pH 8.0) was added to the sample prior to loading on a 1% agarose gel for electrophoresis overnight at 20 mA. Electrophoresis was performed in a tank containing Tris-acetate running buffer (40 mM Tris-acetate pH 8.0, 1 mM EDTA). The DNA was visualised by staining the gels in a solution of ethidium bromide (0.5 μg/ml) on a rocking platform. Lambda DNA digested with *Hind*111 was used as a molecular weight standard.

The gel was then soaked in 200 ml denaturing solution (1.5M NaCl, 0.5M NaOH) for 20 minutes on a rocking platform and placed DNA side up on a sheet of Whatman 3MM filter paper supported by a thick sponge lying in a reservoir of 10 x SSC (1.5M NaCl, 0.15M trisodium citrate). A nylon membrane (Hybond N^+, Amersham) soaked in water for 1 minute followed by 10 x SSC for 1 minute was placed on top of the agarose gel. A second sheet of 3MM filter paper was placed on top of the membrane followed by a 5 cm stack of paper towels and a glass plate. The transfer proceeded by capillary blotting for 5 hours after which the membrane was rinsed in 5 x SSC, dried and fixed by placing the membrane DNA side up on a

sheet of 3MM filter paper soaked in 0.4M NaOH. The membrane was neutralised by rinsing in 50 ml of neutralising solution (1.5M NaCl, 0.5M Tris-HCl, 1 mM EDTA Na_2, pH 7.2) for 5 minutes and then sealed in a plastic bag for storage.

2.5.1.3 Oligolabelling of DNA probes

Two µl of plasmid DNA (approximately 50 ng), 3 µl 9mer random primer mix (0.1 µg/µl) and 3 µl water were placed in a 1.5 ml Eppendorf tube and heated in a beaker of boiling water for 5 minutes. The tube was quickly chilled in ice water and placed on ice, after which 12.5 µl oligo buffer (dATP, dTTP, dGTP 60 µM each, 150 mM Tris-HCl, 150 mM NaCl, 30 mM $MgCl_2$, 300 µg/ml BSA), 0.1 unit Klenow enzyme and 3.5 µl [α-^{32}P]dCTP (~10µCi/µl, Amersham) were added. This mix was incubated for 1 hour at 37°C and then passed through a Sephadex G-100 mini-column saturated with TE buffer to separate the labelled probe from the unicorporated [α-^{32}P]dCTP. 200 µl salmon sperm DNA (5 mg/ml) was then added to the sample.

2.5.1.4 Hybridisation of DNA probes to membranes

The membranes were soaked in 5 x SSC for 5 minutes before being rolled up into a cylinder and placed in a hybridisation bottle (Hybaid) along with 9.8 ml of hybridisation solution comprising 1.8 ml water, 3 ml 5 x HSB (3M NaCl, 100 mM PIPES, 25 mM Na_2EDTA, pH 6.8 with 4M NaOH), 3 ml Dextran sulphate (25% w/v), 2 ml Denhardt's III (2% BSA, 2% Ficoll 400, 2% PVP) and 200 µl salmon sperm DNA (5 mg/ml). Pre-hybridisation was carried out for 4-6 hours at 65°C in a hybridisation oven.

The labelled probe was boiled for 5 minutes, chilled on ice, added to the hybridisation solution in the bottle and left to hybridise to the membranes overnight. The membranes were taken out

of the bottle and washed for 20 minutes at 65°C in 200 ml 2 x SSC, 0.1% SDS. This was followed by three further washes in 1 x SSC, 0.5 x SSC and 0.2 x SSC under the same conditions. Membranes were sealed in plastic wrap and placed in a film cassette containing intensifying screens with the DNA facing up. A sheet of Fuji RX medical X-ray film was placed on top and exposed for 4-5 days at -80°C.

Labelled probe DNA was stripped from the membranes following autoradiography by pouring boiling stripping solution (0.1% SDS, 2 mM Na_2EDTA) over the membranes and rocking on a platform for 30 minutes. Membranes were then dried on 3MM Whatman paper and sealed in a plastic bag and stored at 4°C.

2.5.2 RFLP probes for partial map construction

Total genomic DNA was digested with *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV or *Hind*III. RFLP probes obtained through the Australian Triticeae Mapping Initiative were hybridised to Southern blots of the 150 wheat lines and the results scored as 'Schomburgk' allele, 'Yarralinka' allele or heterozygous according to the genotype at each locus. A total of 215 probes were used to screen the parental lines for polymorphisms.

2.6 Microsatellite analysis

2.6.1 Normal reactions

Polymerase chain reaction (PCR) conditions contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 100 μM each dNTP, 1.5 mM $MgCl_2$, 900 nM each primer, 0.5 units Taq polymerase and 80 ng genomic DNA in a total volume of 25 μl. Temperature conditions for PCR were 95°C for 3

minutes, followed by 35 cycles of 96°C for 1 minute, 55°C or 58°C for 1 minute 30 seconds, 72°C for 1 minute 50 seconds with a final step of 72°C for 10 minutes and 25°C for 5 minutes.

2.6.2 [α-^{32}P]dCTP labelled reactions

Polymerase chain reaction (PCR) conditions were as above except for the addition of 0.1 µl [α-^{32}P]dCTP (~10µCi/µl, Amersham). Temperature conditions for PCR were as stated for non-radioactive reactions.

2.6.3 Polyacrylamide gel electrophoresis

The front glass plate used for the sequencing gels was rinsed with distilled water and dried. When it was completely dry it was treated with Repel-Silan ES (Dimethyldichlorosilane 2% (w/v) in octamethyl-cyclotetrasiloxane) (Pharmacia Biotech) according to the manufactures instructions. This was done to allow the removal of the plate without breaking or tearing the gel.

Amplified fragments from the normal reactions were separated on 7% polyacrylamide gels containing 7% bis-acrylamide (w/v), 90 mM Tris HCl, 88 mM boric acid, 2 mM EDTA, 0.1% ammonium persulphate (w/v) in a total volume of 45 ml. After mixing the acrylamide solution, 50 µl TEMED was added before pouring.

The amplified fragments from reactions containing [a-^{32}P]dCTP labelled fragments were separated on 6% polyacrylamide sequencing gels (10 ml SequaGel buffer, 40 ml Monomer

Solution, 400 µl ammonia persulphate 10% (w/v)) (National Diagnostics) using a Hoefer SQ3 sequencer gel tank (Pharmacia Biotech) at 40 watts for 150 minutes. The gels were then dried on Whatman 3MM paper and exposed to Fuji RX medical X-ray film at room temperature for 24-48 hours.

PUC19 DNA restricted with *Msp*1 was used as a molecular weight standard. The digested fragments were end labelled with [α-^{32}P]dCTP. The labelling reaction contained 25 ng digested Puc19 DNA, 2 µl [α-^{32}P]dCTP (~10µCi/µl, Amersham), 25 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM DTT, 0.5 mM spermidine and 2 units of polynucleotide kinase in a total volume of 10 µl. The mix was incubated at 37°C for 30 minutes after which and equal volume (10 µl) of loading buffer was added (98% deionised formamide, 10 mM Na_2EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol).

2.6.4 Microsatellite markers used in map construction and genetic similarity studies

Thirty one wheat microsatellite (WMS) primer pairs developed by Plaschke *et al.* (1995) and Röder *et al.* (1995) were analysed. 11 (33%) of the 31 microsatellite markers tested identified a polymorphism between the parents. The polymorphic microsatellite makers were mapped on the 150 SSD lines and added to the partial map created using the RFLP markers. The microsatellite markers were also used in genetic similarity studies to assess relationships between 124 Australian wheat cultivars.

2.7 AFLP analysis

2.7.1 Genomic digestion, adaptor ligation and pre-amplification

The AFLP method developed by Vos *et al.* (1995) was followed with some modifications. Genomic DNA (1 μg) was digested with the restriction endonucleases *Pst*1 and *Mse*1 at 37°C. Double stranded adaptors were ligated to the ends of the restriction fragments of each sample in a reaction mix containing 5 μM *Mse*1 annealed adaptors, 0.5 μM *Pst*1 annealed adaptors, 10 mM Tris-HCl, 10 mM magnesium acetate, 50 mM potassium acetate, 5 mM DTT, 1.2 mM ATP and 1 unit of T4 DNA ligase in a total volume of 10 μl. The mix was incubated at 37°C for three hours and then stored at 4°C overnight. The DNA was recovered by ethanol precipitation and re-suspension in 60 μl 0.1M TE (10 mM Tris-HCl pH 8.0, 0.1 mM Na_2EDTA). Pre-amplification was performed using primers specific for the *Pst1* and *Mse1* adaptors including one selective nucleotide. The pre-amplification mix contained 3 ng *MseC* primer, 3 ng *PstA* primer, 10 mM Tris-HCl (pH 8.4), 25 mM KCl, 100 μM each dNTP, 1.5 mM $MgCl_2$, 1U Taq polymerase and 4 μl of the ligated template mix in a total volume of 25 μl. Pre-amplification PCR conditions consisted of 20 cycles of 94°C for 30 seconds, 56°C for 1 minute and 72°C for 1 minute.

2.7.2 Selective amplification and electrophoresis

The pre-amplification template DNA was diluted 1:5 in water before the selective amplification was performed. Selective amplification was carried out using primers containing three selective bases at the 3' end. The amplification mix contained 1.5 ng each selective primer, 10 mM Tris-HCl (pH 8.4), 25 mM KCl, 100 μM each dNTP, 1.5 mM $MgCl_2$, 1U Taq polymerase and 2 μl

of the diluted pre-amplification template DNA in a total volume of 20 µl. PCR reaction conditions for selective amplification consisted of one cycle at 94°C for 30 seconds, 65°C for 30 seconds and 72°C for 1 minute followed by 9 cycles over which the annealing temperature was decreased by 1°C per cycle with a final step of 25 cycles of 94°C for 30 seconds, 56°C for 30 seconds and 72°C for 1 minute. The Pst1 primer used in selective amplification was end-labelled with [γ–^{32}P]dATP (~10µCi/µl, Amersham) by incubating 5 ng primer, 25 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM DTT, 0.5 mM spermidine and 2 units of polynucleotide kinase in a total volume of 10 µl at 37°C for 30 minutes.

Amplified fragments were separated on 6% denaturing polyacrylamide gels (10 ml SequaGel buffer, 40 ml Monomer Solution, 400 µl ammonia persulphate 10% (w/v)) (national diagnostics). Samples were heated to 90°C for two minutes and then chilled on ice prior to loading on the gel. PUC19 DNA restricted with *Msp*1 was used as a molecular weight standard as outlined in Section 2.6.1. The gels were transferred to Whatmann 3MM paper for drying and auto-radiography was performed out using Fuji RX medical X-ray film at room temperature for 24-48 hours.

2.7.3 AFLP markers used for partial map construction

Bulked segregant analysis (Michelmore *et al.* 1991) was performed using AFLPs to develop additional markers for fine mapping major QTLs associated with the traits of interest. The bulks were screened with a total of 64 AFLP primer combinations to identify polymorphisms (Table 2.1). AFLP primer combinations that identified polymorphisms between the bulks were screened on the 150 SSD lines.

Table 2.1 Selective *Pst*1 and *Mse*1 primers used to screen bulks of F_4 SSD lines for polymorphisms associated with flour colour and milling yield (selective bases in bold). A total of 64 possible combinations were used with P1M1 representing the first combination, P1M2 the second combination and P8M8 the 64th combination.

*Pst*1 selective primers	Primer designation	*Mse*1 selective primers	Primer designation
GACTGCGTACATGCAG**AAC**	P1	GATGAGTCCTGAGTAA**CAA**	M1
GACTGCGTACATGCAG**AAG**	P2	GATGAGTCCTGAGTAA**CAG**	M2
GACTGCGTACATGCAG**ACA**	P3	GATGAGTCCTGAGTAA**CAT**	M3
GACTGCGTACATGCAG**ACC**	P4	GATGAGTCCTGAGTAA**CTG**	M4
GACTGCGTACATGCAG**ACG**	P5	GATGAGTCCTGAGTAA**CCA**	M5
GACTGCGTACATGCAG**ACT**	P6	GATGAGTCCTGAGTAA**CCT**	M6
GACTGCGTACATGCAG**AGC**	P7	GATGAGTCCTGAGTAA**CGA**	M7
GACTGCGTACATGCAG**AGG**	P8	GATGAGTCCTGAGTAA**CTA**	M8

2.8 Field trials

The mapping population was sown in field experiments at sites in South Australia during the 1995 and 1996 seasons. The 1995 trial was sown at Winulta (loam over clay) while the 1996 trial was sown at Walkers Flat (sandy loam over clay). A randomised complete block design was used in both years. Plots of each of the 150 lines measured 4.2 metres x 0.75 metres with seed being harvested and stored for QTL analysis. Limited seed restricted the 1995 trial to one replicate while two replicates were sown in 1996.

2.9 Genetic analysis

Data obtained from scoring segregation patterns of the 147 RFLP, microsatellite and AFLP markers were analysed with MapManager QT (Version 8.0) software (Manly and Elliott 1993) using the Kosambi mapping function (Kosambi 1944; Lander *et al.* 1987). Interval mapping based on data obtained from sets of linked markers (Lander and Botstein 1989) was used to obtain further information on QTLs identified by single point analysis. A LOD score of 3.0 was chosen as the threshold for detecting QTLs (Lander and Botstein 1989). A graphical display of the QTL associations was generated using Q-gene (Nelson 1997).

2.10 Analysis of variance and heritability

Analysis of variance (ANOVA) of trait data was performed with the computer program Genstat 5 (Lane 1988) with the three field data sets treated as replicates. The results of the ANOVA were used to obtain an estimate of the broad-sense heritability (H^2) of flour colour (Hartl *et al.* 1988). Broad-sense heritability was calculated as: $H^2 = \sigma^2_g / \sigma^2_t = \sigma^2_g/(\sigma^2_g + \sigma^2_e)$ where σ^2_g is the genotypic variance, σ^2_e is the environmental variance and σ^2_t is the total variance for the trait. The genotypic variance (σ^2_g) can be calculated using the value of the mean squares

between the SSD lines from the field data: m.s. = σ^2_e +b σ^2_g where m.s. is the mean squares, σ^2_e is the residual mean squares and b is the number of replicates. The amount of genetic variation accounted for by a QTL in a segregating population can then be calculated by dividing the phenotypic variation by H^2.

Chapter III

General Mapping

3.1 Introduction

A large number of studies have been conducted in recent years aimed at developing genetic maps in different crop species. These include crops such as maize (Helentjaris *et al.* 1985; Austin and Lee 1996), rice (McCouch *et al.* 1988; Causse *et al.* 1994; Mackill *et al.* 1996), barley (Heun *et al.* 1991; Becker *et al.* 1995; Langridge *et al.* 1995), and wheat (Röder *et al.* 1995; Van Deynze *et al.* 1995; Nelson *et al.* 1995a, 1995b, 1995c). Mapping in diploid species such as barley and rice has developed to a stage where detailed maps covering large proportions of the genome are now available for a number of different crosses. However, the development of genetic maps in polyploid cereal crops such as wheat has been slowed by the lack of intra-specific polymorphism (Nelson *et al.* 1995a).

Genetic maps of the 21 wheat chromosomes have been recently developed using RFLP markers (Table 1.2). The development of these and other detailed maps in related species is laborious and time consuming and is generally undertaken by large research groups. The construction of a detailed genetic map of all 21 wheat chromosomes was beyond the scope of this project. The primary goal of the research reported here was to identify associations between molecular markers and a number of quality traits and disease resistance genes segregating in a population of hexaploid wheat (*Triticum aestivum*).

To avoid the time consuming and difficult task of developing a detailed linkage map, an alternative approach was developed to quickly identify markers linked to loci associated with the traits of interest. This approach was based on the development of a 'partial linkage map' with the aim of establishing a skeletal map using RFLP and microsatellite or simple sequence repeat (SSR) markers. This map could then be used to identify regions of the genome that showed some level of association with the segregating traits in the mapping population.

Having identified regions of interest, bulked segregant analysis could then be implemented to rapidly place additional AFLP markers in these regions, and to identify closely linked flanking marker loci. Using this approach, linked markers could be quickly identified in special purpose populations without having to develop a detailed linkage map.

In this study a partial map was developed for each chromosome group of hexaploid wheat using RFLP, SSR and AFLP markers. Maps were created based on molecular data obtained from a mapping population of 150 F_4 derived SSD lines generated from a cross between the cultivars 'Schomburgk' and 'Yarralinka'.

3.2 Results

Of the 215 RFLP markers screened on the parental lines, a total of 107 (49.8%) detected a polymorphism with at least one of the five restriction enzymes used. Of these 107 markers, 70 were mapped in the 150 SSD lines and formed the skeletal linkage map. Of all the RFLP probes screened, the barley *Xmwg* genomic clones showed the highest level of polymorphism between the two parents (100%), while the barley *Xabc* cDNA clones showed the lowest level of polymorphism (23.5%) (Table 3.1). The second, larger, group of barley genomic clones (*Xabg*) were also highly polymorphic, with 80% of clones detecing a polymorphism with at least one of the restriction enzymes. In general, the genomic clones detected higher levels of polymorphism compared to the cDNA clones (Table 3.1).

A total of five endonuclease restriction enzymes were used to digest the genomic DNA in order to identify polymorphisms. The results show that *EcoRV* was the most effective enzyme in revealing polymorphisms with the genomic clones (33.9%),

Table 3.1 Summary of polymorphisms identified between two wheat cultivars 'Schomburgk' and 'Yarralinka' after screening with 215 genomic and cDNA clones.

Clones	Source	Total number of clones screened	Polymorphic clones	Percent polymorphism	Reference
Xcdo	oat cDNA	35	16	45.7	Heun *et al.* 1991
Xabc	barley cDNA	17	4	23.5	Kleinhofs *et al.* 1993
Xbcd	barley cDNA	36	13	36.1	Heun *et al.* 1991
Xawbma	barley root cDNA	15	9	60.0	Murphy *et al.* 1995
Xmwg	barley genomic	3	3	100	Graner et al. 1991
Xabg	barley genomic	15	12	80.0	Kleinhofs *et al.* 1993
Xpsr	wheat genomic	63	34	54.0	Chao *et al.* 1989
Xwg	wheat genomic	25	12	48.0	Heun *et al.* 1991
Xksu	*T. tauschii* genomic	6	4	66.7	Gill *et al.* 1991
Total		215	107	49.8	

while *DraI* was most effective in showing polymorphisms for the cDNA clones (29.1%) (Table 3.2). Overall, *DraI* was the most effective enzyme, followed by *EcoRV*, *EcoR1*, *BamH1* and *HindIII*. However, the difference between *DraI* and *EcoRV* was negligible. An example of one of the RFLP clones mapped in the population is shown in Figure 3.1.

The SSR markers detected lower levels of polymorphism than the RFLPs, with only 11 of the 31 markers (36%) identifying a scorable polymorphism between the parental lines. The 11 polymorphic SSR markers were then mapped on the 150 SSD lines. An example of one of the SSRs mapped in the SSD lines is shown in Figure 3.2.

Due to the shortage of available SSR markers, AFLP analysis was performed to fine map regions of interest and to increase the number of markers on the map using 64 primer combinations (Table 3.3). The average level of polymorphism identified with the primer combinations screened was only 11.1% (percentage of polymorphic bands compared to the total number of bands), which was lower than both the RFLP and SSR markers. Of the selective *Pst* and *Mse* primers used to screen the parents, *Pst* ACG and *Mse* CAG identified the highest levels of polymorphism (16.5% and 14.4%, respectively) (Table 3.3). Primer pairs that detected a polymorphism between bulks were mapped on the entire population generating an average of 5.2 polymorphic bands per reaction. The additional unlinked markers identified in the screen were added to the map with a total of 43 AFLP marker loci scored in the 150 SSD lines. The number of amplification products generated with each primer pair ranged from 22 to 75, with an average of 49 bands per reaction. An example of a polymorphism identified between the bulks generated for flour colour, and its segregation among the SSD lines is shown in Figure 3.3. Although AFLP primers detect lower levels of polymorphism than SSR or RFLP markers, they have a much

Table 3.2 Percentages of polymorphisms identified between the wheat cultivars 'Schomburgk' and 'Yarralinka' digested with five restriction enzymes and screened with 215 genomic and cDNA clones.

Clones	*EcoR1*	*EcoRV*	*BamH1*	*HindIII*	*Dra1*
Genomic	28.6	33.9	27.7	27.7	25.9
cDNA	19.4	19.4	19.4	17.5	29.1
Total	24.2	27.0	23.7	22.8	27.4

Figure 3.1 Segregation pattern of the RFLP marker *Xabg484* between the parental lines 'Schomburgk' and 'Yarralinka' digested with five restriction enzymes (polymorphisms indicated by arrows).

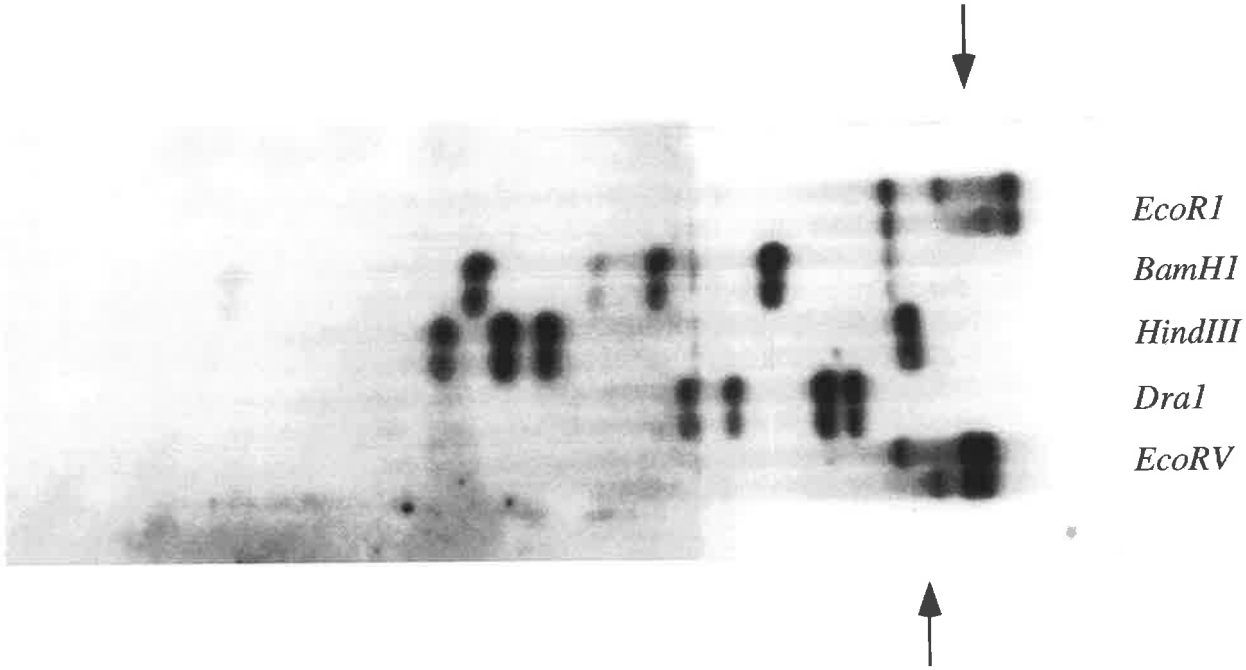

EcoR1
BamH1
HindIII
Dra1
EcoRV

Figure 3.2 Segregation pattern of the microsatellite marker *Xgwm155-3* between the parental lines 'Schomburgk' and 'Yarralinka', and a number of SSD lines.

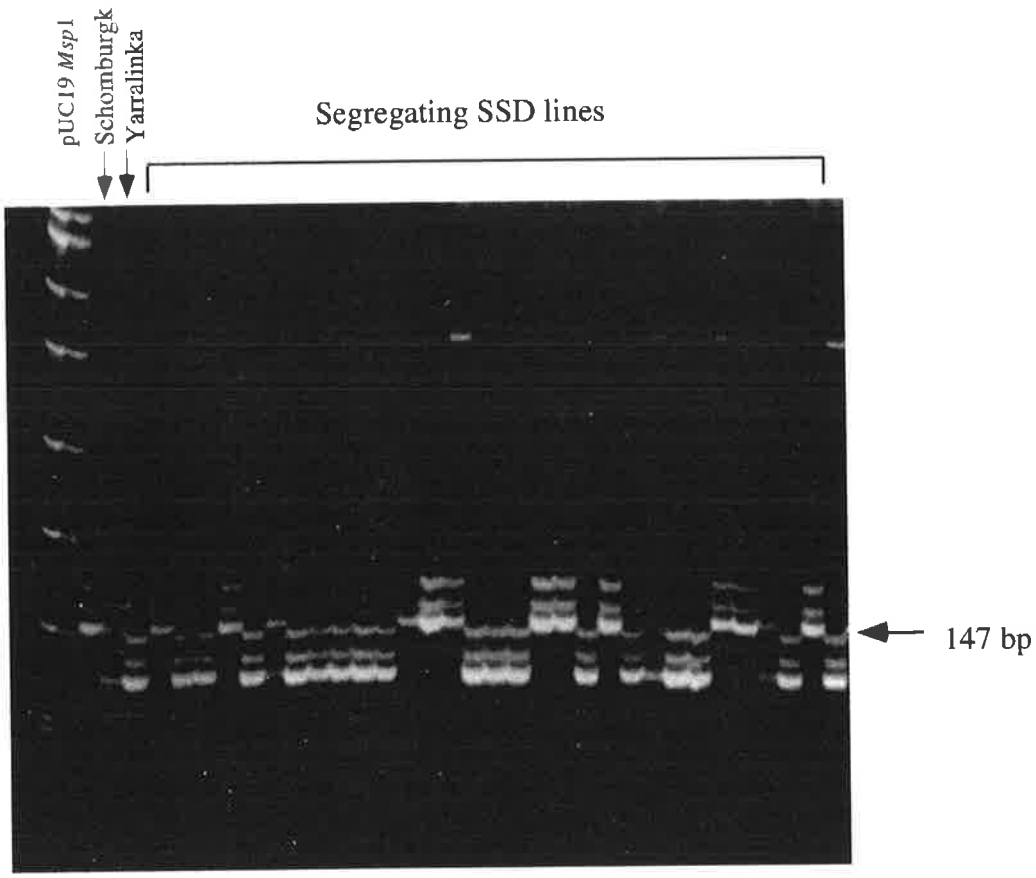
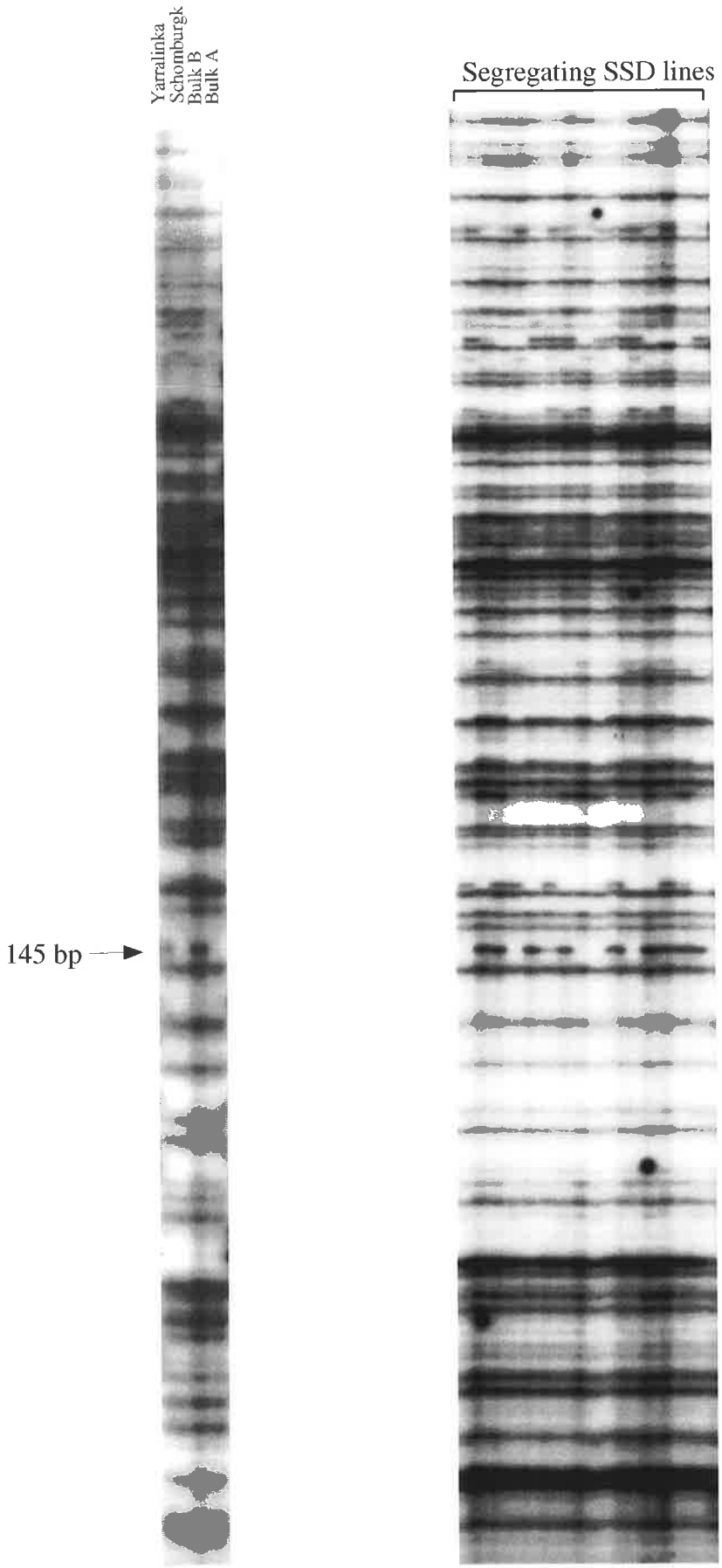

pUC19 Msp1
Schomburgk
Yarralinka
Segregating SSD lines
147 bp

Table 3.3 Total numbers of bands and levels of polymorphism generated from a screen of the two parental lines 'Schomburgk' and 'Yarralinka' using 64 combinations of *Pst* 1 and *Mse* 1 selective AFLP primers.

*Pst*1 and *Mse*1 selective primers	Primer designation	Total number of bands	Average number of polymorphic bands	Average level of polymorphism
Pst **AAC**	P1	46-68	3.4	6.1%
Pst **AAG**	P2	35-75	5.0	9.0%
Pst **ACA**	P3	44-61	6.0	11.5%
Pst **ACC**	P4	22-46	3.6	11.6%
Pst **ACG**	P5	34-48	6.8	16.5%
Pst **ACT**	P6	32-61	6.3	12.6%
Pst **AGC**	P7	46-70	6.0	11.1%
Pst **AGG**	P8	42-54	4.7	10.6%
Mse **CAA**	M1	44-72	5.6	10%
Mse **CAG**	M2	33-59	6.6	14.4%
Mse **CAT**	M3	34-75	3.7	6.8%
Mse **CTG**	M4	34-63	5.3	10.3%
Mse **CCA**	M5	34-68	6.4	13.1%
Mse **CCT**	M6	36-52	4.3	9.7%
Mse **CGA**	M7	22-48	5.1	13.2%
Mse **CTA**	M8	32-65	5.0	10.9%

Figure 3.3 Polymorphism identified between the bulks created for flour colour using the selective primers *Pst*AAG *Mse*CTA and the segregation of the marker (*Xwua16.5-7A*) in a number of SSD lines. The polymorphic band in the bulks and SSD lines is indicated by the arrow.


Yarralinka
Schomburgk
Bulk B
Bulk A
Segregating SSD lines
145 bp

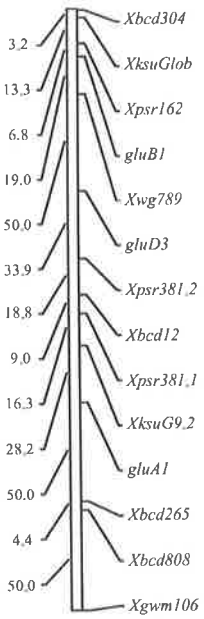
higher multiplex ratio (Qi and Lindhout, 1997; Waugh *et al.*, 1997), which enables a large number of polymorphic markers to be identified per reaction.

The 'partial' consensus map of the 7 wheat chromosomes generated in this study is shown in Figure 3.4. The consensus map was generated by grouping all the markers that mapped to each genome of the 7 chromosome groups. The markers were ordered at an exclusion Lod score of 3 with unlinked markers separated by a distance of 50 cM. A total of 143 scorable marker loci were used to create the partial map. Markers were assigned to chromosomes based on linkage data generated using MapManager QT (Manly and Elliott 1993), as well as established marker information derived from previously published wheat maps (listed in Table 1.2).

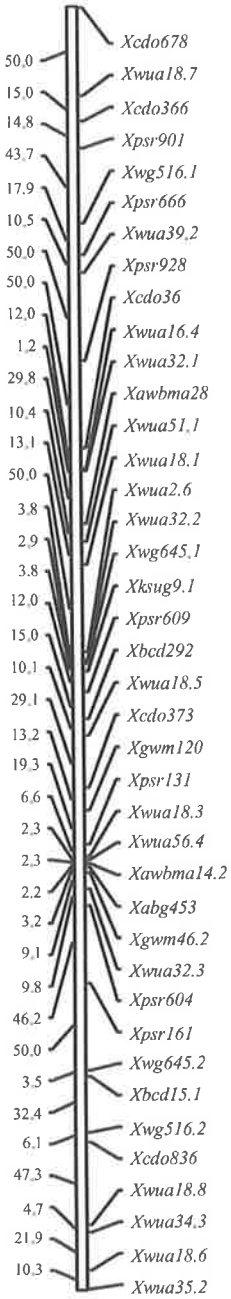
Due to the low number of markers used to create the partial map, some markers could not be assigned to specific linkage groups within a chromosome. The low number of markers contributed in part to the uneven distribution of markers across the genome (Figure 3.5), making it difficult to estimate the total genetic distance of chromosomes based on the marker data. In general the RFLP and SSR markers were well distributed on individual chromosomes containing larger numbers of marker loci. Similarly, the AFLP markers were generally well dispersed on individual chromosomes, although some regions appeared to contain small clusters of markers. These included a region on chromosome 2 and two regions on chromosome 7 (Figure 3.4), one of which was targeted using BSA.

A number of markers showed distorted segregation ratios in the mapping population. These may have arisen as a result of gametocidal effects resulting in unequal transmission of gametes. The pooling of F_1 seed containing different genotypes may also have contributed to the distorted segregation's at some loci. In general, the

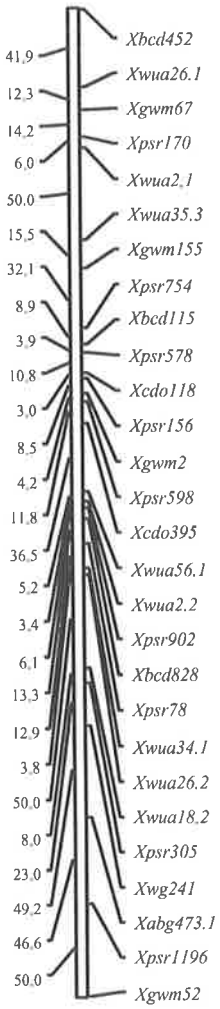
Figure 3.4 Partial consensus map of the 7 wheat chromosome groups from the 'Schomburgk' x 'Yarralinka' mapping population containing 143 loci generated using 70 RFLP, 11 SSR and 10 AFLP primer pairs (genetic distance in centimorgans). Unlinked markers are separated by a distance of 50 cM.



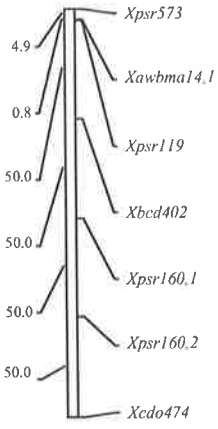
Group 1
(14 markers)



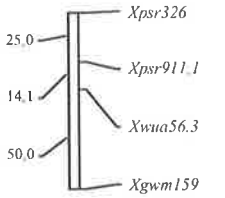
Group 2
(40 markers)



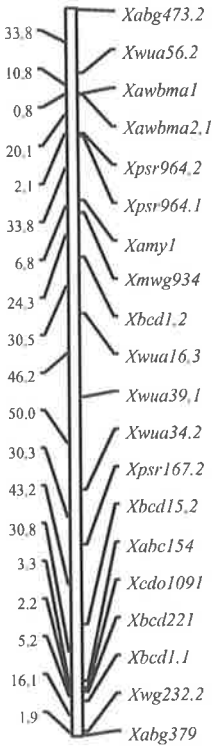
Group 3
(28 markers)



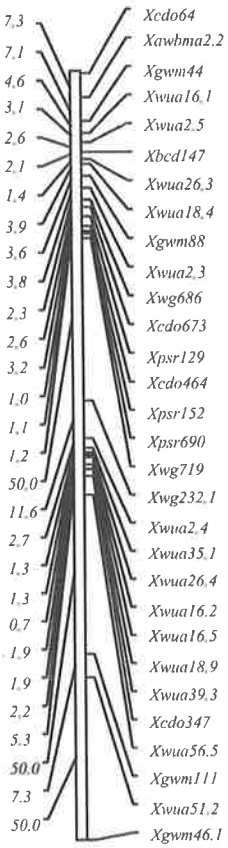
Group 4
(7 markers)



Group 5
(4 markers)

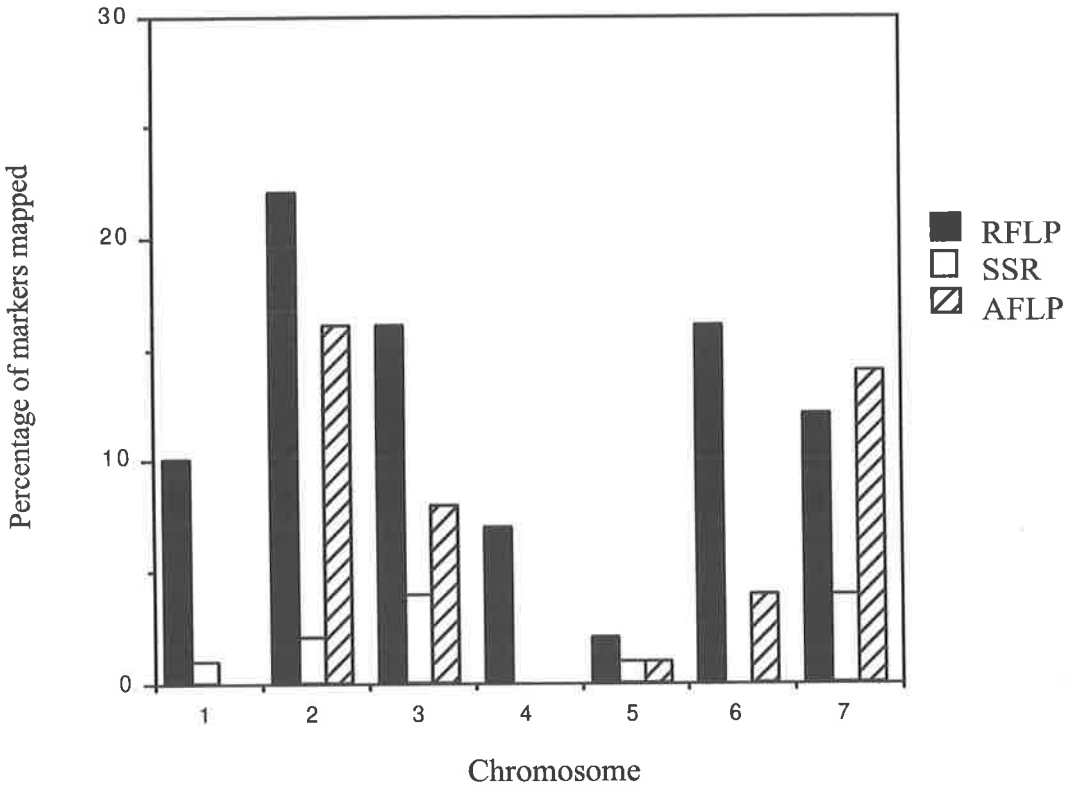


Group 6
(20 markers)



Group 7
(30 markers)

Figure 3.5 Distribution of RFLP, SSR and AFLP markers across the seven chromosome groups in wheat. Markers were mapped in a population of 150 F_4 derived SSD lines generated from a cross between 'Schomburgk' and 'Yarralinka'.


30
20
10
0
Percentage of markers mapped
1
2
3
4
5
6
7
Chromosome
RFLP
SSR
AFLP

deviations from the expected segregation ratios were not sustained over adjacent markers, indicating that there are no large regions showing skewed segregation.

3.3 Discussion

The 'partial' consensus map generated in this study was produced with the aim of identifying molecular markers linked to economically important traits segregating in the population. The skeletal nature of the map made it difficult to compare with more detailed maps that have been generated in wheat, as there were few markers in common between this map and other published maps. However, the literature published to date has demonstrated that there is a high degree of colinearity between the genomes and homoeologous groups within the Triticeae (Devos *et al.* 1992; Bennetzen and Freeling 1993; Van Dynze *et al.* 1995).

The percentage of RFLP clones that identified a polymorphism between the parents (49.8%) is comparable to results obtained by Champaux *et al.* (1995) in rice (45%), Blanco *et al.* (1996) in tetraploid wheat (45%), and Nelson *et al.* (1995b) in a wide cross of wheat (50%). Higher percentages of polymorphic clones have been identified in rice (Li *et al.* 1995) and barley (Graner *et al.* 1991), but these species generally show higher levels of intraspecific polymorphism than wheat (Plaschke *et al.* 1995; Röder *et al.* 1995; Yang *et al.* 1994). Overall, the percentage of polymorphic markers identified with the genomic clones was higher compared to the cDNA clones (Table 3.1). Graner *et al.* (1991) also found that a higher percentage of genomic clones identified polymorphisms compared to cDNA derived clones in barley. It is probable that the higher level of polymorphism observed with the genomic markers in this study, reflects the targetting of non-coding regions. It also

indicates that there may be more variation between the parental lines in non-coding regions compared with conserved regions.

In this study the percentage of SSRs that were polymorphic between the parents (36%) was lower than the RFLPs. This result is comparable with that of Nagaoka and Ogihara (1997) who identified a polymorphism rate of 33% for SSR primers between two wheat accessions. Similarly, Kojima *et al.* (1998) identified a polymorphism rate of 33% when SSRs were mapped in wheat. However, in other crops such as rice and maize, SSRs have been found to show higher levels of heterozygosity than RFLPs (Taramino and Tingey 1996; McCouch *et al.* 1997). The application of SSRs in this study was limited by the low number of sequences available. The cost and time required to develop SSRs has inhibited their application to some extent, however, once established they provide reliable site specific markers that are easy to implement in mapping programs.

The percentage of AFLP primer pairs that identified a polymorphism between the parents in this study was lower than that for both the RFLPs and SSRs. Other studies have also identified relatively low levels of polymorphism with AFLP markers (Schut *et al.* 1997; Qi and Lindhout 1997). However, the high multiplex ratio of AFLPs makes them a particularly useful marker system for identifying large numbers of polymorphisms. The number of amplification products generated using AFLP in this study are comparable with the findings of Waugh *et al.* (1997). They identified 60-80 products per reaction in three populations of barley digested with *EcoR1* and *Mse1* and amplified using primers with three selective bases. In contrast, Qi and Lindhout (1997) identified much higher numbers of amplification products in barley (50 to 180 bands) using the same restriction enzymes and primers with three selective bases. Additionally, Lu *et al.* (1996) only identified 22-39 bands per AFLP

primer combination in pea after digesting with *Pst1* and *Mse1* enzymes, and amplifying with primers containing two and three selective bases, respectively.

When AFLP markers were initially developed it was unclear whether similar sized products were actually homologous. This led to uncertainty about whether markers were transferable between populations or whether results would be reproducible between laboratories. Waugh *et al.* (1997) assessed a number of AFLP primer pairs in three separate populations of barley with the aim of establishing whether similar sized products in different genetic backgrounds were actually homologous. A total of 81 products were mapped in all three populations with each marker mapping to a similar location in each population. They identified only minor changes in marker order, which often occur in mapping experiments and are attributed to computational inaccuracies in estimating marker order (Waugh *et al.* 1997). Based on the results they concluded that the products they mapped in different populations were homologous and could be used as chromosome specific markers. This would reduce the need to map alternative single-locus markers, such as RFLPs, for chromosome alignment. AFLPs could be used as scaffold markers at known locations for rapid genome coverage in mapping populations. The use of AFLP markers in such an approach would be extremely beneficial as very little DNA is required, the assay is extremely fast and large numbers of loci are detected.

The group 1 chromosome map developed in this study consists of 14 evenly distributed markers spanning more than 300 cM (Figure 3.4). This is significantly larger than the group 1 maps generated by Van Deynze *et al.* (1995) which covered around 170 cM using more than double the number of markers. Chromosome 2 contained the highest number of markers (42) covering an estimated distance of more than 730 cM. The map of group 2 chromosomes generated by Nelson *et al.* (1995a) contained more markers yet spanned a shorter genetic distance. The marker

distribution on chromosome 2 is generally even although some minor regions of clustering are apparent (Figure 3.4). A total of 28 markers mapped to group 3 covering a genetic distance of more than 430 cM, compared to a maximum distance of 260 cM mapped on 3B by Nelson *et al.* (1995b) with 62 markers. Groups 4 and 5 contained very few markers (7 and 4 respectively) which prevented the establishment of clear linkage groups. Chromosome 6 was assigned 22 markers covering a total genetic distance of more than 260 cM. This was slightly larger than the map developed by Marino *et al.* (1996) which spanned a maximum distance of 206 cM on chromosome 6D. A total of 32 markers mapped to group 7 spanning more than 230 cM. This was also larger than the map generated by Nelson *et al.* (1995c), which covered a maximum of 120 cM on chromosome 7A.

The genetic distance measures of the maps generated in this study were larger compared with other maps published to date. This increase in genetic distance may have arisen in part with the use of PCR based markers. Other studies have found that the addition of AFLP markers to established RFLP maps increased the total map length, with markers mapping to more distal regions of the chromosome arms (Van Eck *et al.* 1995; Schondelmaier *et al.* 1996; Mackill *et al.* 1996). Another factor that may have led to the large estimations of genetic distance in this study was the low number of markers mapped. This resulted in a number of unlinked markers and markers groups across the genome. In addition, a number of markers showed distorted segregation ratios, which may have led to larger estimations of genetic distance.

One of the most important requirements of this 'parital' map development approach is the placement of evenly distributed 'scaffold' markers across the whole genome. In this study the RFLP and SSR markers were not evenly distributed across all chromosomes (Figure 3.5). The RFLP markers used to screen the parents for

polymorphisms were initially selected based on their availability and distribution. However, chromosomes 4 and 5 contained only 7 and 4 markers, respectively indicating that these chromosomes may not be very polymorphic in this cross. Although this reduced the probability of identifying all the regions of interest, major QTLs associated with the segregating traits could still be successfully identified and mapped.

Chapter IV

Wheat Protein

4.1 Introduction

Grain protein concentration is an important factor affecting both the milling and baking quality of flour, and plays a major role in determining end product use (Costa and Kronstad 1994; Payne 1987; Stoddard and Marshall 1990). Grain protein is measured and discussed under two major categories, quantity and quality. Quantity is easily measured and is based on standard laboratory procedures, whereas quality is more complex, involving characteristics inherent in the protein (Swanson 1938). Protein quality is determined by the type of subunits that are present, including both low and high molecular weight glutenins, as well as gliadins. A significant amount of work has been carried out studying these subunits and their effects on grain quality (for review see Payne 1987; Autran 1993). However very little is understood about the genetic control of total protein in the grain. The percentage of grain protein in the flour affects many properties of bread baking including dough mixing requirement, extensibility, loaf volume and water absorption. The requirements for each of these properties varies depending on the nature of the end product, with high protein levels required for the production of breads and pasta (12-15%), while low protein levels are desirable in the production of noodles (11-13%) and biscuits (8-9%) (Crosbie 1989; Yamazaki *et al.* 1981). Therefore, breeders need to select for different levels of protein in their breeding lines in order to produce cultivars that meet the requirements set by the industry.

Factors which contribute to protein percentage in the harvested grain include both genetic and environmental components. The ability of the plant to take up nitrogen during the growing period, and its partitioning or redistribution during grain development are important factors that affect grain protein content (Darlling *et al.* 1976; Desai and Bhatia 1978; Cox *et al.* 1986). Other physical traits such as the size of the grain and the amount of starch in the endosperm, will affect the percentage of protein in the mature seed. These physical characteristics are

significantly influenced by environmental conditions throughout the growing season. The time of maturity or heading date also plays a critical role in determining the protein levels in the harvested grain as it affects many factors related to grain filling. Despite the complexity and variability of protein levels, it is an important selection trait in the development of new cultivars.

In order to select for superior breeding lines, protein content must be measured to allow segregating lines to be ranked. Grain protein can be measured in different ways with a number of tests varying in speed and accuracy. The determination of wet and dry gluten content of flour by washing has been used to estimate protein quantity. However, the experimental error associated with this test is large. In contrast, Near Infrared spectroscopy (NIR) (Williams 1975; Rosenthal 1975) is a reliable and accurate method commonly used for the rapid determination of protein levels in small samples of crushed grain or flour. This method is used in breeding programs to assay protein levels in large numbers of samples. However, the most accurate measure of protein quantity is the Kjeldahl nitrogen analysis method. This approach is based on the assumption that there is a constant relationship between total nitrogen, and the amino acids which make up proteins. The Kjeldahl method uses a wet oxidation in concentrated sulfuric acid, and was first published by Kjeldahl in 1883. It has since been modified to include many types of nitrogen compounds in various crop species (Bradstreet 1954). The Kjeldahl method is generally used by breeders to accurately measure protein levels in smaller numbers of breeding lines which have been carried through the program. However, the test is time consuming and expensive.

The complex interactions between an unknown number of genes and the environment make it difficult for breeders to select for protein accurately. Many trials and replicates need to be assessed over different sites and seasons to determine the genetic variation in protein levels for different lines. Therefore, the development of an inexpensive reliable assay that could be used to select for protein, would provide breeders with a useful tool for selection early in the breeding program. The aim of this study was to identify molecular markers linked to QTLs associated with grain protein percentage. Maturity was also assessed in one of the field trials to determine whether it affected protein levels in the segregating population. The partial

consensus map described in the previous chapter was used to identify putative QTLs associated with both protein percentage and maturity.

4.2 Materials and methods

4.2.1 Determination of protein using the Kjeldahl method

Grain used for measuring protein percentage was harvested from the two field trials conducted in 1995 and 1996 (see Section 2.8). The Kjeldahl method was used to measure protein percentage. Using this method, a known weight of burr milled grain (approximately 1 g) was digested with two Kjeldahl catalyst tablets in 15 ml of sulphuric acid (0.1M) at 400°C for 1 hour and 20 minutes in a heating block. After cooling, 80 ml of water was added to each sample before the analysis was performed using a Kjeldahl 1030 Auto Analyser. A blank containing a small amount of digested sugar was run before each batch of samples for background calibration. To calculate the protein percentage for each sample, the following formula was used: Percentage protein = [(titre-blank)/sample weight] x 0.1401 x N(0.79857), where N is a calibration factor (N = 5.7 for wheat and flour). This calculation was performed according to the calibration established by the Cereal Chemistry Division of the South Australian Research and Development Institute.

4.2.2 Analysis of variance and heritability

Analysis of variance was performed on the field trial data in order to calculate an estimate of broad sense heritability for protein, as outlined in Section 2.10.

4.2.3 Maturity

Maturity scores for each of the 150 SSD and parental lines were obtained from the 1996 field trial by assessing the heading date of plots at the end of the season. Each line was scored from 1 – 6, with 1 being very early and 6 being very late.

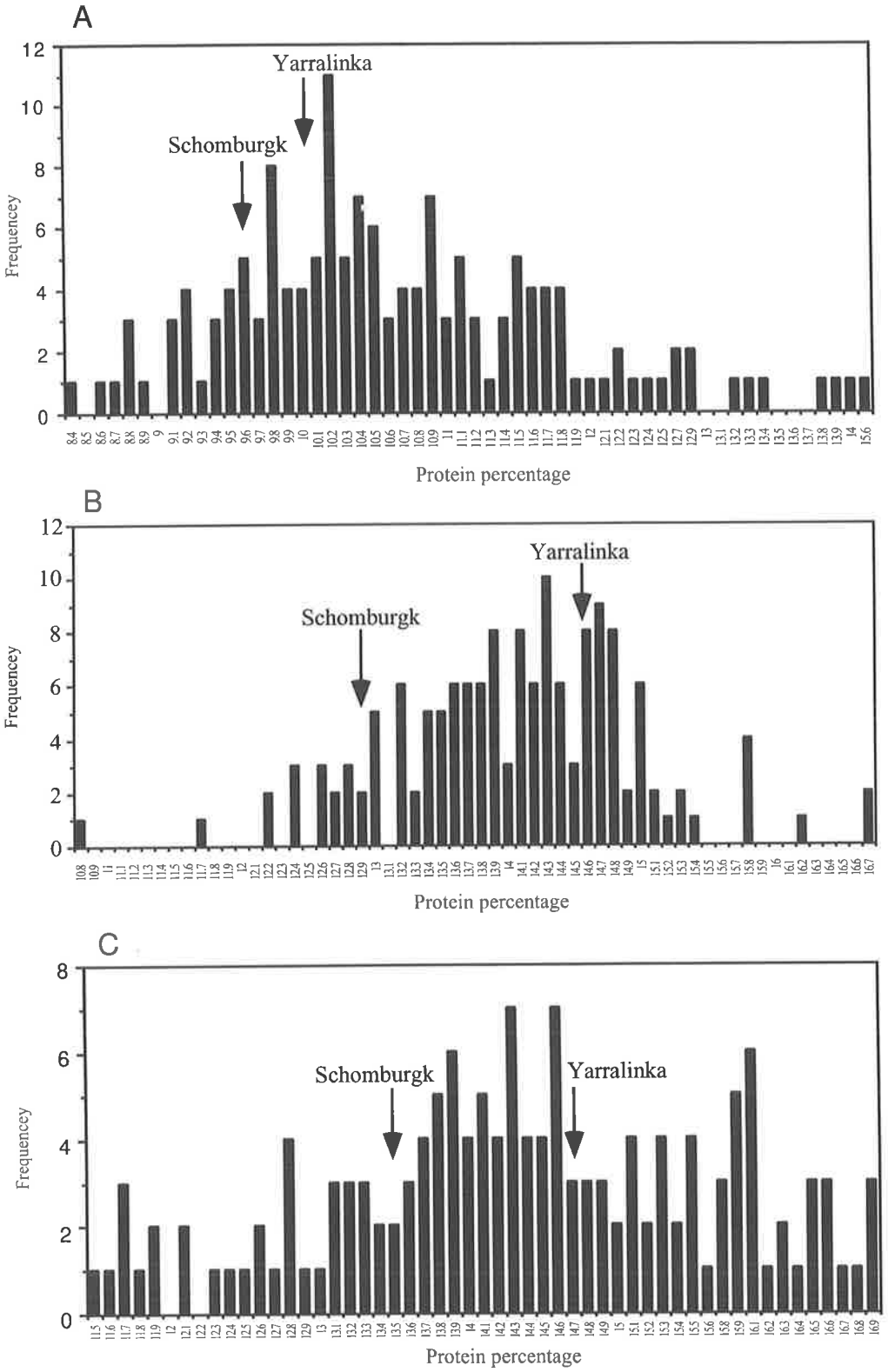
4.2.4 Identification of markers linked to QTLs associated with protein and maturity

The associations between markers and QTLs were tested using single-point regression analysis with MapManager QT (Version 8.0) (Manly and Elliott 1993), based on the likelihood ratio statistic. Single-point regressions were the primary method for detecting associations between markers and QTLs. Groups of two or more closely linked markers with significant associations were assumed to identify the same QTL. Interval mapping, which searches for the effects of a QTL using sets of linked markers (Lander and Botstein 1989), was used to obtain further information on QTLs identified by single point analysis. A LOD score of 3.0 was chosen as the threshold for detecting QTLs (Lander and Botstein 1989). A graphical display of the QTL associations was generated using Q-gene (Nelson 1997).

4.3 Results

Plots of each of the 150 SSD lines were harvested from the field trials in both the 1995 and 1996 seasons. After crushing 30g of seed, a small sample was assayed to measure protein percentage. The protein scores of the 150 SSD lines obtained from each field trial data set were plotted as histograms to examine the complexity of inheritance (Figure 4.1). These plots reveal a large amount of variation in protein levels within the segregating population. Furthermore, in all three data sets, the range in variation observed in the population extended well beyond the mean parental scores for 'Schomburgk' and 'Yarralinka'. This extended range in variation indicates that there is evidence of transgressive segregation for the trait in this population,

Figure 4.1 Histograms of protein percentages of the 150 SSD lines from (A) the 1995 field trial, (B) Replicate 1 of the 1996 field trial, and (C) Replicate 2 of the 1996 field trial. Protein percentages were calculated using the Kjeldahl method from ground flour samples.


A
Yarralinka
Schomburgk
Frequencey
Protein percentage
B
Yarralinka
Schomburgk
Frequencey
Protein percentage
C
Schomburgk
Yarralinka
Frequencey
Protein percentage

suggesting that both 'Schomburgk' and 'Yarralinka' contain alleles associated with high and low levels of grain protein. The results also show that 'Yarralinka' produced consistently higher protein levels (~1%) than 'Schomburgk' in the field. This supports the results reported when 'Yarralinka' was first released which showed that 'Yarralinka' produced consistently higher levels of protein compared with other commonly grown South Australian varieties, including 'Schomburgk' (Rathjen *et al.* 1995). It was also noted that the distribution and the position of the parents with the distribution range varied across all three data sets. The parental means for 'Schomburgk' and 'Yarralinka' in both Replicate 1 (12.9% and 14.6%) and Replicate 2 (13.5% and 14.7%) of the 1996 trial were higher than the 1995 data. This increase in protein was also reflected in the mapping population and was attributed to site and seasonal effects.

Using the genetic map described in Chapter 3, a total of seven markers were found to be associated with protein (Table 4.1). However, each of the associated markers were identified as being linked to protein in only one data set, with no markers consistently linked to protein in all three data sets. In 1995, only one RFLP marker, (*Xbcd1-6D.1*) located on chromosome 6D, was identified that was associated with protein ($P \leq 0.01$). In contrast, four RFLP markers and one SSR marker were associated with protein in the first replicate of the 1996 trial, including *Xcdo64-7*, *Xawbma2-7.2*, *Xwg719-7*, *Xcdo464-7* and *Xgwm88-7*. However, only one AFLP marker on chromosome 2 (*Xwua56-2.4*) was associated with protein in the second replicate. The failure to identify consistently linked markers was attributed to the low heritability of the trait in this population, and the large amount of transgressive segregation. From the regression coefficients outlined in Table 4.1, it appears that all except one of the linked markers (*Xbcd1-6D.1*) were associated with putative QTLs contributing to lower levels of protein. This may be an artefact of the uneven distribution of markers across the entire genome, such that loci contributing to higher protein levels were not identified.

Table 4.1 Summary of 7 molecular markers identified linked to protein percentage in at least one of the three field trial data sets (P≤0.01). The likelihood ratio statistics, % total variation accounted for, and the regression coefficients for each marker are listed.

Marker	Likelihood ratio statistic			% Total Variation			Regression coefficient		
	1995	1996 rep1	1996 rep2	1995	1996 rep1	1996 rep2	1995	1996 rep1	1996 rep2
Xbcd1-6D.1	9	-	-	6	-	-	0.82	-	-
Xcdo64-7	-	7.4	-	-	6	-	-	-0.47	-
Xawbma2-7.2	-	9.4	-	-	7	-	-	-0.50	-
Xwg719-7	-	8.6	-	-	8	-	-	-0.55	-
Xcdo464-7	-	6.7	-	-	7	-	-	-0.52	-
Xgwm88-7	-	7.3	-	-	5	-	-	-0.47	-
Xwua56-2.4	-	-	10.1	-	-	6	-	-	-0.36

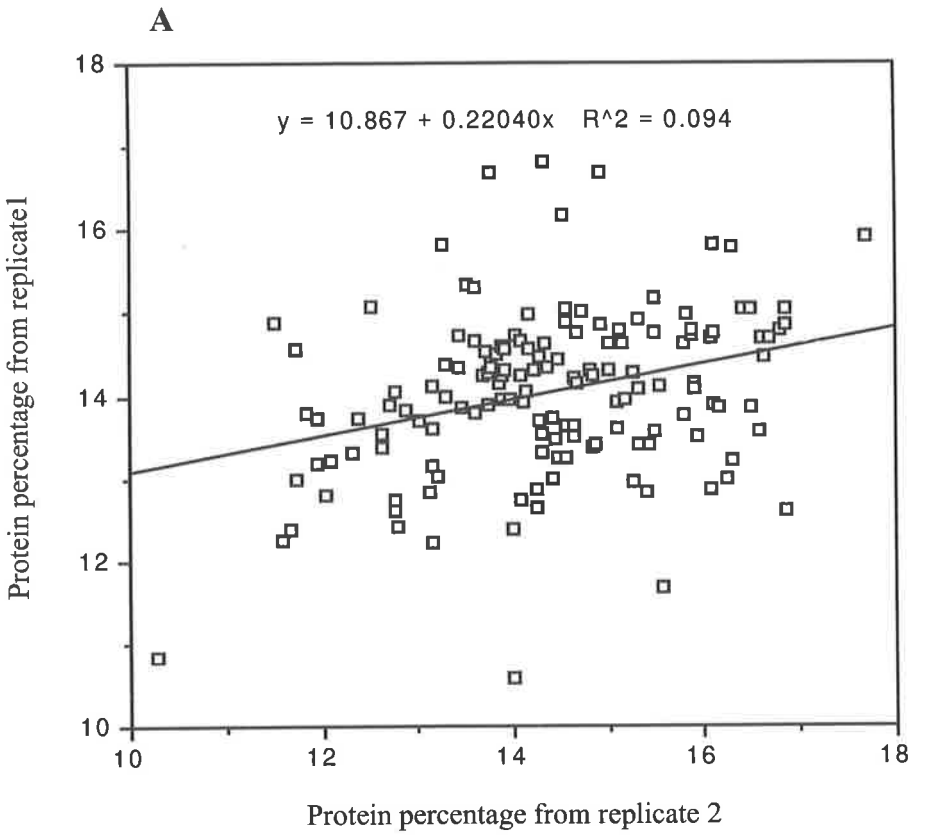
The heritability of protein content in this population was found to be low (H^2 = 0.11), indicating that a large proportion of the observed variation has arisen as a result of environmental influences or other genetic factors such as maturity or heading date. Linear regression was performed to compare the protein scores of the 150 lines between the three field data sets (Figure 4.2). The results showed that there was virtually no correlation in protein levels between the protein percentages of each of the 150 SSD lines for the two 1996 replicates (A) (r^2 = 0.094). Furthermore, the comparison between the protein scores for the 1995 field data and the average of the two 1996 replicates (B), revealed an even poorer correlation in protein scores (r^2 = 0.011). Additionally, the ANOVA results (Table 4.2) show that there was no significant difference in protein content between the 150 lines (F probability = 0.014).

To determine whether variation in maturity was affecting protein percentage, maturity scores were obtained from the 1996 field trial. The results of the maturity scores showed that there was a considerable level of variation in heading date within the population, with a difference of approximately 3 weeks between the earliest and latest lines. When the maturity scores were plotted as a histogram there was evidence of transgressive segregation within the 150 SSD lines (Figure 4.3), indicating that both 'Schomburgk' and 'Yarralinka' contain alleles associated with both early and late maturity. When interval mapping was carried out using Q-gene (Nelson 1997), a QTL associated with maturity was identified on the short arm of chromosome 2 (Figure 4.4). A number of markers were linked to this locus with the closest marker being *Xwua56.4*, which accounted for 10% of the total variation in maturity between the 150 SSD lines. This marker was also linked to a putative QTL associated with protein in the second replicate of the 1996 field trial data set, which accounted for 6% of the total variation in protein.

4.4 Discussion

The selection of lines with desirable protein levels in the breeding program requires multiple field trials to determine real genetic differences which may otherwise be masked by environmental effects. Moreover, a small amount of flour is required to perform the

Figure 4.2 Comparison of protein percentages of the 150 SSD lines obtained from (A) the two 1996 field trial replicates, and (B) the 1995 and 1996 field trials. Protein percentages were calculated using the Kjeldahl method from ground flour samples.


A
y = 10.867 + 0.22040x R^2 = 0.094
Protein percentage from replicate1
Protein percentage from replicate 2
18
16
14
12
10
10
12
14
16
18

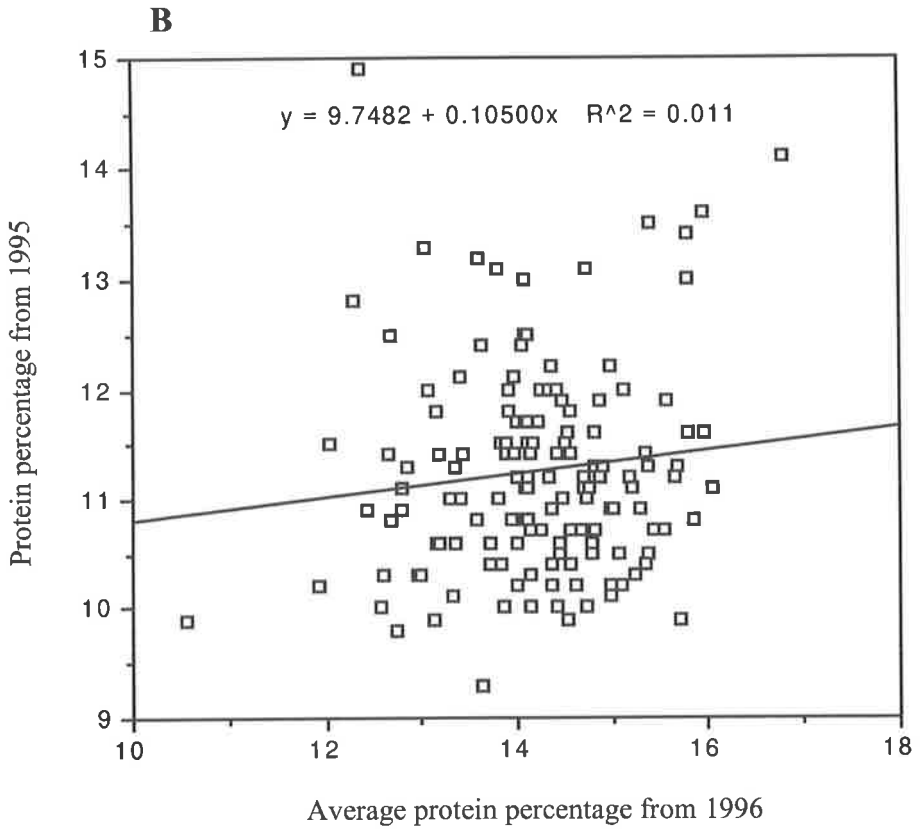

B
y = 9.7482 + 0.10500x R^2 = 0.011
Protein percentage from 1995
Average protein percentage from 1996
15
14
13
12
11
10
9
10
12
14
16
18

Table 4.2 Analasis of variance results for protein percentages from 150 segregating SSD lines sown over two seasons at different field sites.

Source of variation	df	Sums of squares	Mean squares	Variance ratio	F pr.
Between parents	1	26.9	26.9	50.59	<0.001
Between lines	149	260.15	1.746	1.36	0.014
Residual	294(4)	378.15	1.286		

Figure 4.3 Histogram of Maturity scores of the 150 SSD lines from the 1996 field trial. Plots were scored in the field at the end of the growing season. Maturity scores were ranked from $1-6$, with 1 being very early and 6 being very late. The parental scores are indicated by arrows.

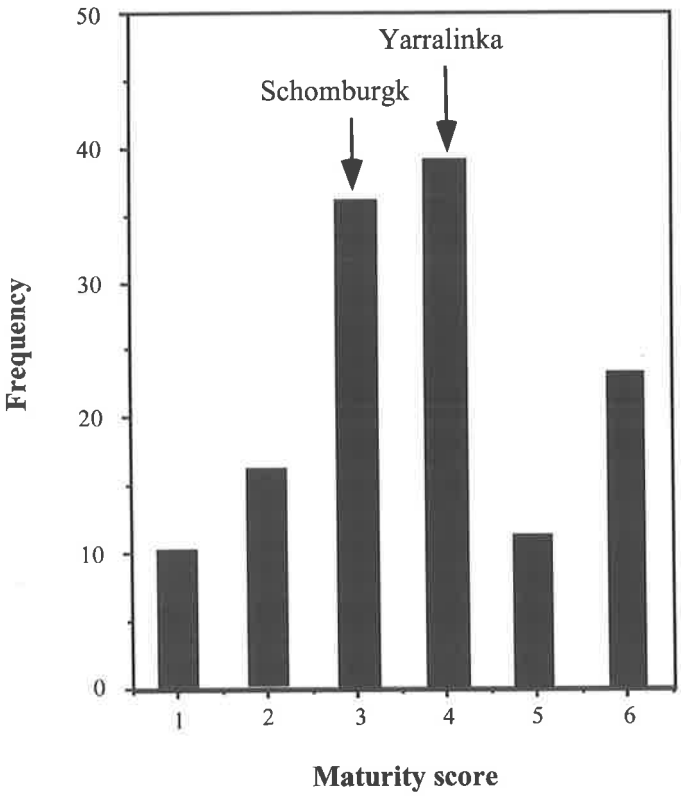
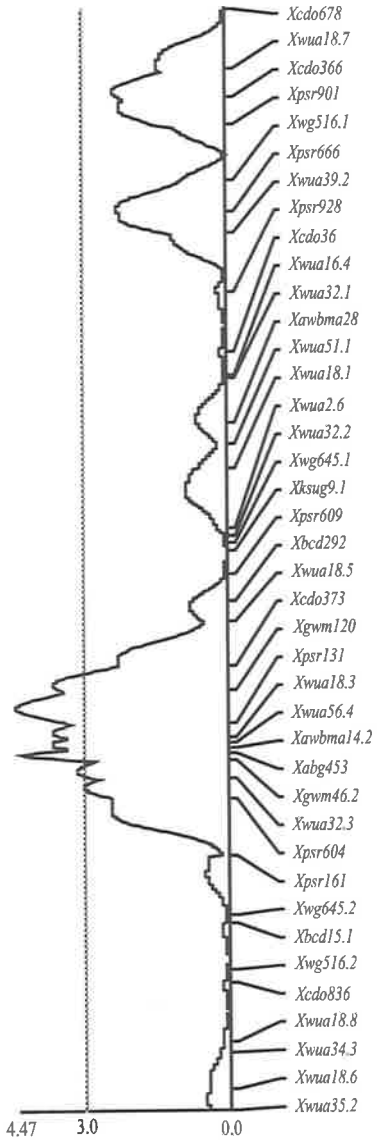

Yarralinka
Schomburgk
Frequency
50
40
30
20
10
0
1
2
3
4
5
6
Maturity score

Figure 4.4 LOD plot of chromosome 2 showing the location of a QTL on the short arm associated with maturity. Maturity scores were obtained from the 1996 field trial. This locus accounted for 10% of the total variation in maturity between the 150 SSD lines.


Xcdo678
Xwua18.7
Xcdo366
Xpsr901
Xwg516.1
Xpsr666
Xwua39.2
Xpsr928
Xcdo36
Xwua16.4
Xwua32.1
Xawbma28
Xwua51.1
Xwua18.1
Xwua2.6
Xwua32.2
Xwg645.1
Xksug9.1
Xpsr609
Xbcd292
Xwua18.5
Xcdo373
Xgwm120
Xpsr131
Xwua18.3
Xwua56.4
Xawbma14.2
Xabg453
Xgwm46.2
Xwua32.3
Xpsr604
Xpsr161
Xwg645.2
Xbcd15.1
Xwg516.2
Xcdo836
Xwua18.8
Xwua34.3
Xwua18.6
Xwua35.2
4.47
3.0
0.0
Lod Score

appropriate NIR or Kjeldahl assays, which are laborious and expensive when dealing with large numbers of samples. Therefore, the identification of molecular markers linked to major genes controlling protein is a major goal in wheat mapping. Previous studies have shown that grain protein generally exhibits a large range of continuous variation and is affected by several factors, including environmental conditions and agricultural practice (Stoddard and Marshall 1990; Payne *et al.* 1987). Researchers studying the inheritance of protein in the past have proposed varying types of control, ranging from complex genetic control, to a few major genes with unknown numbers of minor genes (Sampson and Flynn 1982).

In this study a partial linkage map of hexaploid wheat was generated from a population of 150 SSD lines with the aim of identifying major QTLs associated with grain protein. From the ANOVA results of the field trial data, the heritability of protein percentage in the population was found to be low (H^2=0.11). Consequently, it was difficult to obtain reproducible protein scores for the 150 SSD lines. This was highlighted by the lack of a significant correlation in protein scores between seasons and replicates over the three field data sets. Furthermore, the lower the overall broadsense heritability of the trait, the lower the heritabilities of each QTL involved, making them difficult to map (Kearsey and Farquhar 1998). A total of seven markers associated with protein percentage were identified on chromosome groups 2, 6 and 7. The locus on group 6 chromosomes (linked to *Xbcd1-6D.1*) may be the same as that identified by Blanco *et al.* (1996), who identified a QTL associated with total protein on chromosome 6 in wheat. However, the results are inconclusive.

The results obtained in this study suggest that the variation in protein in this population is significantly influenced by other environmental and genetic factors, making it difficult to detect significant associations between markers and QTLs. One of the main factors which affected protein percentage, was the variation in maturity observed between the 150 SSD lines. A QTL associated with maturity was identified on the short arm of chromosome 2. This QTL was located in the same position as a putative QTL identified associated with protein in the second replicate of the 1996 field trial data. This region is of particular interest given the apparent pleitropy exhibited by the locus. A total of three classes of genes associated with maturity have

been identified in cereals, including genes associated with vernalisation response, photoperiod response, and genes which affect flowering independently of these environmental conditions (known as earliness per se genes, or *eps*) (Laurie *et al.* 1995).

Laurie *et al.* (1994, 1995) used glasshouse experiments to identified two major photoperiod genes in barley with different modes of action. The gene with the largest effect was an *eps* locus located on the short arm of chromosome 2 (2H) (*Ppd-H1*), while the second gene was located on the long arm of chromosome 5 (1H) (*Ppd-H2*). When field experiments were carried out, *Ppd-H1* on chromosome 2 had a highly significant effect on flowering time while *Ppd-H2* showed no significant effect. Loci associated with vernalisation (*Sh, Sh2, Sh3*) were also identified on chromosome 4(4H), 7(5H) and 5(1H). The major *eps* locus on chromosome 2 was thought to be related to the *Ppd* series of photoperiod genes on the short arms of chromosome 2 in wheat previously reported by Welsh *et al.* (1973) and Law *et al.* (1978). In this study a QTL associated with maturity was identified close to the centromere on the short arm of chromosome 2. This QTL may be the same locus as that previously identified in wheat, and may be homoeologous to the (*Ppd-H1*) locus identified in barley.

The results from field trials in the breeding program initially showed that 'Yarralinka' consistently produced higher protein percentages (~1%) compared with 'Schomburgk' (Rathjen *et al.* 1995). These results were supported by the findings of this study which also demonstrated that 'Yarralinka' produces consistently higher levels of protein (~1%) than 'Schomburgk', indicating that there is a real genetic difference between the parental lines. However, the results show that the majority of the variation in protein within this population is controlled by other genetic and environmental factors, including maturity. Further assessment and more detailed statistical analysis of the data may be able to account for some of the variation in protein scores contributed by the high level of variation in maturity between the 150 SSD lines.

Chapter V

Milling yield

5.1 Introduction

The wheat grain is made up of three main structural tissues: the embryo or germ which amounts to 2-3% by weight, the starch rich endosperm which fills 81-84% of the grain, and the aleurone layers and seed coat which account for 14-16% by weight and make up the bran (Peterson 1965). In the process of milling, the bran and germ are separated from the endosperm and the endosperm is crushed to fine flour as the grain passes through a series of rollers. The main aspects of milling quality include the quantity of moisture required for conditioning, the rate at which the grain can be milled, the flour yield, and the colour of the flour (Simmonds 1989). The yield of flour is referred to as the milling yield, and is an important criterion in both domestic and international markets, with every 1% increase in milling yield increasing the value of the grain by $2 per tonne (Marshall *et al.* 1986). The proportion of endosperm in the grain confers an upper limit to the maximum flour yield that can be obtained from milling.

In addition there are several physical factors that affect milling yield, including the proportion of germ, thickness of the bran, size of the grain and the depth of the crease (Marshall *et al.* 1984). Grain hardness also plays an important role in flour milling, affecting the endosperm fracture pattern, starch damage, break flour release, particle size and the ease of separating bran from endosperm (Stenvert 1972). In the past a range of different phenotypic predictors of milling performance have been tested, with the aim of finding suitable selection criteria for use in breeding programs. Shuey (1960) assessed kernel size, and found that it was a better predictor of milling yield than test weight. Similarly, Hook (1984) evaluated test weight and found that it was a poor predictor of wheat milling potential, while Marshall *et al.* (1986) confirmed that grain size was associated with an increase in flour yield.

Although plant breeders have been able to significantly increase milling yields using various milling assays, further increases may be possible with the development of more efficient selection methods. Non-invasive methods for assessing grain quality are important early in the breeding program so that samples are left intact for further seed multiplication. Berman *et al.* (1996) evaluated the use of image analysis to predict the milling quality of grain samples. They were able to account for 66% of the variation in milling yield in the samples using a linear equation based on four measurements: means of area of the grain, minor and major axis, ellipsoidal volume and test weight. This method would be useful for screening breeding lines due to its non-destructive nature and the large proportion of variation accounted for. However, the traits being measured were influenced by environmental conditions during the growing period. Therefore, in order to accurately select for milling yield early in the breeding program, some understanding of the genetic control of the trait is required.

The development of molecular marker techniques in the last decade has led to the construction of genetic maps in many crop species such as wheat (Röder *et al.* 1995; Van Deynze *et al.* 1995; Nelson *et al.* 1995a, 1995b, 1995c; Marino *et al.* 1996). Using these maps and their corresponding mapping populations, the number, significance and location of quantitative trait loci (QTL) associated with a variety of phenotypic characteristics have been identified (Table 1.2). In addition, it has been found that the selection of traits at the genetic level, combined with selection based on phenotypic scores, results in improvements in breeding programs (Lande and Thompson, 1990; Gimelfarb and Lande, 1995). The primary goal of this study was to identify QTLs controlling milling yield, with the aim of identifying markers that could be used in marker assisted selection (MAS).

5.2 Materials and Methods

5.2.1 Milling yield evaluation

Grain hardness of harvested grain was measured from a sample of burr milled grain using near infrared spectroscopy (NIR) (Williams, 1975; Rosenthal, 1975). Grain used for milling was

harvested from the two field trials conducted in 1995 and 1996 (see Section 2.8). Samples of 100 g of grain for each of the SSD lines were conditioned 24 hours prior to milling with conditioning levels determined based on grain hardness. Milling was performed on a Quadramat Junior Mill (Bass 1988) fitted with a 70gg silk reel sieve. The weight of the flour and bran were recorded for each sample and the percentage flour yield calculated. The temperature of the mill was recorded for each sample at the time of milling allowing the data to be corrected according to the calibration established by the Cereal Chemistry Division of the South Australian Research and Development Institute: % yield = Raw yield - (17.72 - (Temperature x 0.55)).

5.2.2 Bulked segregant analysis

Bulked segregant analysis (Michelmore *et al.* 1991) was performed to identify markers linked to additional QTLs not detected with RFLP or SSR markers. Two bulks were constructed by combining aliquot's of DNA (2 μg) from two sets of ten SSD lines representing the two extremes of the distribution for milling yield. The bulks were screened with 64 AFLP primer combinations (Table 2.1) to identify polymorphisms. Polymorphisms identified between the bulks represent potential markers linked to QTLs associated with the milling yield.

5.2.3 Analysis of variance and heritability

Analysis of variance was performed on the field trial data in order to calculate an estimate of broad sense heritability for protein, as outlined in Section 2.10.

5.2.4 Identification of markers linked to QTLs associated with milling yield

The associations between markers and QTLs were tested using single-point regression analysis with MapManager QT (Version 8.0) (Manly and Elliott 1993), based on the likelihood ratio statistic. Single-point regressions were the primary method for detecting associations between markers and QTLs. Groups of two or more closely linked markers with significant

associations were assumed to identify the same QTL. Interval mapping, which searches for the effects of a QTL using sets of linked markers (Lander and Botstein 1989), was used to obtain further information on QTLs identified by single point analysis. A LOD score of 3.0 was chosen as the threshold for detecting QTLs (Lander and Botstein 1989). A graphical display of the QTL associations was generated using Q-gene (Nelson 1997). Wheat nullisomic-tetrasomic lines obtained from Mr Terry Miller (John Innes Centre, Norwich) were used to assign linked markers to specific chromosomes.

5.3 Results

In order to examine the level of variation for milling yield in the population, histograms were generated from the milling scores obtained in each of the three field trial data sets (Figure 5.1). From the histograms, the level of variation observed within the mapping population was greater than 10% in each of the data sets. The results also show that 'Schomburgk' consistently produced higher milling yields than 'Yarralinka' (~3%) indicating that there is a significant genetic difference between them. Furthermore, the variation observed in the population extended well beyond the parental mean scores. This evidence of transgressive segregation indicates that both 'Schomburgk' and 'Yarralinka' contain alleles associated with high and low milling yields.

From the skeletal map initially developed using RFLP and SSR markers (Chapter 3), a total of seven markers were identified that were associated with milling yield in at least one of the three data sets ($P \leq 0.01$) (Table 5.1). Two RFLP markers on chromosome 3A, *Xbcd115-3A* and *Xpsr754-3A*, were found to be linked to a QTL associated with milling yield in all three data sets. Two additional RFLP markers were also identified linked to this QTL (*Xpsr578-3* and *Xcdo118-3*), but were only associated with the trait at this level of significance in two of the three data sets. This locus accounted for an average of 10.3% of the phenotypic variation in milling yield over both seasons. In order to obtain an estimate of the broad-sense heritability of milling yield, an ANOVA was performed on the three data sets (Table 5.2). The results

Figure 5.1 Histograms of frequency of SSD lines versus average milling yield scores for samples harvested from field trials grown over two seasons at different sites. Three data sets were obtained from the trials; (A) 1995, (B) 1996 Replicate 1 and (C) 1996 Replicate 2. Parental means are indicated by arrows.

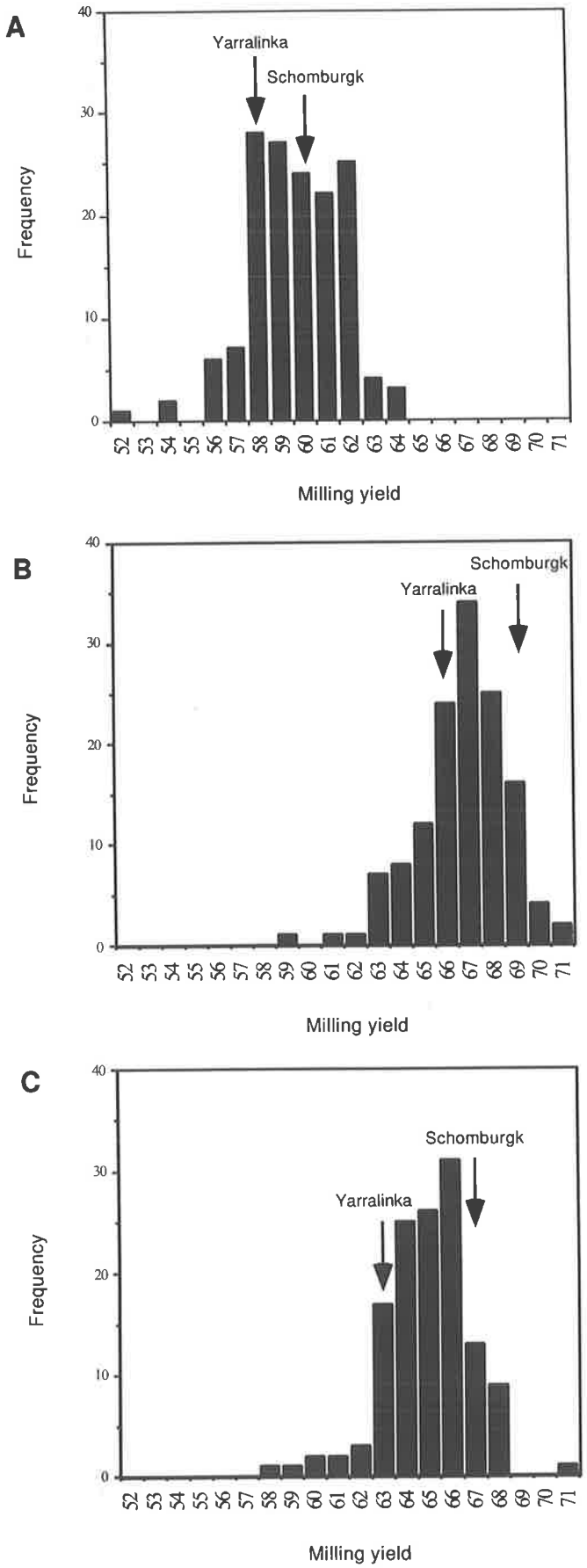

A
Yarralinka
Schomburgk
Frequency
Milling yield
B
Schomburgk
Yarralinka
Frequency
Milling yield
C
Schomburgk
Yarralinka
Frequency
Milling yield

Table 5.1 Summary of 6 RFLP, 1 microsatellite and 3 AFLP markers linked to 5 QTLs (P≤0.01) associated with milling yield. The likelihood ratio, percentage of phenotypic variation accounted for, and regression coefficients are reported for each marker in the three field data sets.

Marker	Likelihood ratio statistic			% Variation			Regression coefficient		
	1995	1996 rep1	1996 rep2	1995	1996 rep1	1996 rep2	1995	1996 rep1	1996 rep2
Xpsr754-3A	13.9	14.3	8.9	10	11	7	-1.30	-1.28	-1.00
Xbcd115-3A	14.0	8.7	16.2	11	7	13	-1.30	-0.97	-1.37
Xpsr578-3A	15.3	-	8.9	11	-	7	-1.33	-	-0.93
Xcdo118-3A	7.6	7.1	-	6	6	-	-0.94	-0.89	-
Xpsr156-3A	8.7	-	-	6	-	-	-0.99	-	-
Xbcd452-3A	7.8	-	-	6	-	-	1.07	-	-
Xwua2-3A.2	-	8.8	-	-	6	-	-	-0.92	-
Xwua51-7A.2	10.1	8.5	-	11	10	-	-1.33	-1.07	-
Xgwm111-7A	16.1	8.5	-	11	7	-	-1.43	-0.97	-
Xwua56-5.3	-	12.1	9.2	-	9	7	-	1.14	1.02

Table 5.2 Analysis of variance results for milling yield scores from 150 segregating SSD lines sown over two seasons at different field sites.

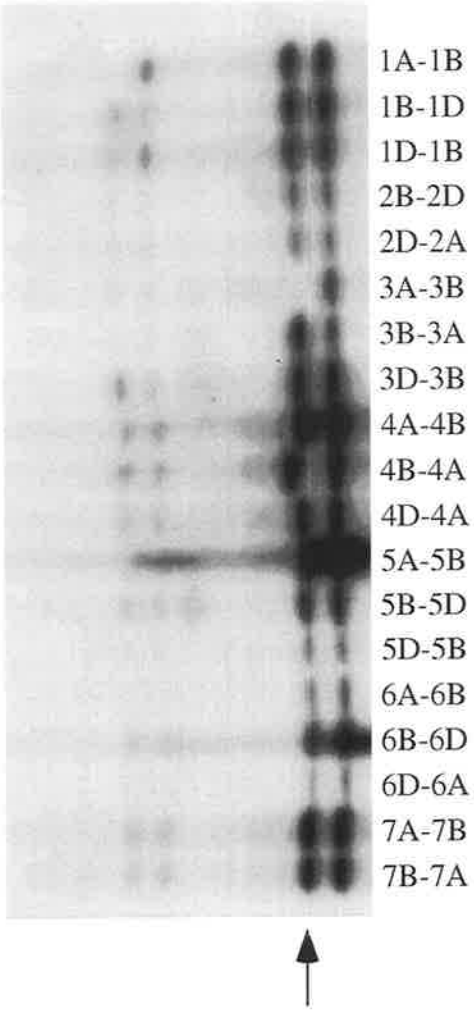
Source of variation	d.f.	Sums of squares	Mean squares	Variance ratio	F pr.
Between parents	1	151.8	151.8	117.5	<0.001
Between lines	149	1104.8	7.415	3.72	<0.01
Residual	271(27)	539.6	1.991		

showed that that there was a significant difference in milling yield between the two parents, and between the 150 SSD lines. From the ANOVA, the heritability of the trait was calculated as 0.48. Based on this level of heritability, the major QTL on chromosome 3 accounted for 21.5% of the genetic variation of milling yield in the mapping population. The genome location of the QTL was confirmed by mapping *Xpsr578-3* to chromosome 3A using nullisomic-tetrasomic analysis (Figure 5.2). One other RFLP marker on chromosome 3A was also linked to milling yield, *Xbcd452-3A* (Table 5.1). However, this marker was only associated with milling yield in one of the three data sets, and was not significant at a Lod score of 3. Additionally, an SSR marker on chromosome 7 (*Xgwm111-7*) was found to be linked to milling yield in two of the three data sets (Table 5.1). This marker accounted for an average of 9% of the phenotypic variation, and 18.8% of the genetic variation of the trait over both seasons. Further fine mapping was required to confirm the presence of a QTL in this region, and to identify additional markers in the regions of interest.

In order to fine map the regions of interest, bulked segregant analysis (BSA) was conducted using AFLP markers. DNA samples from 20 individual SSD-lines representing the two extremes (10 individuals per extreme) of the distribution for milling yield were pooled for analysis. Of the 64 primer combinations tested, three generated amplification products that were present in only one of the bulks. These primer combinations were then screened on the whole population to confirm their association with milling yield. After mapping, one of the markers (*Xwua51-7.2*) was found to be linked to the QTL on chromosome 7, previously identified by the SSR marker *Xgwm111-7*. A second marker identified in the BSA (*Xwua56-5.3*) mapped to group 5 and was associated with milling yield in the two 1996 replicates only ($P \leq 0.01$) (Table 5.1). This marker accounted for an average of 17% of the genetic variation between the two replicates. The third marker identified (*Xwua2-3.2*) was linked to the QTL on group 3 but was only associated with milling yield in Replicate 1 of the 1996 trial.

When milling yield scores from the field trial data were subjected to interval analysis using Q-gene software (Nelson 1997), only the QTL on 3A was significant in all three data sets at a

Figure 5.2 Southern analysis of 19 nullisomic-tetrasomic (NT) lines probed with the RFLP marker *Xpsr578*. The polymorphic band indicated by the arrow, is absent in the NT 3A-3B lane, confirming the location of the marker on chromosome 3A of wheat.


1A-1B
1B-1D
1D-1B
2B-2D
2D-2A
3A-3B
3B-3A
3D-3B
4A-4B
4B-4A
4D-4A
5A-5B
5B-5D
5D-5B
6A-6B
6B-6D
6D-6A
7A-7B
7B-7A

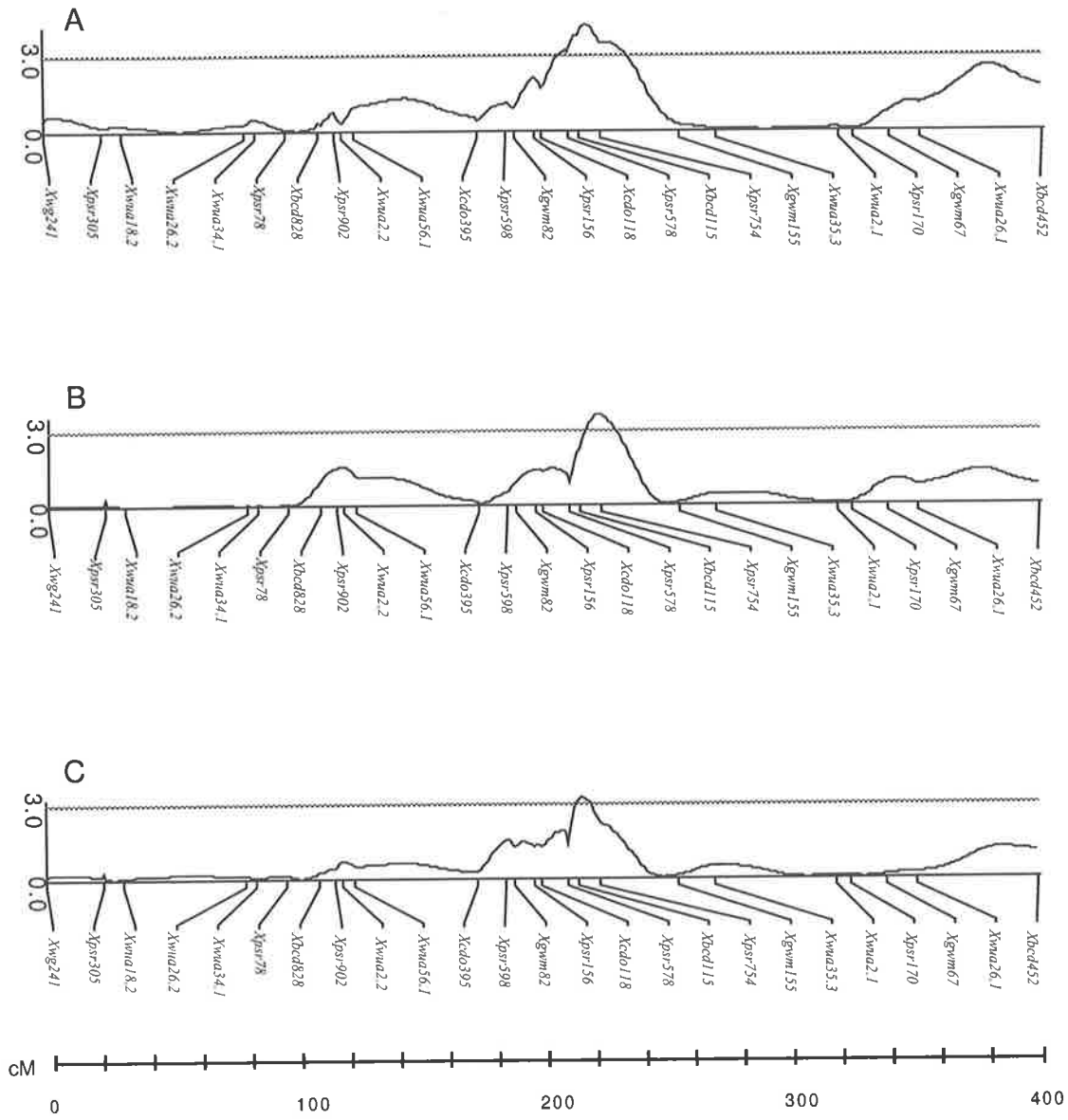
LOD score of 3. The LOD plots of the three data sets were drawn to localise the milling yield locus more precisely (Figure 5.3). *Xbcd115-3A* and Xpsr754-3A were the most closely associated flanking markers and are separated by a distance of 3.9 cM. The average percentage of phenotypic variance explained by this QTL was 10.3%, which is equivalent to 22% of the genetic variance based on the estimated heritability. Overall, the two QTLs identified on chromosomes 3 and 7 accounted for more than 40% of the genetic variation of milling yield in the mapping population.

5.4 Discussion

Currently, selection for high milling yields in breeding programs can only be carried out after several years of field trials when enough seed is available for milling. The milling assay is also time consuming and expensive when dealing with large numbers of samples. Therefore, the identification of markers linked to major loci associated with the trait would allow breeders to begin selection much earlier in the breeding program.

In this study, three regions of the genome were identified that were associated with milling yield in at least two of the three data sets ($P \leq 0.01$). However, the major locus on chromosome 3 was the only QTL identified that was highly significant in all three data sets, indicating that the other regions may be more sensitive to environmental influences. The QTLs on chromosomes 7 and 3 combined, accounted for more than 40% of the genetic variation in milling yield over the two seasons. Additionally, the linked marker on chromosome 5 accounted for 17% of the genetic variation. This region is of particular interest as previous research by Sourdille *et al.* (1996) identified a major QTL on chromosome 5 associated with kernel hardness in wheat. The QTL they identified accounted for 63% of the phenotypic variation in hardness in their mapping population. The significance of this region for milling yield in this population needs to be confirmed. From the results in shown in Table 5.1, three of the five regions of interest had negative regression coefficients, suggesting that the 'Schomburgk' parent was the source of most of the increasing effects on milling yield.

Figure 5.3 LOD plots of chromosome 3A showing the location of a QTL associated with milling yield. Data were obtained from 150 SSD lines sown in field trials over two seasons at different sites. Results include data from (A) 1995, (B) 1996 Replicate 1 and (C) 1996 Replicate 2.


A
3.0
0.0
Xwg241
Xpsr305
Xwna18.2
Xwua26.2
Xwua34.1
Xpsr78
Xbcd828
Xpsr902
Xwua2.2
Xwua56.1
Xcdo395
Xpsr598
Xgwm82
Xpsr156
Xcdo118
Xpsr578
Xbcd115
Xpsr754
Xgwm155
Xwua35.3
Xwua2.1
Xpsr170
Xgwm67
Xwua26.1
Xbcd452
B
3.0
0.0
Xwg241
Xpsr305
Xwna18.2
Xwua26.2
Xwua34.1
Xpsr78
Xbcd828
Xpsr902
Xwua2.2
Xwua56.1
Xcdo395
Xpsr598
Xgwm82
Xpsr156
Xcdo118
Xpsr578
Xbcd115
Xpsr754
Xgwm155
Xwua35.3
Xwua2.1
Xpsr170
Xgwm67
Xwua26.1
Xbcd452
C
3.0
0.0
Xwg241
Xpsr305
Xwna18.2
Xwua26.2
Xwua34.1
Xpsr78
Xbcd828
Xpsr902
Xwua2.2
Xwua56.1
Xcdo395
Xpsr598
Xgwm82
Xpsr156
Xcdo118
Xpsr578
Xbcd115
Xpsr754
Xgwm155
Xwua35.3
Xwua2.1
Xpsr170
Xgwm67
Xwua26.1
Xbcd452
cM
0
100
200
300
400

The use of AFLP markers (Zabeau and Vos 1993; Vos *et al.* 1995) in combination with BSA provided an alternative approach for rapidly identifying markers linked to the trait of interest. Although this approach was successful, only three additional markers were identified linked to milling yield. Further screening of the bulks with additional AFLP primer combinations could detect more polymorphic markers in these areas. Additionally, new bulks could be created on the basis of milling yield scores, as well as the segregation of the linked markers, to fine map specific loci. Any polymorphisms identified in such an analysis should map to these regions. This would be particularly useful for further analysing the putative QTL on chromosome 5. Overall, the combination of RFLP, SSR and AFLP markers in this study proved to be an efficient way of rapidly identifying markers linked to QTLs associated with the trait of interest.

The use of a SSD population in this study enabled the trait to be assessed over sites and seasons. From the results, the estimated broadsense heritability of milling yield (0.48) was larger than the heritability calculated for protein content (0.11) in the previous chapter. Although breeders have been able to successfully increase milling yields in the past, traits that exhibit a lower heritability are still difficult to analyse because of the large environmental component. Milling yield exhibited a reasonably high level of heritability in this study, allowing more accurate field data to be obtained compared to protein. Lande and Thompson (1990) concluded from their studies on MAS that under individual selection, the relative efficiency of MAS is greatest for characters with a lower heritability. Even though traits with low heritability may benefit more using MAS, a moderate fraction of the additive genetic variance must be significantly associated with the marker loci for MAS to be effective. From the results obtained in this study, a large amount (57%) of the genetic variation in milling yield was accounted for by the QTLs identified on chromosomes 3, 5 and 7. Therefore, milling yield would be a suitable trait for applying MAS.

The dissection of the trait and the identification of markers linked to major QTLs provides breeders with the tools for manipulating the desirable alleles. The evidence of transgressive segregation in the population means that breeders have the potential to maximise gains in

milling yield by selecting for lines containing desirable alleles from both parents using MAS. The three QTLs identified in this study may also help to provide a better understanding of the physiology or biochemistry of milling yield. The loci identified may be associated with some physiological trait such as the thickness of the seed coat and aleurone layer, or the depth of the crease in the grain. Some of these traits could be measured in this population to determine whether they are associated with the QTLs that have been identified.

Chapter VI

(In press in: Theoretical and Applied Genetics)

Title: Mapping loci associated with flour colour in wheat (*Triticum aestivum* L.)

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Summary

An RFLP map constructed using 150 single seed descent (SSD) lines from a cross between two hexaploid wheat varieties ('Schomburgk' x 'Yarralinka') was used to identify loci controlling flour colour. Flour colour data were obtained from field trials conducted over two seasons at different sites. The estimated heritability of this trait was calculated as 0.67. Two regions identified in the preliminary analysis on chromosomes 3A and 7A, accounted for 13% and 60% of the genetic variation respectively. A detailed analysis of the major locus on 7A was conducted through fine mapping of AFLP markers identified using bulked segregant analysis (BSA). Seven additional markers were identified by the BSA and mapped to the region of the 7A locus. The applicability of these markers to identify wheat lines with enhanced flour colour is discussed.

Key Words Wheat, Flour colour, QTL mapping, RFLP, AFLP

Introduction

Flour colour is one of the most important considerations in the assessment of flour quality and is of particular importance in determining the quality of the end product. White flour, lacking significant pigmentation, is considered a pre-requisite for bread production and flour is often bleached with bezoyl peroxide before use to eliminate colour defects (Kruger and Reed, 1988). Alternatively, flour with high levels of yellow pigmentation is preferred for Chinese alkaline noodle production, and in many Asian countries noodles are prepared from flour specifically selected to enhance the colour of the final product (Kruger *et al.* 1994). Yellow alkaline noodles and white salted noodles are generally made from wheats with mixed grain hardness varying

between soft and hard classes, with protein levels ranging from 10-12% (Simmonds, 1989).

Flour carotenoids, principally xanthophyll and flavone compounds, are responsible for the yellow colour of the flour (Miskelly, 1984). The natural colour of the flavones is advantageous for the production of noodles in Japan and South East Asia where traditionally an alkaline-salt mixture of sodium and potassium carbonates and phosphates called 'Kansui' is added during production (Kruger *et al.* 1992). The highly alkaline pH (between 9 and 11) of the dough mixture causes the disassociation of flavones from the polysaccharides, which enhances the yellow pigmentation. As well as enhancing the colour, the alkaline salts contribute to the flavour of the noodles and toughen the dough, improving the bite texture of the final product.

In commercial noodle production, alkaline salts are frequently substituted with a sodium hydroxide solution. The addition of increasing concentrations of alkali results in noodles that have a brighter, more intense yellow colour than noodles made with salted dough. However, alkali treatment results in a deterioration of dough quality, particularly the loss of gluten elasticity and pasting properties which may result in an undesirable soft textured product (Terada *et al.* 1981). An increase in flour pigment levels would lower the concentrations of alkali required to produce the desired yellowness, resulting in an improvement in noodle texture.

Miskelly *et al.* (1984) detected a significant positive correlation between flour colour and noodle sheet yellowness in both Japanese and Chinese noodles. This finding offers the opportunity to enhance the yellow pigmentation of the dough by breeding for improved flour colour. While significant genetic diversity exists within cultivated wheat for flour colour (Moss 1967), this trait is expressed as a quantitative character and has proved difficult to manipulate in breeding programs. Selection for quantitative trait characters is often difficult due to variation associated with environmental factors that adversely affect accurate measurement of the trait. Indirect

selection for linked markers offers an alternative approach that avoids multiple evaluations in replicated trials over sites and seasons.

The development of molecular marker techniques has provided an additional tool for the construction of genetic maps in many crop species including wheat (Devos and Gale 1993). Using these maps and their corresponding mapping populations, the number, significance and location of quantitative trait loci (QTL) associated with a variety of phenotypic characteristics have been identified (Paterson *et al.* 1988). In rice, QTLs for yield related traits, blast resistance and root morphology have been located on genetic linkage maps (Xiao *et al.* 1995; Wang *et al.* 1994; Champoux *et al.* 1995). QTLs associated with important traits have also been identified in other cereals including agronomic traits, grain yield and disease characters in barley (Thomas *et al.* 1995; Backes *et al.*1995; Oziel *et al.* 1996), as well as resistance to pre-harvest sprouting, kernel hardness and grain protein content in wheat (Anderson *et al.*1993; Sourdille *et al.*1996; Blanco *et al.*, 1996). Marker-assisted strategies may be useful for dissecting the genetic control of quantitative phenotypes such as flour colour. The goal of this research is to identify associations between QTLs for flour colour and molecular markers so that such markers may be used as indirect selection criteria in wheat breeding programs.

Material and Methods

Plant Material

A recombinant inbred population derived from hexaploid wheat that segregated for flour colour was used in this analysis. This population consisted of 150 F_4 derived lines developed through single seed descent (SSD) from a cross between the Australian cultivars 'Schomburgk' and 'Yarralinka'. The mapping population was sown in field experiments with the University of Adelaide's wheat breeding trials at sites in South Australia during the 1995 and 1996 seasons. The 1995 trial was sown at Winulta while the 1996 trial was sown at Walkers Flat. Both sites have a similar

soil type of sandy loam over clay. A randomised complete blocks design was used in both years. Limited seed restricted the 1995 trial to one replicate while two replicates were sown in 1996. Wheat nullisomic-tetrasomic lines obtained from Mr Terry Miller (John Innes Centre, Norwich) were used to assign linked markers to specific chromosomes.

Flour colour evaluation

One hundred grams of the harvested seed from each line was milled on a Quadramant Junior Mill (Bass 1988) and flour samples were scored for colour using a Minolta Chroma CR-200 meter (Symons and Dexter 1991; Wutscher and McCollum 1993). This probe uses diffuse illumination produced by a pulsed xenon arc lamp and is commonly used for measuring colour in a variety of products ranging from wheat milling products to soil (Symons and Dexter 1996; Wutscher and McCollum 1993). Colour readings are expressed as L* (lightness), a* (red-green chromaticity) and b* (yellow-blue chromaticity), with the b* values a measure of the yellowness of the flour sample.

RFLP analysis

A total of 217 RFLP markers were screened for their ability to identify polymorphisms between the parents. These DNA clones were obtained through the Australian Triticeae Mapping Initiative and were selected to give uniform genome coverage. The majority of these markers have been mapped in wheat and their chromosomal locations identified (Gale *et al.* 1995; Van Deynze *et al.*1995; Nelson *et al.*1995a, 1995b, 1995c; Marino *et al.*1996). DNA extraction, restriction enzyme digestion, Southern blotting and hybridisation were carried out as described by Guidet *et al.* (1991). Total genomic DNA was digested with *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV or *Hind*III. Southern blots of the 150 wheat lines were hybridised to RFLP

probes and the results scored as 'Schomburgk' allele, 'Yarralinka' allele or heterozygous according to the genotype at each locus.

AFLP analysis

The AFLP method developed by Vos *et al.* (1995) was followed with some modifications. Genomic DNA (1 μg) was digested with the restriction endonucleases *Pst*1 and *Mse*1. Double stranded adaptors were then ligated to the ends of the restriction fragments followed by ethanol precipitation and re-suspension in 60 μl 0.1M TE. Pre-amplification was performed using primers specific for the *Pst*1 and *Mse*1 adaptors including one selective nucleotide, followed by selective amplification using similar primers with three selective bases. A total of 8 *Pst*1 and 8 *Mse*1 primers were used giving a total of 64 possible primer combinations (Table 1). The pre-amplification mix was diluted 1:5 in water before being used in the selective amplification step. Pre-amplification PCR conditions consisted of 20 cycles of 94°C for 30 seconds, 56°C for 1 minute and 72°C for 1 minute. PCR reaction conditions for selective amplification consisted of one cycle at 94°C for 30 seconds, 65°C for 30 seconds and 72°C for 1 minute followed by 9 cycles over which the annealing temperature was decreased by 1°C per cycle with a final step of 25 cycles of 94°C for 30 seconds, 56°C for 30 seconds and 72°C for 1 minute. The *Pst*1 primer used in selective amplification was end-labelled with [γ^{32}P] ATP (Freinberg and Vogelstein 1983). Amplified fragments were separated on 6% denaturing polyacrylamide gels. The gels were transferred to 3MM paper for drying and auto-radiography was carried out with Fuji RX medical X-ray film at room temperature for 24-48 hours.

Microsatellite analysis

Thirty one wheat microsatellite (WMS) primer pairs developed by Plaschke *et al.* (1995) and Röder *et al.* (1995) were analysed. Polymerase chain reaction (PCR)

Table 1. Selective *Pst*1 and *Mse*1 primers used to screen bulks of F_4 SSD lines for polymorphisms associated with flour colour (selective bases in bold). A total of 64 possible combinations were used with P1M1 representing the first combination, P1M2 the second combination and P8M8 the 64th combination.

*Pst*1 selective primers	Primer designation	*Mse*1 selective primers	Primer designation
GACTGCGTACATGCAG**AAC**	P1	GATGAGTCCTGAGTAA**CAA**	M1
GACTGCGTACATGCAG**AAG**	P2	GATGAGTCCTGAGTAA**CAG**	M2
GACTGCGTACATGCAG**ACA**	P3	GATGAGTCCTGAGTAA**CAT**	M3
GACTGCGTACATGCAG**ACC**	P4	GATGAGTCCTGAGTAA**CTG**	M4
GACTGCGTACATGCAG**ACG**	P5	GATGAGTCCTGAGTAA**CCA**	M5
GACTGCGTACATGCAG**ACT**	P6	GATGAGTCCTGAGTAA**CCT**	M6
GACTGCGTACATGCAG**AGC**	P7	GATGAGTCCTGAGTAA**CGA**	M7
GACTGCGTACATGCAG**AGG**	P8	GATGAGTCCTGAGTAA**CTA**	M8

contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 100 μM each dNTP, 1.5 mM $MgCl_2$, 900 nM each primer, 0.5 units Taq polymerase and 80 ng genomic DNA in a total volume of 25 μl. Temperature conditions for amplification were 95°C for 3 minutes, followed by 35 cycles of 96°C for 1 minute, 55°C or 58°C for 1 minute 30 seconds, 72°C for 1 minute 50 seconds with a final step of 72°C for 10 minutes and 25°C for 5 minutes. Amplified products were separated on 7% non-denaturing polyacrylamide gels and stained with ethidium bromide as described by Röder *et al.* (1995).

Bulked segregant analysis

Bulked segregant analysis (Michelmore *et al.*1991) was performed to develop additional markers for fine mapping the major locus on chromosome 7A in order to identify tightly linked flanking markers which could be used in marker assisted selection. Two bulks were constructed by combining aliquots of DNA (2 μg) from two sets of ten SSD lines representing the two extremes of the distribution for flour colour with alternate alleles at the *Xcdo347-7A* locus. The bulks were screened with the 64 AFLP primer combinations to identify polymorphisms.

Genetic analysis

Data obtained from scoring segregation patterns of DNA markers among recombinant inbreds was analysed with MapManager QT (Version 8.0) software (Manly and Elliott 1993) using the Kosambi mapping function (Kosambi 1944; Lander *et al.*1987). Analysis of variance (ANOVA) was performed with the computer program Genstat 5 (Lane 1988) with the three field data sets treated as replicates. The results of the ANOVA were used to obtain an estimate of the broad-sense heritability (H^2) of flour colour (Hartl *et al.*1988). Broad-sense heritabiltiy was calculated as: $H^2 = \sigma^2_g / \sigma^2_t = \sigma^2_g/(\sigma^2_g + \sigma^2_e)$ where σ^2_g is the genotypic variance, σ^2_e is the environmental

variance and σ^2_t is the total variance for the trait. The genotypic variance (σ^2_g) can be calculated from the value of the mean squares between the SSD lines: m.s. = σ^2_e +b σ^2_g where m.s. is the mean squares, σ^2_e is the residual mean squares and b is the number of replicates. The amount of genetic variation accounted for by a particular QTL is calculated by dividing the phenotypic variation by H^2.

The associations between markers and QTLs for flour colour was tested with a single-point regression analysis using MapManager QT based on the likelihood ratio statistic. Single-point regressions served as the primary method of detecting associations between markers and QTLs. Groups of two or more closely linked markers with significant associations were assumed to identify the same QTL. A second method, interval mapping, which searches for the effects of a QTL using sets of linked markers (Lander and Botstein 1989) was used to obtain further information on QTLs identified by single point analysis. A LOD score of 3.0 was chosen as the threshold for detecting QTLs (Lander and Botstein 1989). A graphical display of the QTL associations was generated using Q-gene (Nelson 1997).

Results

Partial map construction

A partial genetic linkage map was constructed based on the segregating RFLP, microsatellite and AFLP data from the 150 SSD lines at a LOD threshold of 3. Of the 217 RFLP probes screened, 70 (32%) identified polymorphisms between the two parents. 11 (33%) of the 31 microsatellite markers tested identified a polymorphism between the parents. AFLP primer combinations that identified polymorphisms between the bulks were also screened on the 150 SSD lines, identifying 5.2 polymorphisms on average per primer pair. A total of 147 RFLP, microsatellite and AFLP marker loci were used to create the partial map (not shown).

Analysis of agronomic traits

The two parents of the population differed significantly in their flour colour scores (Table 3). The distribution of flour colour scores from the three field data sets is shown in Figure 1, including the means for the parents. The distribution of the SSD lines in the mapping population appeared to be bimodal in the 1995 trial (Figure 1A) and more normal in the 1996 trial (Figure 1B and 1C). The potential association of flour colour with markers was tested with single-point regression analysis. Three marker loci *Xcdo347-7A*, *Xwg232.1-7A* and *Xbcd828-3A* were found to be significantly associated with flour colour. The chromosomal locations of the segregating bands detected by these markers were identified using nullisomic-tetrasomic wheat lines.

Associations of flour colour with the linked markers on chromosome 7A (*Xcdo347-7A* and *Xwg232.1-7A*) and 3A (*Xbcd828-3A*) were highly significant ($P \leq 0.001$) over both seasons at the two sites (Table 2). Individually these markers accounted for an average of 37% and 9% of the phenotypic variation observed for flour colour. In order to obtain an estimate of the broad-sense heritability of flour colour, ANOVA was performed with the three data sets treated as replicates (Table 3). From the ANOVA, the heritability of the trait was calculated as 0.68. Based on these results the amount of genetic variation accounted for by the RFLP markers on chromosomes 7A and 3A is estimated at 54% and 13%, respectively.

Bulked segregant analysis

To identify additional markers linked to the QTL for flour colour and to enable interval mapping of the region around *Xcdo347-7A*, a bulked segregant analysis was conducted. DNA samples from 20 individual SSD-lines representing the two extremes (10 individuals per extreme) of the distribution for flour colour and having alternate alleles at the *Xcdo347-7A* locus were analysed. Of the 64 primer

Figure 1 Histograms of frequency of F_4 and F_5 SSD lines versus average flour colour score for samples collected from field trials grown over two seasons at different sites. Three data sets were obtained from the trials; (A) 1995, (B) 1996 Replicate 1 and (C) 1996 Replicate 2. Parental means are indicated by arrows.

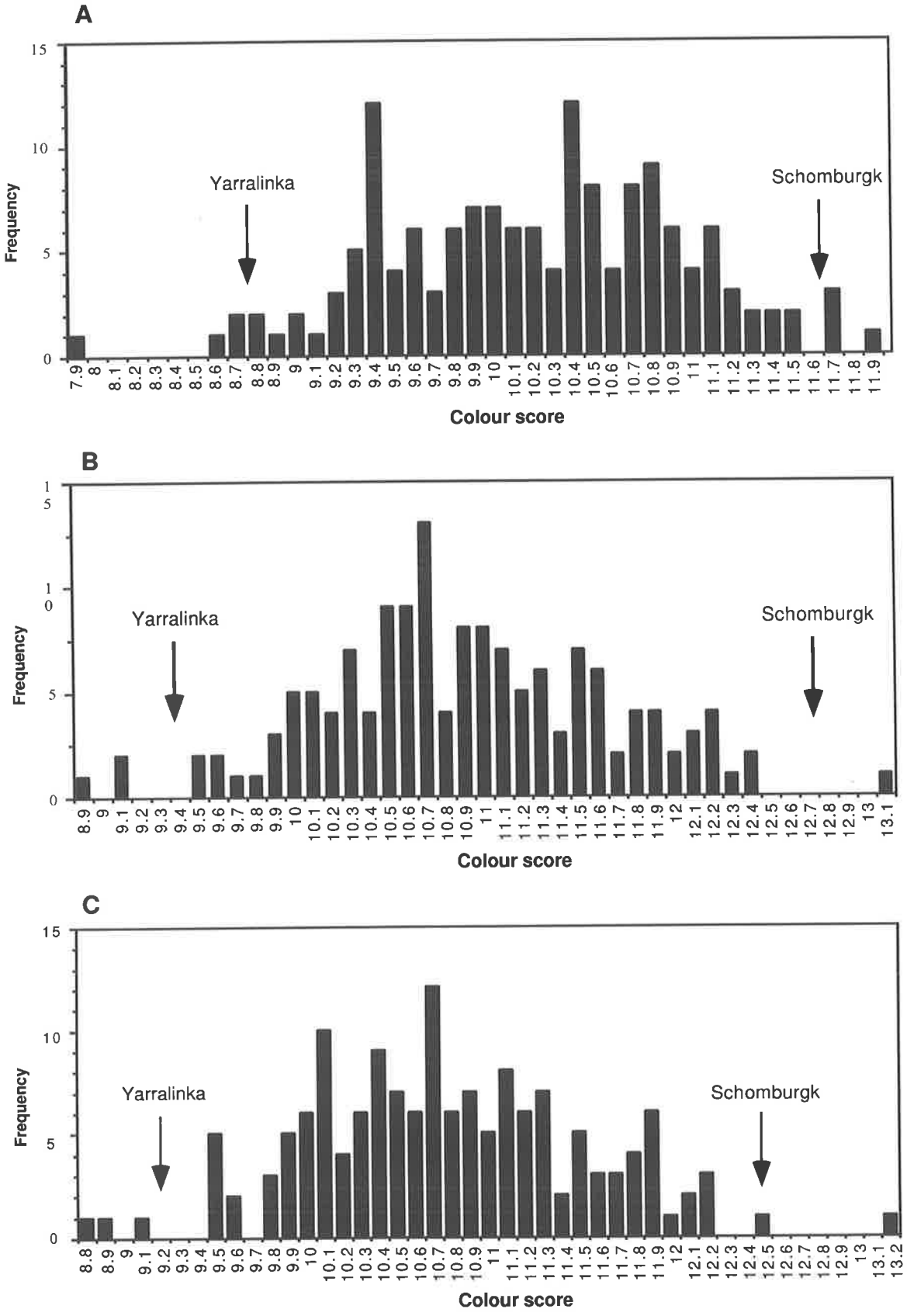

A
Frequency
Yarralinka
Schomburgk
Colour score
B
Frequency
Yarralinka
Schomburgk
Colour score
C
Frequency
Yarralinka
Schomburgk
Colour score

Table 2. Summary of 3 RFLP and 7 AFLP markers linked to a major locus on chromosome 7A associated with flour colour (P≤0.001). The likelihood ratio, percentage of phenotypic variation accounted for, and regression coefficients are reported for each marker in three field data sets.

Marker	Likelihood ratio statistic			% Variation			Regression coefficient		
	1995	1996 rep1	1996 rep2	1995	1996 rep1	1996 rep2	1995	1996 rep1	1996 rep2
Xbcd828-3A	9	8	12	7	6	8	-0.37	-0.35	-0.43
Xwg232-7A	26	29	22	17	19	15	-0.62	-0.65	-0.59
Xcdo347-7A	36	43	45	27	32	33	-0.78	-0.84	-0.90
Xp3m2/9-7A	40	57	55	24	34	32	-0.73	-0.85	-0.84
Xp5m3/1-7A	45	58	57	27	34	33	-0.77	-0.88	-0.88
Xp2m8/5-7A	45	58	56	28	35	34	-0.78	-0.89	-0.89
Xp4m2/4-7A	43	57	55	26	34	32	-0.74	-0.87	-0.85
Xp5m7/3-7A	37	45	44	24	29	27	-0.71	-0.79	-0.80
Xp1m2/4-7A	26	34	33	17	22	21	-0.62	-0.72	-0.71
Xp7m8/5-7A	24	32	34	16	21	22	-0.58	-0.70	-0.72

Table 3. Analysis of variance results for flour colour scores from 150 segregating F_4 and F_5 SSD lines sown over two seasons at different field sites.

Source of variation	df	Sums of squares	Mean squares	Variance ratio	F pr.
Between parents	1	219.6	219.6	3219	<0.001
Between lines	149	198.0	1.33	7.26	<0.001
Residual	290(8)	53.07	0.183		

combinations tested to identify AFLP markers linked to *Xcdo347-7A*, six primer combinations produced amplification products that were present in only one of the bulks. Three of these bands were associated with the 'Schomburgk' allele and three with the 'Yarralinka' allele. A seventh polymorphism was also identified which was co-dominant. Linkage between the *Xcdo347-7A* locus and the amplified products was confirmed by monitoring the segregation products of each informative primer combination in the 150 SSD lines. All 7 polymorphic amplification products were linked to flour colour and the *Xcdo347-7A* locus on chromosome 7 when mapped on the whole population. The 7 AFLP primer combinations also identified additional polymorphisms not associated with the *Xcdo347-7A* locus which were scored and included in the mapping data set. The flour colour data was re-analysed after the additional AFLP markers had been added to the partial map, but no other QTLs were identified.

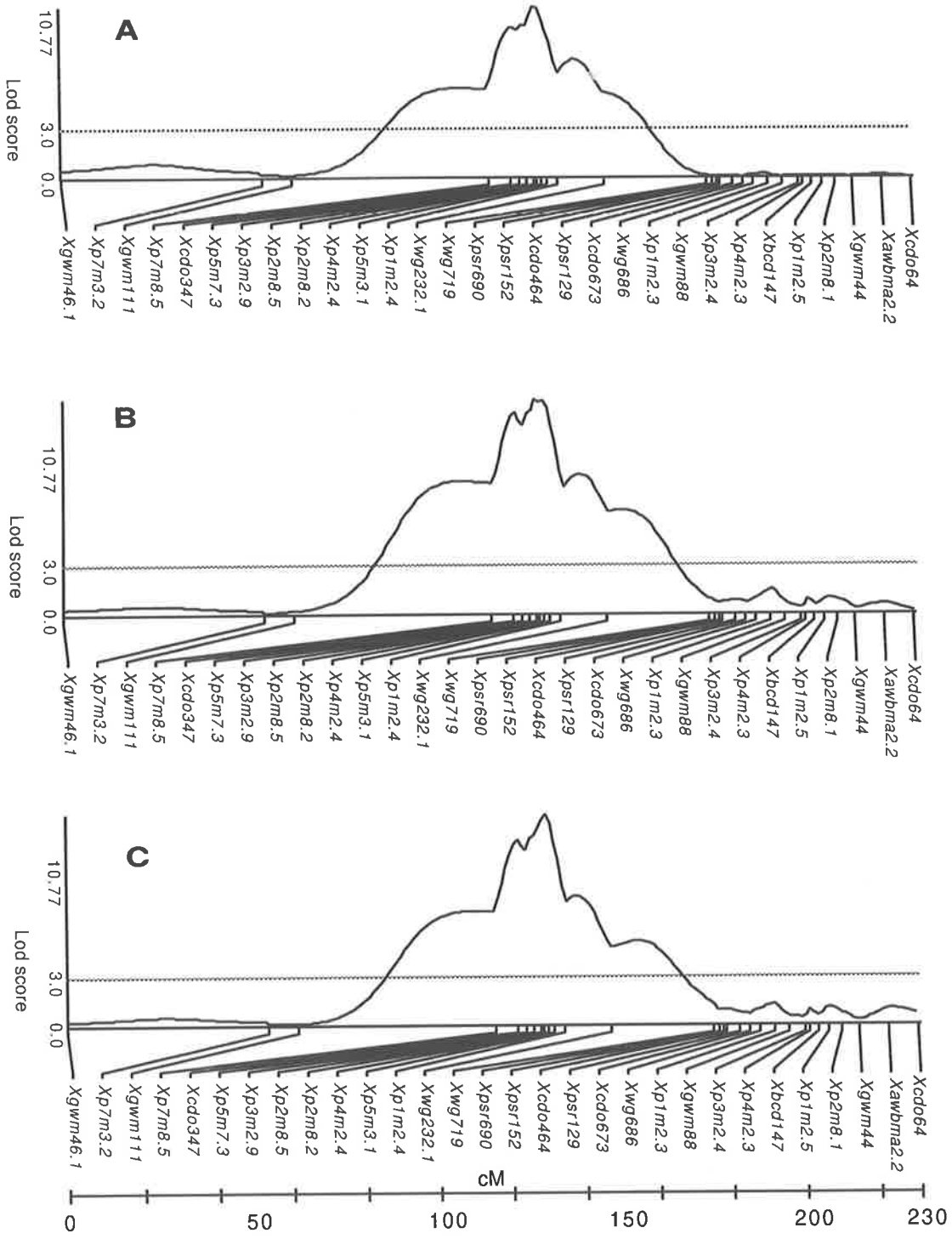
Interval Analysis

When flour colour scores from the field trial data were subjected to interval analysis using Q-gene software (Nelson 1997), only one QTL on 7A was detected (LOD>3.0). The LOD score plots of the three data sets were drawn to localise the genes for flour colour more precisely (Figure 2). The AFLP locus *Xwua16.5-7A* was the most closely associated with the flour colour locus (0.7cM). The average percentage of phenotypic variance explained by this QTL was 41%, which is equivalent to 60% of the genetic variance based on the estimated heritability.

Discussion

The primary goal of this research was to identify associations between QTLs for flour colour and molecular markers so that such markers could be used as an indirect selection criterion in wheat breeding programs. Using a SSD population of 150 lines,

Figure 2 LOD plots of chromosome 7A showing the location of a major locus associated with flour colour. Data were obtained from 150 F_4 and F_5 SSD lines sown in field trials over two seasons at different sites. Results include data from (A) 1995, (B) 1996 Replicate 1 and (C) 1996 Replicate 2. The AFLP marker prefix *Xwua* indicates a locus detected by a primer combination at the University of Adelaide's Waite Institute. The number following the prefix corresponds to the primer combination used to identify the locus.


A
B
C
Lod score
10.77
3.0
0.0
Xgwm46.1
Xp7m3.2
Xgwm111
Xp7m8.5
Xcdo347
Xp5m7.3
Xp3m2.9
Xp2m8.5
Xp2m8.2
Xp4m2.4
Xp5m3.1
Xp1m2.4
Xwg232.1
Xwg719
Xpsr690
Xpsr152
Xcdo464
Xpsr129
Xcdo673
Xwg686
Xp1m2.3
Xgwm88
Xp3m2.4
Xp4m2.3
Xbcd147
Xp1m2.5
Xp2m8.1
Xgwm44
Xawbma2.2
Xcdo64
cM
0
50
100
150
200
230

a total of 10 marker loci were identified that showed significant associations with QTLs for flour colour located on chromosomes 3A and 7A.

The benefit of using a single seed descent population is that the genetic structure of the population is predominantly fixed. Any heterozygotes that are scored are detected by the MapManager software. Since genotypes are predominantly fixed, SSD lines are valuable for assessing environmental impact on trait expression. In our study, the estimated heritability of flour colour was calculated as 0.68, indicating that a large part of the expression of this trait is genetically controlled making it easier to manipulate at the genetic level in a breeding program. We identified two QTLs for flour colour in this population but only the QTL on chromosome 7A was highly significant in all three replicates. This may indicate that the QTL on chromosome 3A is more sensitive to environmental effects.

All of the regression coefficients of the marker loci linked to yellow flour colour were negative (Table 2). This suggests that the 'Schomburgk' parent was the source of all the increasing effects on flour colour, and largely explains the lack of transgressive segregation observed in the progeny.

Seventy four percent of the genetic variation could be explained by the detected QTLs, with the QTL at *Xwua16.5-7A* alone accounting for 60 percent. The large amount of variation explained by this QTL would suggest that the inheritance of this trait is predominantly controlled by this locus. The inability to explain the remaining genetic variation may result from the moderate size of our mapping population, which did not allow for low-effect QTLs to be identified, or the incomplete coverage of the wheat linkage map.

Ideally a comprehensive map of the wheat genome would be required for complete analysis but this is time consuming to develop. The AFLP technique (Vos *et al.*1995; Zabeau and Vos 1993) has the capacity to assay a much greater number of loci for polymorphism than other currently available PCR-based techniques, and is suitable for detailed mapping exercises. Initially, we used a combination of locus specific

RFLP and microsatellite markers to construct a partial genetic linkage map. Bulked segregant analysis and AFLP markers were used to identify markers closely linked to a major QTL identified on chromosome 7. This approach resulted in more markers being identified in the region around *Xcdo347-7A* and rapidly increased the number of markers scored in the population. Bulked segregant analysis using AFLP markers has proved to be an effective method of identifying markers closely linked to traits of interest. The ability to rapidly identify and map additional markers tightly linked to the *Xcdo347-7A* locus enabled a detailed map of this chromosomal region to be constructed. The combination of RFLP and AFLP markers proved to be an efficient way of identifying markers closely linked to the trait of interest.

Thomas *et al.*(1995) reported on the suitability of converting AFLP markers to allele specific markers and there is clearly the potential to apply this to the markers identified in our study. The identification of molecular markers linked to flour colour has the potential to accelerate wheat breeding for this particular trait. Once suitable genomic regions associated with flour colour have been identified, breeders will be able to select for quantitative traits at the genetic level rather than relying on phenotypic expression. Previously, the early selection of traits at the phenotypic level has been limited due to the small amount of grain available on an individual plant basis. Selection of plants based on their molecular profile will allow the breeder to readily combine preferred alleles at selected loci to maximise expression of the trait.

Although the markers described here are likely to be effective in monitoring the yellow flour colour locus from the variety 'Schomburgk', it is not known if desirable yellow colour from other sources is controlled by the same locus. In this case, the absence of transgressive segregation and the apparent simplicity of inheritance suggests that the 'Schomburgk' locus will have broad applicability. However, future testing is required to confirm this. The behaviour of the allele from Yarralinka that is associated with whiteness should also be further assessed. In some breeding programs this trait is also important but it will again be necessary to analyse the behaviour of the Yarralinka allele in different genetic backgrounds.

Chapter VII

(To be submitted to Theoretical and Applied Genetics)

Title: Molecular mapping of Rust Resistance in Wheat

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Summary

In this study, molecular markers were identified linked to six rust resistance genes in a population of hexaploid wheat. The population consisted of 150 F_4 derived single seed descent lines generated from a cross between 'Schomburgk' and 'Yarralinka'. A molecular map constructed using RFLP, SSR and AFLP markers, enabled markers to be identified linked to *Lr3a, Lr20, Sr5, Sr9e, Sr22* and *Sr36*. The application of these linked markers in marker assisted selection for plant breeding purposes is discussed.

Keywords AFLP RFLP Wheat Leaf-Rust Stem-Rust *Puccinia recondita Puccinia graminis*

Introduction

Leaf rust and stem rust diseases, caused by the fungi *Puccinia recondita* f. sp. *tritici* and *Puccinia graminis* f. sp. *tritici*, are widespread throughout the wheat growing areas of Australia and have caused significant yield losses in susceptible cultivars. Estimates of crop losses vary from 30% in leaf-rust susceptible cultivars (Rees and Platz, 1975) to 55% in wheats susceptible to both stem and leaf-rust (Keed and White, 1971). Growing resistant cultivars is the most economical and environmentally safe method of controlling wheat rusts. Therefore, breeding for rust resistance has been and continues to be a major goal in many wheat improvement programs (Van Ginkel and Rajaram 1993). Currently more than ninety different leaf-rust (*Lr*) and stem-rust (*Sr*) resistance genes have been characterised in wheat (Knot 1989; McIntosh *et al.* 1995). Most of these resistance genes have been identified in cultivated *Triticum aestivum* L. Thell, however, many others have been introgressed from wild relatives (McIntosh *et al* .1995).

Single gene resistance to the rusts has usually not been persistent and this has been attributable to the evolution of new virulence in the pathogen to these genes (Samborski 1985). Durable resistance to leaf rust has only been achieved when multiple resistance genes have been combined in the same line. This has been demonstrated with the combination of *Lr34* with *Lr12* and *Lr13* (Roelfs 1988), *Lr 13* and *Lr 16* (Long *et al.* 1993; Samborski and Dick 1982) and *Lr9* and *Lr24* (Long *et al.* 1994; McVey and Long 1993). An epistatic effect with resistance has also been observed when *Lr24* has been used in combination with other rust resistance genes, with a higher level of resistance observed than when only an individual resistance gene is present (Ezzahiri and Reolfs 1989; German and Kolmer 1992).

The selection of resistant lines in breeding programs has been constrained by limitations in the methods for screening for resistance. The selection of genotypes carrying two or more resistance genes using screens based on the host-pathogen interactions requires time-consuming

greenhouse or field tests that sometimes prove unreliable at the single plant level. Such tests are not always possible due to the lack of isolates with specific virulence genes. Therefore, it is clear that the identification of DNA markers linked to the individual loci conferring resistant phenotypes would allow reliable and efficient indirect screening of segregating populations at an early plant growth stage. The identification of molecular markers that are tightly linked to the resistance genes would allow the selection of multiple disease resistance loci and enable the 'pyramiding' of race specific resistance genes. Most of the *Lr* and *Sr* genes are dominant resistance genes inherited in a Mendelian fashion. Although genetic studies on the inheritance of these genes have been carried out for several decades, information on the precise location of these genes in largely lacking. In wheat, markers linked to several leaf rust resistance genes have been identified (Table 1). However, markers to stem rust resistance loci have only been reported for *Sr22* (Paull *et al.* 1994).

Recently a molecular marker based map of wheat was constructed in a recombinant inbred (RI) population which segregated for both stem rust and leaf rust resistance genes (Parker *et al.* 1998). The objectives of this study were, using this population and dataset, to identify chromosomal regions carrying rust resistance genes. We describe the identification of molecular markers linked to four stem rust and two leaf-rust resistance genes currently of significance to the Australian wheat breeding program. The results indicate that the molecular markers identified are suitable for use in selection for rust resistance.

Material and Methods

Plant Material

A recombinant inbred population derived from hexaploid wheat that was segregating for several stem rust and leaf-rust resistance genes was studied. This population consisted of 150 F_4 derived lines developed through single seed descent (SSD) from a cross between the Australian cultivars 'Schomburgk' and 'Yarralinka'.

Table 1 Summary of leaf rust and stem rust resistance genes mapped in wheat.

Gene	Origin	Chromosomal Location	Reference
Lr1	*T. aestivum*	5DL	Feuillet *et al.* 1995
Lr9	*Ae. umbellulata*	6BL	Autrique *et al.* 1995; Schachermayr *et al.* 1994
Lr10	*T. aestivum*	1AS	Nelson *et al.* 1997; Feuillet *et al.* 1997
Lr19	*A. elongatum*	7DL	Autrique *et al.* 1995; Winzeler *et al.* 1995
Lr23	*T. turgidum*	2BS	Nelson *et al.* 1997
Lr24	*A. elongatum*	3DL	Autrique *et al.* 1995; Dedryver *et al.* 1996
Lr25	*S. cereale*	4BL	Procunier *et al.* 1995
Lr27	*T. aestivum*	3BS	Nelson *et al.* 1997
Lr29	*A. elongatum*	7DS	Procunier *et al.* 1995
Lr32	*T. Tauschii*	3DS	Autrique *et al.* 1995
Lr34	*T. aestivum*	7DS	Nelson *et al.* 1997
Sr22	*T. monococcum*	7AL	Paull *et al.* 1994

Rust cultures and inoculation procedures

Pathotypes of *Puccinia recondita* f. sp. *tritici* used included accession 104-2,3,6,7 (760694) avirulent for *Lr20* and *Lr3* and accession 53-1,6,7,10,11 (810043) avirulent for *Lr3* and virulent for *Lr20*. Pathotypes of *Puccinia graminis* f. sp. *tritici* used included accessions 34-1,23,4,5,6,7 (103) avirulent for *Sr22* and *Sr9e*, 40-1,2,3,6,7 (341) virulent for *Sr9e* and avirulent for *Sr36* and *Sr22*, 40-1,2,3,4,5,6,7 (383) virulent for *Sr9e* and *Sr36* and avirulent for *Sr22*, and 21-2,3,4,7 (60) avirulent for *Sr5*, *Sr9e*, *Sr22* and virulent for *Sr36*. SSD lines and parental cultivars were sown in 9cm pots as four clumps containing 10-12 seeds each and grown in temperature controlled rooms at 20°C. Methods for leaf rust and stem rust inoculation as well as disease scoring were as outlined in Bariana and McIntosh (1993). Seedling infection types (IT) with IT scores of 3 or above were classed as susceptible, while IT scores of 2 or below were classed as resistant.

Genetic analysis

Approximately 150 markers were used from the data set referenced above, distributed over the wheat chromosome maps. Segregation of single genes was analysed by chi-square. Quantitative disease scores were analysed with marker data by interval regression mapping (Haley and Knott 1992). A LOD threshold of 3.2 in this experimental population yields an experimental significance approximating 0.05. All calculations were performed with Q-gene (Version 2.29) software (Nelson 1997).

Results and Discussion

The infection type displayed by the relevant *Lr* and *Sr* gene in the parents tested with the four *Puccinia graminis* and two *Puccinia recondita* pathotypes are presented in Table 2. Both parents demonstrated the extreme range of responses to exposure to the pathotypes. In each case the resistant parent demonstrated either an 'immune' response with no visible uredia or a 'resistant' response with hypersensitive flecks. The susceptible parent, in each case was characterised with a 'susceptible' response with large uredia without chlorosis. Ten individual F_5 seed derived from each of the 150 F_4 SSD derived lines were characterised according to their response to each of the pathotypes. Heterozygous F_4 individuals were identified as those demonstrating a mixed infection type in the F_5 seed tested. A clear distinction between resistant and susceptible phenotypes was observed for each of the pathotypes.

The primary goal of this research was to identify associations between rust resistance genes and molecular markers so that such markers could be used as an indirect selection criteria in wheat breeding programs. The locations of the mapped leaf and stem rust resistance genes based on the associations with molecular markers are in general agreement with cytogenetic analysis (Table 3). Several of the resistance genes showed distorted segregation patterns in the mapping population including Sr5, Sr22, Sr36, Lr3a and Lr20. Such distortions may have resulted from pooling several F_1 lines in the construction of the mapping population. Alternatively, The and McIntosh (1975) have previously observed abnormal segregation ratios associated with rust resistance genes and have attributed this to differential transmission rates of male and female gametes associated with a gametocidal effect.

Sr5

Sr5 was originally identified in *T. aestivum* by Sears *et al.* (1957) conditioning immunity or near immunity to a number of races. Mapping in this population confirms its cytologically derived location on chromosome 6DS (McIntosh *et al.* 1995). The resistance allele in this population comes from 'Schomburgk' with the gene showing a distorted segregation ratio (χ^2 = 15.38) with a higher number of heterozygotes than expected. The AFLP marker *Xwua34-6.2*

Table 2 Summary of infection types displayed by the relevant *Lr* and *Sr* genes in the parental cultivars 'Schomburgk' and 'Yarralinka' when tested with four *Puccinia graminis* and two *Puccinia recondita* pathotypes.

	Infection types		
Gene	Schomburgk	Yarralinka	χ^2
Sr5 *	0;	4	15.38
Sr9e	4	1$^-$	1.28
Sr22	4	1$^-$	6.32
Sr36 **	0;=	4	27.6
Lr3a	0;=	4	17.56
Lr20	4	;N	5.73

* Lowest response occurs at temperatures below 20°C
** Lowest infection type expressed at temperatures greater than 25°C

Table 3 The chromosomal locations of four stem rust and two leaf rust resistance genes in wheat based on cytogenetic analysis.

Gene	Origin	Parental Source	Chromosomal Location	Reference
Sr5	*T. aestivum*	Schomburgk	6DS	McIntosh *et al.*, 1995
Sr9e	*T. turgidum*	Yarralinka	2BL	Knott, 1989
Sr22	*T. monococcum*	Schomburgk	7AL	The and McIntosh, 1975
Sr36	*T. timopheevii*	Yarralinka	2BL	Knott, 1989
Lr3a	*T. aestivum*	Yarralinka	6BL	Haggag and Dyck, 1973
Lr20	*T. aestivum*	Schomburgk	7AL	Sears and Briggle, 1969

showed closest association (13.8 cM) with *Sr 5* and also showed distorted segregation (χ^2 = 5.80) deviating from the expected 1:1 ratio. The group 6 RFLP marker, *Xpsr167-6.2*, also closely associated with the *Sr5* locus, mapped on chromosome 6D, confirming the location of the resistance gene (Figure 1).

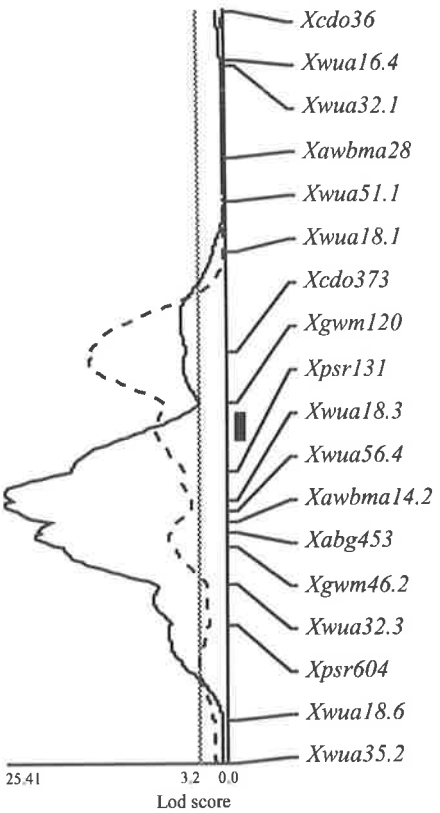
Sr9e

Sr9e located on chromosome 2BL (Sears and Loegering 1968) was transferred from tetraploid Durum (*T. durum*) to hexaploid Vernstein by Luig and Watson (1967). It has not been used to any large extent in breeding programs but is present in some Australian varieties where it is still effective (McIntosh *et al.* 1995). *Sr9e* was mapped at 11 and 18 cM from the 2BL centromere (Sears and Loegering 1968; McIntosh and Baker 1968) and shows a recombination of 0.3 with *Lr23* (McIntosh 1988). The resistance allele from 'Yarralinka' followed the expected 7:2:7 segregation ratio in the mapping population (χ^2 = 1.28) and was most closely associated with the RFLP marker *Xcdo373* (6 cM) (Figure 1).

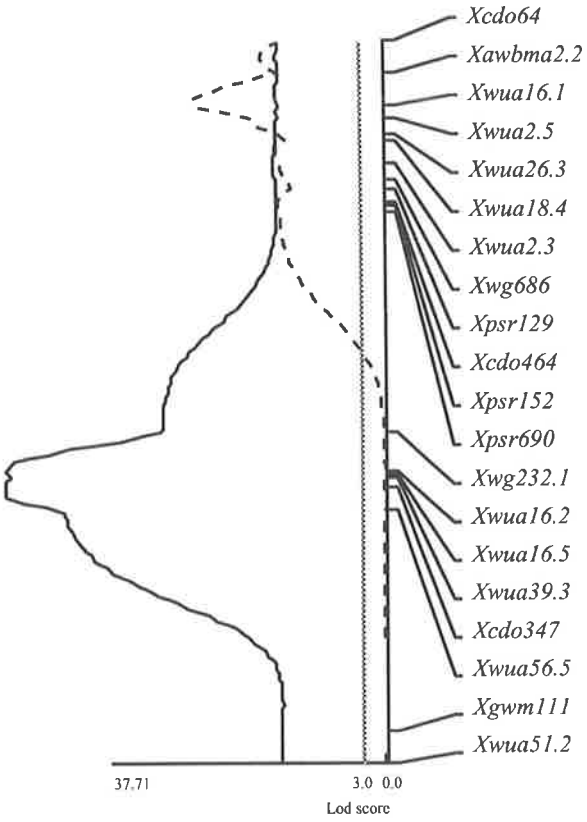
Sr22

Sr22 was originally identified in the A genome of the diploid wheats *Triticum boeoticum* (Gerechter-Amitai *et al.* 1971) and *T. monococcum* (Kerber and Dyck 1973). Gametic and pathological tests found that hexaploid lines containing *Sr22* from both sources contained the same resistance gene (The 1973). *Sr22* was transferred from the *T. boeoticum* source to 'Schomburgk' (Rathjen 1987) and is effective against all pathotypes of stem rust in Australia (Paull *et al.* 1994). The resistance gene was found to be located on chromosome 7AL (The and McIntosh 1975) and several linked RFLP markers have been previously identified including *Xpsr129* and *Xwg686* (Paull *et al.* 1994). *Sr22* in this study deviated from the expected segregation ratio in the mapping population (χ^2 = 6.32) with a significantly higher than expected number of number of heterozygotes. The closest linked marker was *Xawbma2-7.2* (19 cM). Other linked markers included *Xpsr129-7* and *Xwg686-7*, previously identified by Paull *et al.* (1994) as being linked to *Sr22* on chromosome 7AL, confirming the location of the resistance gene (Figure 1).

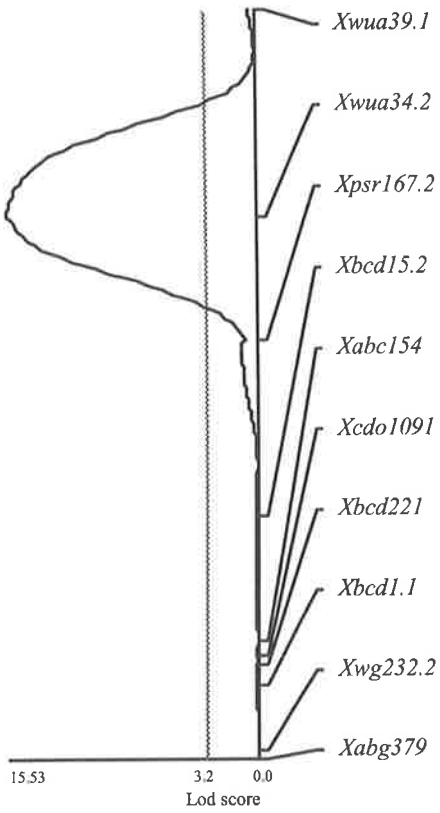
Figure 1 LOD plots showing the location of six rust resistance genes. These include Sr9e (dashed) and Sr36 (solid) on chromosome 2B, Lr3a on chromosome 6BL, Sr5 on chromosome 6DS, and Lr20 (solid) and Sr22 (dashed) on chromosome 7AL.



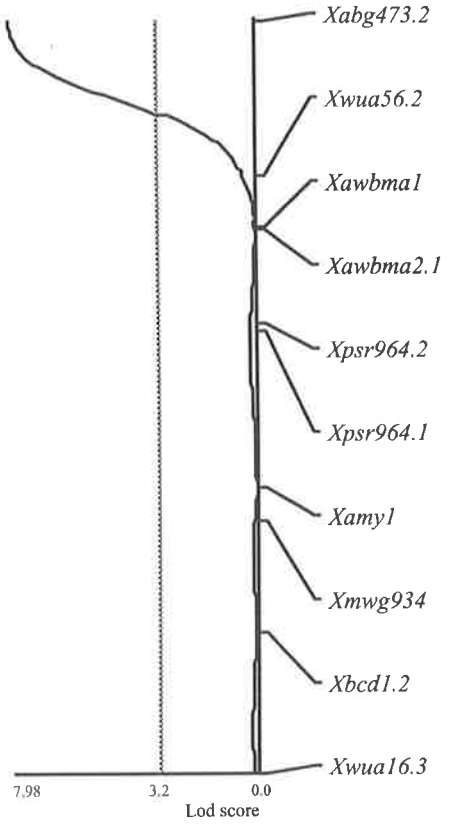
2B



7AL



6DS



6BL

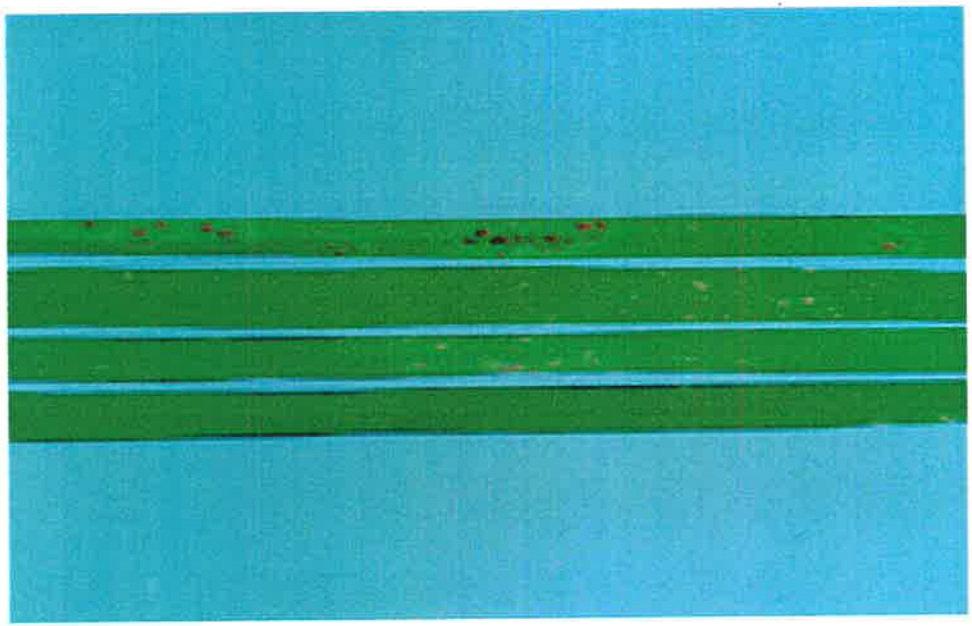
Sr36

Sr36 has proven to be one of the most effective single gene resistances worldwide (Roelfs 1988). The resistance gene was transferred by Allard and Shands (1954) from *T. timopheevii* along with *Pm6* for resistance to powdery mildew. *Sr36* is located on chromosome 2BS (Knott 1989) which is the same chromosome carrying *Sr9e*. The resistance allele in this study comes from 'Yarralinka' and showed a distorted segregation ratio ($\chi^2 = 27.6$) with significantly higher numbers of resistant and heterozygous plants than expected. Early reports indicated that *Sr36* and *Sr9e* were allelic. However, McIntosh and Luig (1973) reported that, in the line Combination III, there was 20% recombination between *Sr36* and *Sr9e*. Further studies of *Sr36* and *Sr9e* in the progeny of a monosomic 2B line derived from Combination III, indicated that *Sr36* and *Sr9e* were located on different chromosome arms. The results from this study confirm that *Sr9e* and *Sr36* are closely linked. Figure 1 shows that the markers most strongly associated with *Sr36* and *Sr9e* were the RFLP markers *Xpsr131* (16 cM) and *Xcdo373* (6 cM), respectively, and the map locations of *Xpsr131* and *Xcdo373* confirm the previous finding that, while the two resistance loci are closely linked, they are on the opposing arms of chromosome 2B.

Lr3a

This resistance gene was initially identified in *T. aestivum* by Mains *et al.* (1926) and was designated *Lr3* by Soliman *et al.* (1964). It is known to have at least 3 alleles and is located on chromosome 6BL (Haggag and Dyck 1973; McIntosh *et al.* 1995). *Lr3a* assigned by Browder (1980) is commonly referred to as *Lr3* and is usually incompletely dominant displaying disturbed segregation ratios (Luig 1964). The resistance allele in this study comes from 'Yarralinka' (Figure 2) with the gene showing a distorted segregation ratio ($\chi^2 = 17.56$) in the mapping population. The RFLP marker, *Xabg473-6.2* was identified as the most closely associated marker with *Lr3a* (19 cM) and mapped to chromosome 6BL confirming the location of the resistance gene (Figure 1).

Figure 2 Examples of leaves sampled from the parental lines 'Schomburgk' and 'Yarralinka', and two resistant SSD lines infected with *Puccinia recondita* f. sp. *tritici* (leaf rust), accession 53-1,1,6,7,10,11 (810043) avirulent for *Lr3*.



Schomburgk (lr3 lr3)

SSD resistant (Lr3 Lr3)

SSD resistant (Lr3 Lr3)

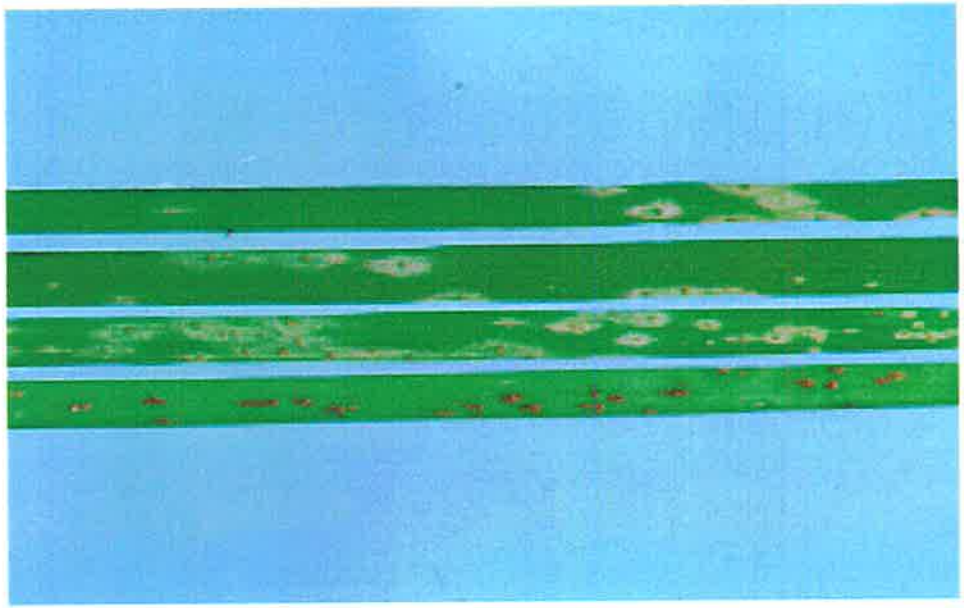
Yarralinka (Lr3 Lr3)

Lr20

The resistance gene Lr20 was identified in *T. aestivum* (Roelfs 1988) and has provided limited protection in Australian wheats (McIntosh *et al.* 1995). Where wheats with Lr20 have been widely grown, resistance has rapidly broken down and Lr20 virulent pathogens have soon become predominant (Park and Wellings 1992). *Lr20* is known to be tightly linked to the *Pm1* locus located on the long arm of chromosome 7A (Watson and Baker 1943; Sears and Briggle 1969) and on the basis of mutation studies, *Lr20* and *Sr15* have been shown to be the same gene (McIntosh 1977). The resistance allele in this study comes from 'Schomburgk' (Figure 3) with the gene showing a distorted segregation ratio in the mapping population ($\chi^2 = 5.72$) with a significantly higher than expected number of heterozygotes. The RFLP marker, *Xcdo347-7* was identified as the RFLP marker most closely associated with *Lr20* (9 cM) and was mapped to chromosome 7AL confirming the location of the resistance gene (Watson and Baker 1943) (Figure 1).

Sources of resistance to the rust diseases are known and have been utilised by wheat breeders. However, achieving durable resistance has been difficult, and the rust fungi continue to evolve and circumvent breeders achievements over time. Although, many single genes have been deployed to provide resistance to rust pathogens the resistance achieved has often been short-lived with the pathogen able to produce single-step mutations for virulence. An example of this was the deployment of the *Lr19* resistance gene which was initially effective worldwide but was overcome in only a few years (Heurta-Espino and Singh 1994). This has led to the implementation of breeding strategies aimed at combining multiple resistance genes in individual varieties. The pyramiding of resistance genes in new wheat varieties will reduce the risk of virulent rust pathotypes arising in the field. Conventional gene-pyramiding techniques rely on field and green house screening with differential rust races. This approach has been difficult in the past due to the epistatic and masking effects of the genes, particularly where a breeding line is already resistant to most races of the pathogen. The development of molecular markers linked to resistance genes is an important advance for plant breeding, as the presence of specific genes can be identified in a segregating population at any stage of plant growth independently of the phenotypic expression of the genes in the plant. The effectiveness of

Figure 3 Examples of leaves sampled from the parental lines 'Schomburgk' and 'Yarralinka', and two resistant SSD lines infected with *Puccinia recondita* f. sp. *tritici* (leaf rust), accession 104-2,3,6,7 (760694) avirulent for *Lr20*.



Schomburgk (Lr20 Lr20)

SSD resistant (Lr20 Lr20)

SSD resistant (Lr20 Lr20)

Yarralinka (lr20 lr20)

marker assisted selection for the accumulation of leaf-rust resistance genes in an individual line has been demonstrated in wheat (Nelson *et al.* 1997). RFLP-assisted backcrossing can also be used to select indirectly for the gene of interest and, at the same time, to reduce the size of the introgressed segment around the gene (Young and Tanksley 1989).

In pyramiding strategies, the identification of molecular markers that are closely linked to each resistance gene is required. We have now identified a series of markers linked to *Sr5, Sr9e, Sr22, Sr36, Lr3a* and *Lr20*. The molecular assay requires only a small amount of DNA from each plant to be tested without destructive sampling, allowing the heterozygotes to be identified and backcrossed in the same generation. The development of reliable PCR-based markers based on the identified molecular markers would further enhance the usefulness of these markers in breeding programs. The conversion of the markers identified in this study to sequence tagged sites (STSs) (Olson 1989) is currently being undertaken and should provide an alternative to the AFLP and RFLP markers that have been identified.

Chapter VIII

Comparison of Microsatellite and RFLP markers

8.1 Introduction

The assessment of genetic variation or relatedness between cultivars is important for plant breeding purposes, as genetic similarity (GS) estimates allow breeders to make more informed decisions regarding the selection of parents for new crosses (Liu and Furnier 1993; Messmer *et al.* 1993; dos Santos *et al.* 1994). They also provide a more accurate analysis of the variation available in germplasm collections, as well as the level of variability present in wild populations (Russell *et al.* 1997). In the past, the estimation of relatedness between cultivars was based on co-ancestry and pedigree records. However, pedigree records are not always accurate or reliable, especially when large numbers of breeding lines are being assessed. Early GS estimates were obtained by measuring the resemblance between lines using biochemical markers such as protein subunits. In recent years GS estimates have been obtained for a whole range of species using different molecular marker techniques.

A number of studies have been carried out to compare coefficient of co-ancestry and molecular marker estimates of GS in different plant species, with varying results. Tinker *et al.* (1993) identified a relatively high level of similarity between relationships derived from GS estimates based on RAPD data, and coefficient of co-ancestry values in barley. Schut *et al.* (1997) carried out a similar study using a group of barley accessions to compare relationships derived from coefficient of co-ancestry, with estimates of GS based on AFLP data. The values of the correlation coefficients between the two methods were significant but not very high. However, Messmer *et al.* (1993) found that estimates of genetic relatedness between maize genotypes based on coefficient of co-ancestry and GS estimates derived from RFLP data were highly correlated (R^2=0.70). Although in some populations the coefficient of co-ancestry may give accurate estimates of genetic relatedness, the estimation of GS using maker data is more

accurate, as the polymorphisms detected are a direct measurement of the variation at the DNA level.

The success of any molecular marker system is based on its ability to detect polymorphism between individual plants or within a mapping population. A number of markers systems are currently being used in GS studies including RAPDs, RFLPs, SSRs and AFLPs. These marker techniques detect polymorphisms by assaying subsets of the total DNA sequence in the genome. Each approach is based on different principles for the detection of mutations, and insertions or deletions in the genome, with different advantages and limitations in terms of cost, time and ability to identify polymorphisms. The relationships generated using different marker systems will vary depending on the number of markers used, the level of genome coverage, and the type of sequence variation detected. With the range of different marker assays available, it is important to determine whether they generate similar patterns of genetic relationships between cultivars.

SSRs are an important source of molecular markers, with their abundance, codominant nature, and high level of variability making them a suitable assay for detecting variation in studies of population genetics, linkage analysis and genetic similarity (Lagercrantz *et al.* 1993; Grimaldi and Crouau-Roy 1997; Schug *et al.* 1997; Harr *et al.* 1998). A wide variety of simple repetitive motifs are commonly found in eukaryote genomes (Tautz and Renz 1984; Levinson and Gutman 1987; Lagercrantz *et al.* 1993), with short and long regions of simple repetitive DNA found at a variety of chromosomal locations. The flanking sequences of SSRs are often unique allowing primers to be designed resulting in tagged SSR markers representing a single locus. The majority of the allelic variation of SSRs is thought to arise as a result of slip strand mispairing (SSM) during replication (Levinson and Gutman 1987; Tautz and Schlötterer 1994), as well as through unequal crossing over (UCO), or by insertions or deletions in the sequence flanking the SSR (Grimaldi and Crouau-Roy 1997). This variation is detected by PCR amplification using primers complimentary to the flanking sequences. Unlike RAPDs, SSRs as a marker system have been found to be highly reproducible between laboratories (Jones *et al.* 1997).

RFLPs have also been extensively used as markers both in mapping studies, as well as in generating GS estimates in different species (Paterson *et al.* 1988; Graner *et al.* 1991; Graner *et al.* 1994; Melchinger *et al.* 1994; Langridge *et al.* 1995). Paull *et al.* (1998) used RFLPs to characterise the genetic diversity of Australian wheat germplasm using 124 cultivars comprising all major wheat varieties. A total of 98 probes were screened against genomic DNA digested with five restriction endonucleases, generating over 1950 polymorphic fragments. In this study SSR markers were used to screen the same 124 wheat accessions to determine whether similar patterns of variability and genetic similarity would be generated using an alternative marker assay. Dendrograms were generated from the marker data and the genetic relationships derived using the two marker systems were compared.

8.2 Materials and methods

8.2.1 Plant Material

124 wheat cultivars and breeding lines were analysed (Table 8.1). These cultivars, originally selected by Paull *et al.* (1998), included accessions of historical significance to wheat breeding programs in Australia, varieties which accounted for over 2% of deliveries to silos, and recently released varieties and parental lines carrying traits of particular interest.

8.2.2 Microsatellites

A total of 31 SSR primer pairs developed by Plaschke *et al.* (1995) and Röder et al. (1995) and two sequence tagged site (STS) markers (LMW glutenin and γ-gliadin) (Devos *et al.* 1995) were used to screen the 124 wheat accessions were. Primer pairs that identified polymorphisms between the 124 cultivars were used to generate GS estimates.

Table 8.1 Details of accessions tested, including source of seed, pedigree, origin and year of registration (Paull *et al.* 1998).

Accession	Source[a]	Pedigree
3Ag#14/5*Condor	PBI, U of S	3Ag#14/5*Condor
3Ag#3/4*Condor	PBI, U of S	3Ag#3/4*Condor
Ag-Amigo/4*CSP44	PBI, U of S	Ag-Amigo/4*CSP44
Aroona	AUS 20992	WW15/Raven
Avocet	AUS 20601	WW119/WW15//Egret
Avocet R	PBI, U of S	seln from Avocet, YrA differential
Banks	AUS 20599	Thatcher/Ag.el//4*Heron(PWTH)/3/Cndr sib/4/2*Cndr
Barunga	AUS 25602	Halberd/Aroona//3*Schomburgk/3/2*Molineux
Bass	AUS 21960	Flinders sib/2*Cook
Batavia	QWRI	Brochis "S"/Banks
Bencubbin	AUS 1924	Gluyas Early/Nabawa
Bindawarra	AUS 20621	Mexico120/Koda//Raven
Bungulla	AUS 77	Bencubbin selection
Chinese Spring	Waite	
Cocamba	AUS 22605	AUS10894/4*Condor
Combination III	AUS 15358	Vernstein/CI12632
Condor	AUS 16036	WW80/2*WW15
Cook	AUS 20275	Timgalen/Condor sib//Condor
Corella	AUS 22655	Complex cross based on Egret
CS(Hope3B)	PBI, U of S	Intervarietal substitution line
CSP44	PBI, U of S	Condor selection plant 44
Cunningham	QWRI	3Ag#3/4*Condor//Cook
Currawa	AUS 2229	Northern Champion/Cretan/2/Little Club
Dagger	AUS 22255	Sabre/Mec3//Insignia
Diaz	AUS 23326	CombIII/3*Oxley/3/3*Cook
Dirk	AUS 3662	Ford/Dundee
Dollarbird	AUS 23824	Wren/Gaboto//Kalyansona/Bluebird
Du Toits	AUS 2310	19th century introduction
Dundee	AUS 2300	Hard Federation/Cleveland/2/Sands
Early Purple Straw	AUS 183	19th century landrace from Tuscany
Early Purple Straw	AUS 2315	19th century landrace from Tuscany
Egret	AUS 16037	Heron/2*WW15
Egret FDN seln	PBI, U of S	seln from Egret homogeneous for YrA
Eradu	AUS 21110	Ciano/Gamenya
Excalibur	AUS 25292	RAC177/Uniculm 492//RAC311S
Falcon	AUS 206	Gular//Dundee/Gular/3/Bencubbin
Federation	AUS 218	Purple Straw/Yandilla
Festiguay	AUS 6113	Webster/Uruguay C10837
Flinders	AUS 99077	Thatcher/Ag.el/4*Heron(PWTH)/Cndr sib//2*Cndr
Florence	AUS 10411	White Naples*2/Fife//Fife/Eden
Ford	AUS 3591	Fan/Comeback//Zealand/Tardent's Blue
Frame	AUS 25601	Molineux/3*Dagger

Free Gallipoli	AUS 2441	Club wheat/Yandilla King
Gabo	AUS 246	Bobin*2/Gaza (possibly)
Gamenya	AUS 256	Gabo/3/Gabo*5/Mentana//Gabo*2/Kenya117A
Gatcher	AUS 11621	Thatcher/SantaCatalina//Mayo48/3/Gabo*3/Charter
Ghurka	AUS 2494	Gallipoli/3/Currawa//Indian4E/Federation
Glenwari	AUS 279	Nabawa//Riverina/Hope
Gluyas Early	AUS 172	Wards Prolific selection
Grebe	AUS 23352	Skorospelka/3*Egret
Gutha	AUS 99084	Gamenya//Gabo*3/Khapstein/3/Falcon*3/Chile1B
Halberd	AUS 11612	Scimitar/KenyaC6042//Bobin/3/Insignia49
Harrier	AUS 99062	Norin10/Brevor(seln14)//Kite sib/3/Kite
Hartog	AUS 21533	Vicam71//Ciano'S'/SieteCer/3/Kalyansona//Bluebird
Heron	AUS 322	Doubbi/2*Ranee/2/Insignia/3/Insignia 49
Hudsons Early Pple Str	AUS 2565	19th century landrace from Tuscany
Insignia	AUS 2642	Ghurka/Ranee
Janz	AUS 24794	3Ag#3/4*Condor//Cook
Kewell	AUS 99027	Peanut Oil Olympic Mutant132A/South African184
Kite	AUS 16035	N10/Br//4*Eureka2/3/T-A/3*Flcn/4/T-A/4*Flcn/5/T-A/5*Flcn
Koda	AUS 6116	Dundee/Kenya745//2*Bobin39/3/Gaza
Kulin	AUS 23163	Bodallin//Gamenya/Inia
Machete	AUS 23038	Mec-3/2*Gabo(RAC177)//Madden
Madden	AUS 16170	Gamenya//Gabo*3/Khapstein
Matong	AUS 21821	Kalyansona/Olympic
MeA4	Roseworthy	Pitic 'S' (Norin10/Brevor14//Yaktana54)
Mec3	Roseworthy	Sonora 64 (Yaktana54//Norin10/Brevor/3/Yaqui54)
Meering	AUS 22606	Condor selection
Mentana	AUS 2997	Rieti/3/Rote Dikkop/Zeeuwse Witte//Squarehead/4/Akagomughi
Mexico120	AUS 546	Yaktana52//Norin 10/Brevor14
Millewa	AUS 20597	Sonora64/Yaqui50E//Gaboto/Mexico8156
Molineux	AUS 24457	Pitic62/Festiguay//2*Warigal
Nabawa	AUS 673	Gluyas Early/Bunyip
Norin 10	Roseworthy	Daruma/Fultz//Turkey Red
Norin10/Brevor	Roseworthy	Norin10/Brevor seln14
Olympic	AUS 3117	Baldmin/Quadrat
Osprey	AUS 22110	Cndr*2/WW33B = Thatcher/Ag.el/4*Hrn/2*(7165) WW80/WW15
Oxley	AUS 16461	Pen62/4*Gabo56//TPPN (WW80)/2*WW15
Pelsart	QWRI	Potam 70/4*Cook
Persia20	AUS 5206	landrace
Phoenix	AUS 21907	WW15*2/WW80
Pinnacle	AUS 3168	Pindar selection (Pindar = Ghurka selection)
Pitic 62	AUS 804	Norin10/Brevor14//Yaktana54
Potam 70	AUS 22698	Lerma Rojo64/Sonora64//Napo63
Purple Straw	AUS 885	19th century landrace from Tuscany
Purple Straw	AUS 892	19th century landrace from Tuscany
Quadrat	AUS 3213	Major/Gallipoli/2/Currawa/3/Ghurka
RAC177	Roseworthy	Sonora64/TZPP//Yaqui54/3/2*Gabo
RAC416-1	Roseworthy	Condor/Petit Rojo
Ranee	AUS 1001	IndianF/Federation
Raven	AUS 1009	Dirk48/Orfed//Uruguay1064
Reeves	AUS 24851	Bodallin//Gamenya/Inia66
Rosella	AUS 23165	Farrolunga/Heron//2*Condor/3/Quarrion sib
Rowan	QWRI	Jarral66/Gamut//4*Hartog

Rye-Amigo/4*Condor	PBI, U of S	Rye-Amigo/4*Condor
Schomburgk	AUS 23325	W3589/Oxley//2*Warigal/3/2*Aroona
Shortim	AUS 20276	Sonora64/P41603//Timgalen sib*3
Siete Cerros	AUS 1214	Penjamo62 sib/Gabo55
Skorospelka	Roseworthy	Kanred/Fulcaster//Klein33
Songlen	AUS 17904	Lerma Rojo64/Sonora64A//Timgalen
Spear	AUS 22254	Sabre/Mec3//Insignia
Spica	AUS 1303	Three Seas/Kamburica//Pusa4/Flora
Spring Wheat	AUS 10894	Afghanistan landrace
Stiletto	AUS 25923	Veranapolis/3*RAC177//3*Spear/3/Dagger
Sunco	AUS 23455	SUN9E-27*4/3Ag#14//WW15/3/3*Cook
Suneca	AUS 21820	Ciano//Spica/Amber Mutant Sonora64
Sunkota	AUS 21113	Timson/IRN 67-451
Sunstar	AUS 22177	Condor/4/WW15/3/W199/WC356/2/LaPrevision
Tatiara	AUS 24317	Mexico120/Koda//Raven/3/Mengavi/4/Siete Cer
Tincurrin	AUS 20578	Gluclub/3/Chile1B//Insignia/Falcon
Trident	AUS 25924	VPM1/5*Cook//4*Spear
Vasco	AUS 23053	3Ag#14/4*Condor//5*Oxley
Veranapolis	AUS 1553	Trintecinco/B2017-37
Virest	AUS 11984	EST39-12/Virgilio
VPM/6*Cook	PBI, U of S	VPM1/6*Cook
Vulcan	AUS 23039	Condor/Pitic62//Condor sib
W3589	PBI, U of S	T. boeoticum/2*Spelmar//2*Steinwedel
Waratah	AUS 1613	Hudsons Early Purple Straw/Gluyas Early
Ward's Prolific	AUS 1630	DuToit selection
Warigal	AUS 20593	WW15/Raven
WW15	AUS 6919	Lerma Rojo//Norin 10/Brevor14/3/3*Andes
WW80	AUS 6921	Penjamo 64/4*Gabo56//TZPP/Nainari
Wyuna	AUS 22607	DX3-134/Olympic
Yarralinka	AUS 24350	Mengavi/SieteCer/3/Mengavi/SieteCer//Crim/4/CombIII/2*Wgl

[a] Source of seed tested in this experiment. AUS: Australian Winter Cereals Collection accession number, PBI, U of S: Plant Breeding Institute, University of Sydney; QWRI: Queensland Wheat Research Institute, Toowoomba; Roseworthy Campus Wheat Breeding Program, University of Adelaide; Waite Campus Wheat Breeding Program, University of Adelaide

[b] State, Country or organisation of origin

[c] Year of registration/release

8.2.3 Data Analysis

Polymorphisms were scored on a presence or absence basis and data were analysed using the software program NTSYS-PC (Version 1.8) (Rohlf 1993). The portion of alleles in common between any two varieties over the total number of loci scored was used as the measure of genetic similarity for both marker systems. For codominant markers such as RFLPs and SSRs, this corresponds to the method of Nei and Li (1979). The cluster analyses were performed using the pair-group method with arithmetic average (UPGMA) (Rohlf 1993), based on the similarity matrices generated for each marker system. The relationships derived from the cluster analyses were visualised by plotting them as dendrograms.

8.3 Results

Of the 31 SSRs screened, only 18 generated scorable polymorphic bands in the 124 wheat cultivars. The number of alleles detected with the SSRs ranged from 3 (*Xgwm159*) to 13 (*Xgwm44*), with an average of 9 alleles per marker and a total of 165 loci scored (Table 8.2). The SSR loci detected in the 124 cultivars were well defined and highly reproducible. GS estimates between cultivars based on the SSR data ranged from 0 to 1 with an average of 0.33. This reflects the hypervariability and higher resolving power of SSR loci compared to RFLPs. However, not all of the accessions were able to be uniquely identified, including the closely related cultivars Spear and Stiletto, and Avocet and AvocetR. Using the RFLP data, it was possible to uniquely identify each of the 124 wheat varieties. The GS estimates based on the RFLP data were considerably higher than the SSR data, ranging from 0.6 to 0.99 with a mean of 0.75.

Dendrograms were produced based on the GS estimates, allowing the genetic relationships derived using both marker systems to be visualised (Figure 8.1 and 8.2). These show that the relationships detected between the 124 cultivars using the two marker systems are markedly different. Although there were some small groups of cultivars that were clustered together in a

Table 8.2 Summary of data obtained from the 18 microsatellites and 2 STS markers used to screen the 124 Australian wheat cultivars, including the annealing temperatures, number of alleles, size of the fragments in Chinese Spring and chromosomal location.

Microsatellite	Annealing temp	Number of alleles	Size in C.S.	Location
Xgwm2	50	7	132	2AS
Xgwm5	55	8	172	2AS
Xgwm11	55	9	196	1BL
Xgwm18	55	5	186	4BS
Xgwm33	55	12	123	1BL
Xgwm44	58	13	182	7DS
Xgwm46	50	12	187	7BS
Xgwm52	50	4	150	3DL
Xgwm67	57	5	85	5BS
Xgwm88	57	7	121	6BL
Xgwm95	50	5	121	2AS
Xgwm106	50	9	139	1DS
Xgwm111	50	8	205	7DS
Xgwm120	57	10	139	2BS
Xgwm131	55	11	131	7BL
Xgwm155	57	6	141	3AL
Xgwm159	57	3	192	5DS
Xgwm194	58	5	131	4DL
GG	55	11	285	1B
LMW	55	15	134	1A
Total		165		

Figure 8.1 Dendrogram of 124 Australian wheat cultivars constructed from a matrix of GS estimates generated using 1955 RFLPs.

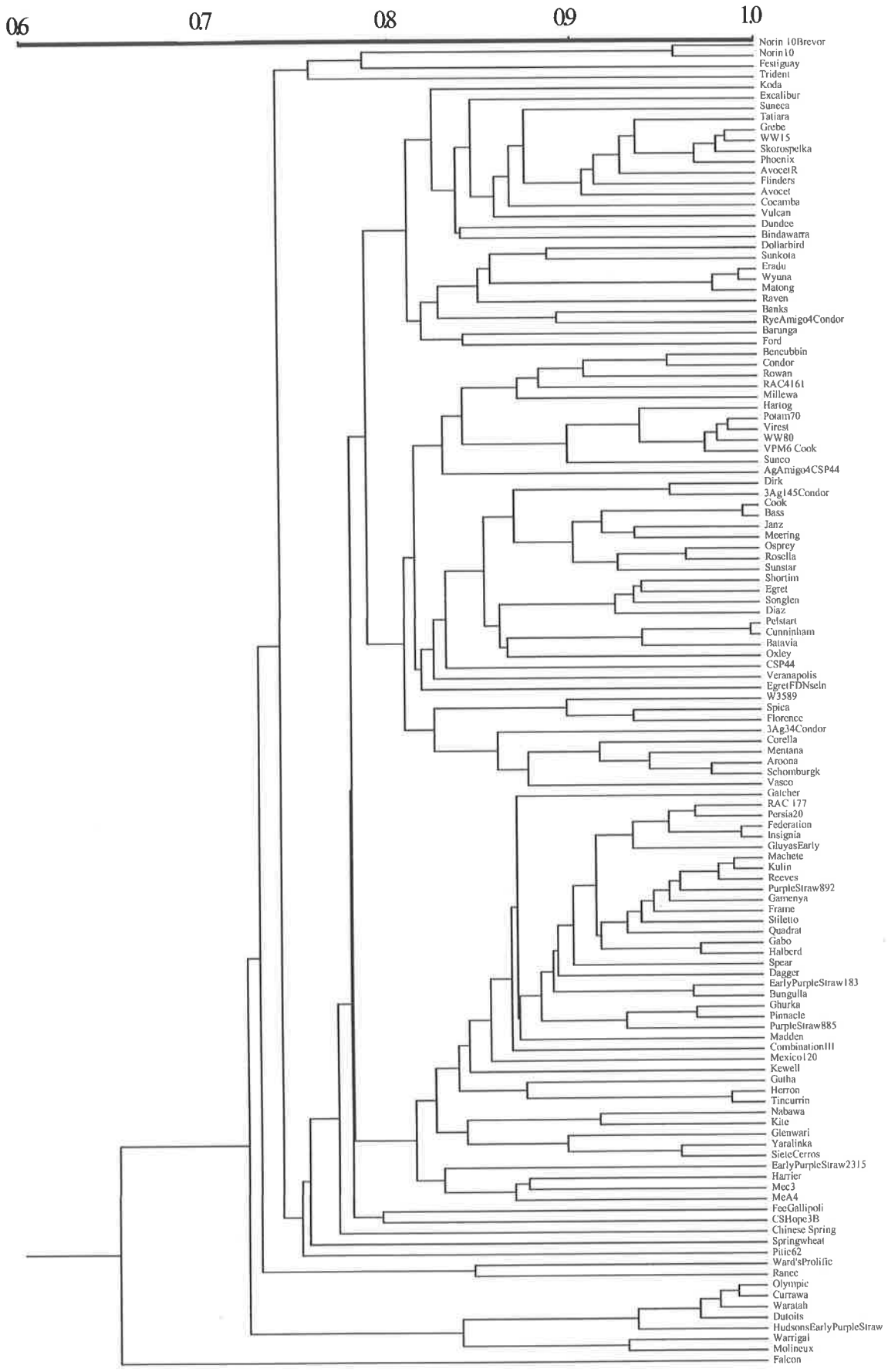
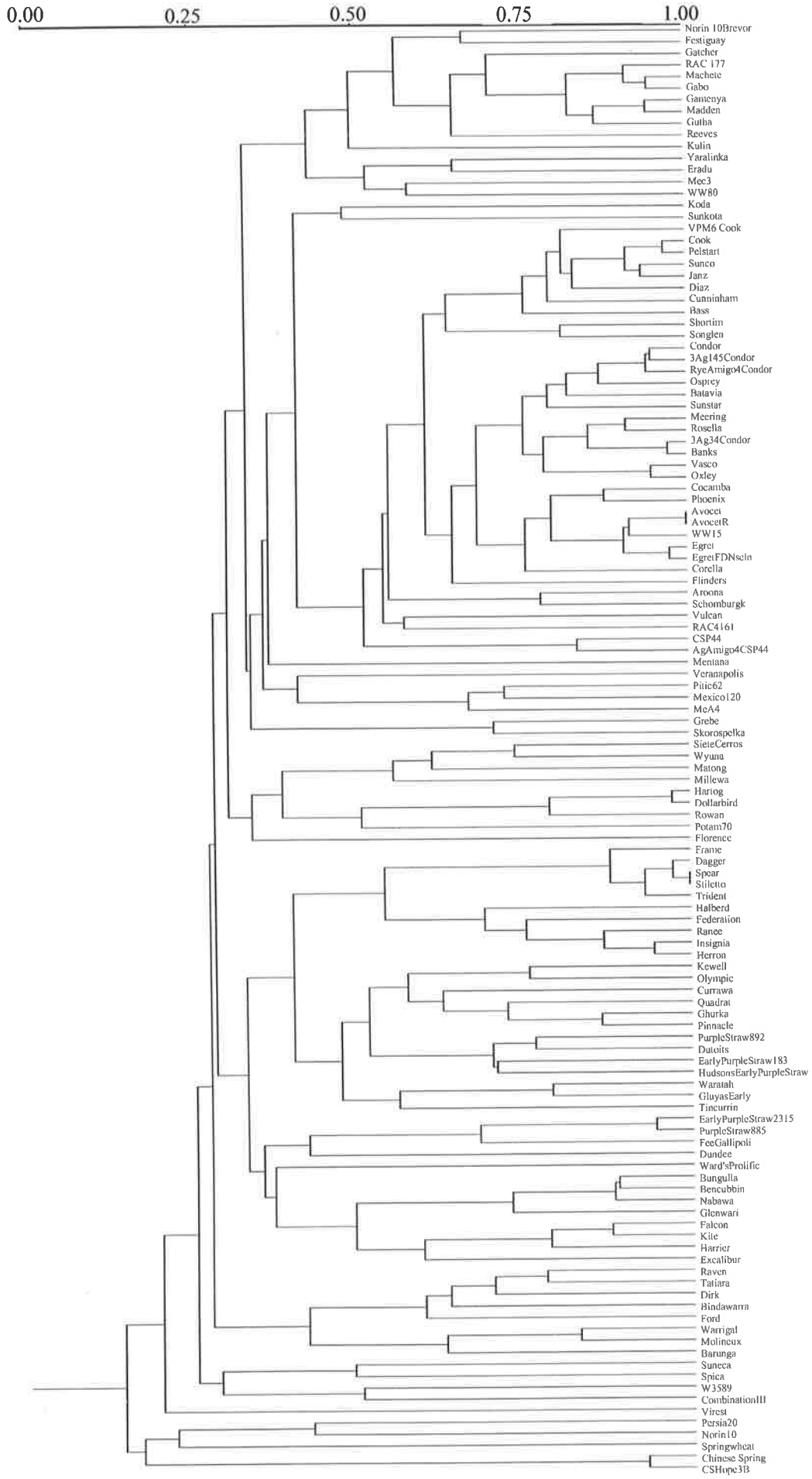

0.6
0.7
0.8
0.9
1.0
Norin 10Brevor
Norin10
Festiguay
Trident
Koda
Excalibur
Suneca
Tatiara
Grebe
WW15
Skoruspelka
Phoenix
AvocetR
Flinders
Avocet
Cocamba
Vulcan
Dundee
Bindawarra
Dollarbird
Sunkota
Eradu
Wyuna
Matong
Raven
Banks
RyeAmigo4Condor
Barunga
Ford
Bencubbin
Condor
Rowan
RAC4161
Millewa
Hartog
Potam70
Virest
WW80
VPM6 Cook
Sunco
AgAmigo4CSP44
Dirk
3Ag145Condor
Cook
Bass
Janz
Meering
Osprey
Rosella
Sunstar
Shortim
Egret
Songlen
Diaz
Pelstart
Cunninham
Batavia
Oxley
CSP44
Veranapolis
EgretFDNseln
W3589
Spica
Florence
3Ag34Condor
Corella
Mentana
Aroona
Schomburgk
Vasco
Gatcher
RAC 177
Persia20
Federation
Insignia
GluyasEarly
Machete
Kulin
Reeves
PurpleStraw892
Gamenya
Frame
Stiletto
Quadrat
Gabo
Halberd
Spear
Dagger
EarlyPurpleStraw183
Bungulla
Ghurka
Pinnacle
PurpleStraw885
Madden
CombinationIII
Mexico120
Kewell
Gutha
Herron
Tincurrin
Nabawa
Kite
Glenwari
Yaralinka
SieteCerros
EarlyPurpleStraw2315
Harrier
Mec3
MeA4
FecGallipoli
CSHope3B
Chinese Spring
Springwheat
Pilic62
WardsProlific
Ranee
Olympic
Currawa
Waratah
Dutoits
HudsonsEarlyPurpleStraw
Warrigal
Molineux
Falcon

Figure 8.2 Dendrogram of 124 Australian wheat cultivars constructed from a matrix of GS estimates generated using 165 SSRs.


0.00
0.25
0.50
0.75
1.00
Norin 10Brevor
Festiguay
Gatcher
RAC 177
Machete
Gabo
Gamenya
Madden
Gutha
Reeves
Kulin
Yaralinka
Eradu
Mec3
WW80
Koda
Sunkota
VPM6 Cook
Cook
Pelstart
Sunco
Janz
Diaz
Cunninham
Bass
Shortim
Songlen
Condor
3Ag145Condor
RyeAmigo4Condor
Osprey
Batavia
Sunstar
Meering
Rosella
3Ag34Condor
Banks
Vasco
Oxley
Cocamba
Phoenix
Avocet
AvocetR
WW15
Egret
EgretFDNsoln
Corella
Flinders
Aroona
Schomburgk
Vulcan
RAC4161
CSP44
AgAmigo4CSP44
Mentana
Veranapolis
Pitic62
Mexico120
MeA4
Grebe
Skorospelka
SieteCerros
Wyuna
Matong
Millewa
Hartog
Dollarbird
Rowan
Potam70
Florence
Frame
Dagger
Spear
Stiletto
Trident
Halberd
Federation
Ranee
Insignia
Herron
Kewell
Olympic
Currawa
Quadrat
Ghurka
Pinnacle
PurpleStraw892
Dutoits
EarlyPurpleStraw183
HudsonsEarlyPurpleStraw
Waratah
GluyasEarly
Tincurrin
EarlyPurpleStraw2315
PurpleStraw885
FeeGallipoli
Dundee
Ward'sProlific
Bungulla
Bencubbin
Nabawa
Glenwari
Falcon
Kite
Harrier
Excalibur
Raven
Tatiara
Dirk
Bindawarra
Ford
Warrigal
Molineux
Barunga
Suneca
Spica
W3589
CombinationIII
Virest
Persia20
Norin10
Springwheat
Chinese Spring
CSHope3B

similar order within both analyses. In general, the two assays appear to have generated relatively independent estimates of relatedness between the 124 wheat cultivars. It was also clear that the variability in GS estimates based on the SSR data was much larger than for the RFLP data, reflecting of the higher levels of variability of SSR loci compared to the RFLPs.

The number of polymorphic loci between all possible pairs of cultivars for both analyses were plotted against each other to determine the correlation between the two marker systems in determining GS (Figure 8.3). The results showed that there was virtually no correlation between the two marker systems in determining GS estimates between the 124 accessions (R^2 = 0.025). The absence of a positive correlation between the SSR and RFLP data was expected given the low level of similarity between the two dendrograms.

As reported by Paull *et al.* (1998), some disease resistance genes from species related to *T. aestivum* and other exotic germplasm have been backcrossed into a number of the 124 Australian cultivars. They were able to identify a number of RFLP markers associated with six chromatin segments carrying resistance genes by comparing the RFLP patterns of the breeding lines carrying the genes with the recurrent parent and derived resistant varieties. Four of the RFLP markers they identified as being potentially linked to *Sr22* (*Xpsr129, Xwg686*) and *Sr36* (*Xpsr131, Xawbma14*) were confirmed to be linked in the rust mapping study described in the previous chapter (Chapter 7). In this study, the SSR patterns of the breeding lines carrying these resistance genes were compared with the recurrent parents and the derived resistant varieties, to determine whether any of the SSRs were potentially linked to these genes. The marker *Xgwm120* was the only SSR that detected an allele unique to specific resistant lines and absent from the recurrent parents. This marker detected an allele in the cultivars known to carrying *Sr 36* (Table 8.3) that was absent in all of the recurrent parents. From the linkage map developed in this study, *Xgwm120* mapped to chromosome 2B and was confirmed as being linked to *Sr36* in the rust study outline in Chapter 7.

Figure 8.3 Comparison of genetic similarity (GS x 100) estimates based on data generated from 595 enzyme-clone RFLP combinations and 18 microsatellites in 124 Australian wheat cultivars.

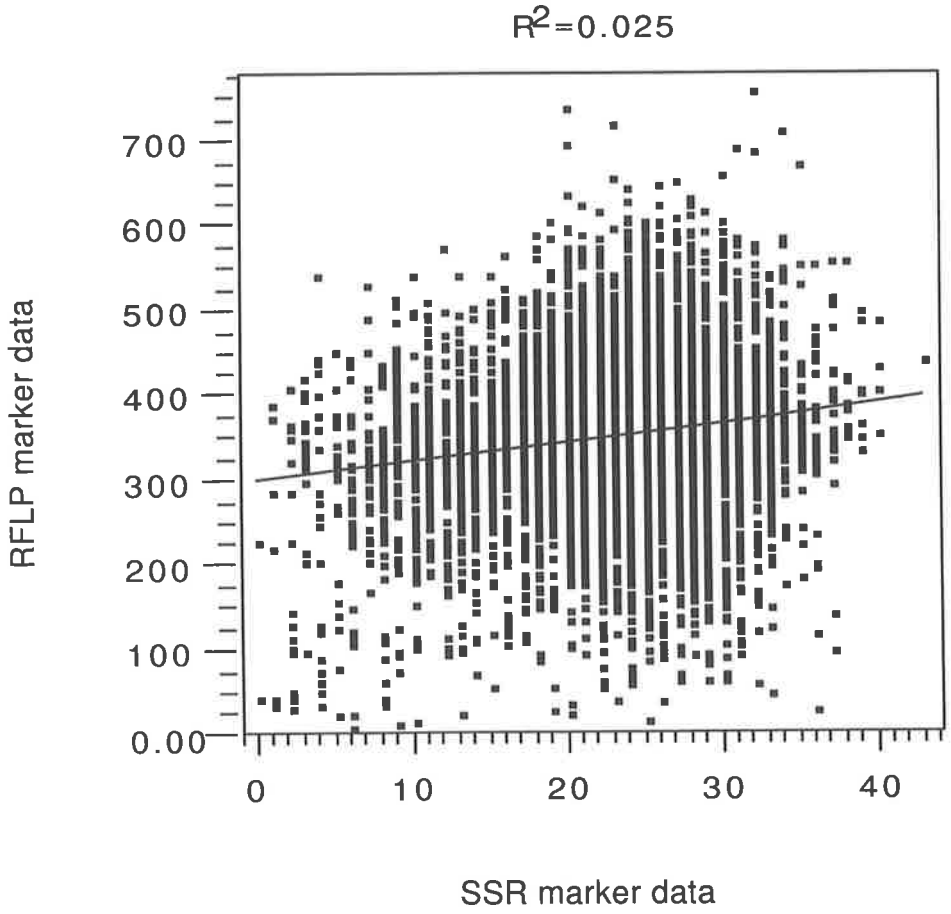

R^2=0.025
RFLP marker data
700
600
500
400
300
200
100
0.00
0
10
20
30
40
SSR marker data

Table 8.3 Associations between markers and cultivars carrying *Sr36* based on pedigree information. Two RFLP markers, *Xpsr131* and *Xawbma14*, were identified by Paull *et al.* (1998) while one SSR marker (*Xgwm120*) was identified in this study.

Resistant cultivar	Donor/ Bridge-parent	Recurrent Parent(s)	Locus	Marker
Bass	Cook	Cook	*Sr36*	*Xpsr131*
Combination	CI 12632			*Xawbma14*
Cook	Timgalen	Condor		*Xgwm120*
Diaz	Combination III, Cook	Cook		
Pelstart	Cook	Cook		
Shortim	Timgalen	Timgalen		
Songlen	Timgalen			
VPM/6*Cook	Cook	Cook		
Yarralinka	Combination III	Warigal		

8.4 Discussion

In this study, SSRs and RFLPs gave independent estimates of genetic relationships between 124 Australian wheat cultivars. The accuracy of GS estimates for any marker system depends on the number of markers, and their distribution of the genome (Schut *et al.* 1997). Although markers represent only a small sample of the plant DNA, they are used to infer relationships over the entire genome. Therefore, the precision of GS estimates generally improves as the number of markers increases (Tivang *et al.* 1994). In this study the number of RFLP loci used to estimate GS among the 124 wheat cultivars was almost 12 fold larger (1955) than the number of SSR loci (165). It is unclear how many SSR loci are required to ensure sufficient accurate marker data to detect reliable genetic relationships. Powell *et al.* (1996) concluded from their studies in soybean, that a relatively small number of SSRs is sufficient to obtain high resolution estimates of relatedness. However, the results of simulation studies by Zhivotovsky and Feldman (1995) may be more realistic. They assessed the effects of SSR mutation rates on their ability to accurately detect genetic relationships between individuals. Their results showed that reliable estimates of tree divergence time and branch lengths in phylogenetic trees involving many taxa may require several hundred loci.

Other studies have also found that SSRs generally generate estimates of GS independent of other data sets. Lu *et al.* (1996) assessed genetic relationships in peas using inter-SSRs, RFLPs, RAPDs and AFLPs. The trees generated using the different marker techniques were all significantly correlated except for the relationships derived from the inter-SSR data. Russell *et al.* (1997) also compared GS estimates derived from RFLP, RAPD, AFLP and SSR data generated using a group of barley cultivars. The GS estimates derived from the SSR data were much lower than those obtained from any of the alternative marker systems, reflecting the unique variability of SSR markers. They found the AFLP and RFLP markers showed the highest similarity of ranking correlation, while the rankings constructed from the SSR data were not well correlated with other marker systems. Powell *et al.* (1996) used soybean to compare the ability of four different marker approaches (RFLP, RAPD, SSR and AFLP) to identify genetic relationships between cultivars. From the GS matrices, the average level of

similarity between genotypes was lowest for SSRs, while estimates for RFLPs, AFLPs and RAPDs were comparable. The RAPD and AFLP clusters were significantly correlated, as were the AFLP and RFLP clusters. However, the SSR clusters were not correlated with any of the other three methods. They concluded that SSRs detected similar relationships at the inter-species level, but at the intra-species level the correlation with other marker systems broke down.

The lack of a correlation between the GS estimates of SSRs and RFLPs may reflect the way in which the markers detect polymorphism in the genome, indicating that different components of DNA variation are being measured with SSRs. Despite their widespread use, the evolutionary dynamics of SSRs are not yet fully understood (Harr *et al.* 1998). SSR alleles evolve in a different manner to the single copy sequence changes or insertions and deletions responsible for producing variation with RFLPs. The mechanism by which SSR alleles are generated is unique, with the majority of variation arising via SSM. The mechanistic basis for SSM involves the local denaturation and displacement of the strands of the DNA duplex followed by mispairing of complementary bases at the site of an existing tandem repeat (Levinson *et al.* 1985). When followed by repair or replication, this can lead to an insertion or deletion of one or more repeat units (Levinson and Gutman 1987). Once expanded, a short repeat region can provide an even more efficient substrate for SSM, increasing the probability of further slippage events. Apart from SSM, unequal crossing over (UCO) can also generate tandem duplications in DNA, and is viewed as an important factor in generating and maintaining multigene families and satellite DNA (Levinson and Gutman 1987).

Although different mechanisms by which allelic variation arises have been reported, it is still unclear why mutation rates differ significantly between different SSR loci. The mutation rate of SSRs has been reported to be quite high, with rates of 10-6 reported in Drosophila, 10-2 - 10-5 in various mammals, 10-4 in Saccharomyces cerevisiae, and 10-3 in humans, (Schug *et al.* 1997; Heyer *et al.* 1997; Deitrich *et al.* 1992; Serikawa *et al.* 1992; Ellegren *et al.* 1995; Henderson and Petes 1992; Jeffreys *et al.* 1988). In humans it has been shown that the mutation rate of SSRs varies from locus to locus with the highest rate being 5.2% in the most

unstable locus Jeffreys *et al.* (1988). However, there was no clear relationship between the length of the allele and the rate of mutation. Although the allele size does not appear to be correlated with the rate of mutation (Jeffreys *et al.* 1988; Harr *et al.* 1998), the DNA sequence flanking the SSR may affect the level of variability. For example, chromatin structures may differ according to the chromosomal location, affecting the mismatch repair efficiency. Cultivars developed from unrelated material may display differences in the level of mutation accumulation in flanking sequences as well as within the SSRs themselves. As a result, material derived from varied genetic backgrounds may show differences in SSR stability and hence allelic diversity.

Additionally, there is a lack of sequence data for the different alleles of SSRs generated within a species. Understanding the mechanism by which new alleles develop is important as it relates to the mechanism of mutation and amplification. Several studies in primates have shown that differences in allele length may not always be due to simple changes in the number of repeats, but to insertion and deletion events in the sequences flanking the SSR (Blanquer-Maumont and Crouau-Roy 1995; Crouau-Roy *et al.* 1996). Grimaldi and Crouau-Roy (1997) in their studies on humans found that alleles identical in size and indistinguishable by conventional PCR typing actually had strikingly different sequences. Studies in mammals and humans have also shown that sequences flanking SSRs may not be as stable as previously thought (Callen *et al.* 1993; Schlotterer 1998). Overall, some of the variability of SSRs appears to arise as a result of sequence variation in the region flanking the repeat, leading to homoplasy. Therefore, sequence analysis of loci may be required to be able to distinguish alleles based on their ancestral relationship for more accurate estimations of genetic relatedness.

In conclusion, the use of SSRs in establishing genetic relationships will not be significantly impeded by the occasional mutation event, as the large number of additional informative markers will either support or deny any relationship changes. However, the use of a low number of hypervariable loci could produce misleading relationship structures between individuals (Jeffreys *et al.* 1988). The results of this and other studies, indicate that small numbers of SSR loci may not detect accurate genetic relationships in some situations. The low

correlation between GS estimates derived from SSR and other marker data suggests that different components of DNA variation, subject to different mechanisms of generating allelic variability, are being measured with SSRs relative to RFLPs. The uniqueness of the SSR assay may limit their usefulness in some applications, where large numbers of loci may need to be screened to compensate for their hypervariability. Although the estimates of GS between cultivars generated from SSR and alternative marker systems may not be well correlated, SSRs are still expected to have a major positive impact on plant genetics.

Chapter IX

General Discussion

The development of molecular marker technology has enabled researchers to begin to understand the genetic control of various traits. A large number of the traits that are used as part of the selection criteria in breeding programs are included based on their economic importance, with little understanding about how they are controlled. The development of genetic linkage maps and other strategies such as bulked segregant analysis have enabled markers to be identified linked to genes that control these traits, providing a basis for the development of alternative plant breeding strategies.

Within the cereals, the generation of genetic maps has been slower in hexaploid wheat compared with diploid barley and rice. Some of the difficulties associated with molecular studies in wheat have been the low levels of intraspecific polymorphism and the large number of linkage groups (Nelson *et al.* 1995a, 1995b; Marino *et al.* 1996). When the research presented in this thesis was initiated, only a limited amount of molecular marker work had been carried out in wheat. The construction of detailed linkage maps in any species is a significant undertaking. The aim of this project was to devise alternative strategies for rapidly identifying markers linked to important quality and disease resistance traits.

The strategy adopted was the rapid development of a 'partial linkage map', combined with bulked segregant analysis. The linkage map was developed by constructing a skeletal map using RFLP and SSR markers, with the aim of identifying markers evenly distributed across the genome. Although the genome coverage of the map was relatively poor, it still enabled markers to be identified linked to major QTLs associated with milling yield and flour colour, in addition to six rust resistance genes (Sr5, Sr9e, Sr22, Sr36, Lr3a and Lr20).

Once regions of interest within the genome had been identified, fine mapping was performed using bulked segregant analysis with AFLPs. The high multiplex ratio of AFLP markers

enabled additional markers to be identified which were placed on the skeletal map, increasing the genome coverage. Additionally, further genome regions associated with the quality traits were identified in the bulked segregant analysis, which had not been detected using the 'partial' map. The failure to identify these other QTLs from the skeletal map was attributed to the poor distribution of markers on some chromosome groups.

This approach to mapping allowed the identification of markers associated with important traits without the development a detailed genetic map. The use of both the 'partial linkage map' and bulked segregant analysis maximised the probability of locating important QTL. Some minor loci linked to flour colour and milling yield identified from the linkage map were not detected in the bulked segregant analysis. Similarly the bulked segregant analysis identified an additional region on chromosome 5 associated with milling yield that was not detected from the linkage map. Therefore, the combination of the two mapping approaches enabled major QTLs associated with these traits to be rapidly mapped. Although the approach taken may not have identified many minor QTL, these loci are of marginal interest in pragmatic breeding. The identification of major loci controlling a large proportion of the genetic variation, represents the main objective of the marker work.

The quality related traits analysed here are some of the first quantitative traits to be mapped in wheat. Previously, only two studies had been carried out aimed at mapping QTL in wheat. Blanco *et al.* (1996) identified a QTL associated with total protein on chromosome 6 in wheat, while Sourdille *et al.* (1996) identified a major QTL on chromosome 5 associated with kernel hardness. The markers identified here linked to QTLs associated with milling yield and flour colour, are important from an economic and breeding perspective. In addition, the population used in this study was generated using current breeding material, so that the results obtained are applicable and relevant to wheat breeding in Australia.

Using linked markers as tags, breeders will be able to begin selecting for high flour yielding lines in the F_2 and F_3 generations. Although the markers have yet to be screened in other populations, the ability to detect and combine desirable alleles from different genetic

backgrounds will increase the probability of developing lines with milling yields exceeding those of 'Schomburgk'. Similarly, the markers linked to flour colour will enable the selection of lines for yellow or white flour earlier in breeding programs. The development of lines containing high levels of flour carotenoids is of particular importance as yellow pigmented flour is required for the production of yellow alkaline noodles in Asia (Kruger *et al.* 1992).

The selection of quantitative traits based on conventional breeding practices is difficult and time consuming, especially for traits of low heritability that are significantly affected by environmental factors. MAS increases the efficiency of selection by allowing breeders to select for the presence of specific marker alleles that are known to be linked to desirable QTL alleles. The selection response can be maximised by using flanking markers to increase the probability of obtaining the required QTL alleles. Further increases in the efficiency of MAS are possible when selection can be exerted on seedlings before the adult phenotype can be measured.

MAS can also be applied in the introgression of superior alleles into desirable genetic backgrounds. Using current procedures this is time consuming and inefficient, as the selection of improved lines is generally based on phenotype rather than genotype. The amount of undesirable donor material can only be reduced by 50% each generation, requiring several years to attain high levels of the recurrent parent background. MAS can help reduce linkage drag by selecting against the donor genome except for the alleles being introgressed.

A number of simulated studies have been carried out to compare the efficiency of pure MAS against pure phenotypic selection. The results of these studies have shown that MAS has the potential to substantially increase the level of selection response for quantitative traits (Lande and Thompson 1990; Hospital *et al.* 1997; van Berloo and Stam 1998). The relative efficiency of MAS appears to be dependent on several factors including population size, the heritability of the trait, the portion of additive genetic variance associated with the marker loci, and the selection scheme. The greatest selection response occurs for traits with low heritability (0.1 - 0.3). At higher heritabilities (0.5 or more) the advantages of pure marker based selection become negligible (Hospital *et al.* 1997; van Berloo and Stam 1998). However, if MAS is

used in combination with phenotypic selection, the selection response is still greater compared with pure phenotypic selection at the higher heritabilities ($H^2 \geq 0.5$). The traits studied here, milling yield, flour colour and protein content, show many of the characteristics required for them to be considered suitable candidates for manipulation by MAS. Therefore, they are ideal targets for marker development and application.

Another important application of MAS is the tagging of disease resistance genes. In order to increase the durability of resistance to a particular pathogen, multiple resistance genes could be pyramided in newly released varieties (Ezzahiri and Reolfs 1989; German and Kolmer 1992). Based on conventional screening techniques, it is difficult to determine whether individual lines carry more than one disease resistance gene when genes from both parents are combined in the progeny. Using MAS, Toojinda *et al.* (1998) were able to rapidly develop barley germplasm carrying multiple stripe-rust resistance alleles. Similarly, Nelson *et al.* (1997) showed that MAS could be effectively used for the accumulation of leaf-rust resistance genes in a line of wheat. In the study, reported here, markers were identified linked to *Sr5, Sr9e, Sr22, Sr36, Lr3a* and *Lr20*. The availability of tightly linked markers for these resistance genes will allow plants carrying more than one gene to be identified without complex pathogen tests in early generations. Five of the SSD lines in the mapping were found to contain at least five of the six resistance genes. These lines are an important source of resistance for breeding programs.

Apart from increasing the selection response for QTLs and allowing resistance genes to be pyramided, MAS is faster and cheaper than many conventional assays, resulting in a saving of time and money for the breeder. Current assays for measuring quality traits such as milling yield, protein, extensibility, and dough strength are time consuming, labour intensive and therefore expensive to perform. Many of these assays are only used to test mid to late generation material that has already passed through several seasons of field trials. The ability to screen lines for these traits early in the program using molecular genotyping will allow undesirable material to be discarded. The use of rapid DNA extraction procedures along with PCR based markers such as STSs or SSRs makes MAS even more attractive. However, molecular markers are not expected to replace phenotypic selection in breeding programs.

Rather, they should be integrated as an additional selection tool to help maximise the selection response. Additional work is required to convert the markers identified in this study into STSs or to find SSR markers close to the traits of interest. The markers should also be screened in other populations to determine whether the QTL they are associated with play an important role in controlling these traits in different genetic backgrounds.

In addition to the mapping of traits, two marker systems were also compared in this study for their ability to reveal genetic relationships between 124 Australian wheat cultivars. The results of the genetic similarity analysis outlined in Chapter 8 demonstrate that both SSRs and RFLPs can be used for characterising and grouping elite wheat breeding cultivars. However, the genetic relationships derived from the SSR data were significantly different to the relationships obtained based on the RFLP data generated by Paull *et al.* (1998). The results of this and other studies demonstrate that SSRs are extremely variable with significant allelic variation (Blanquer-Maumont and Crouau-Roy 1995; Crouau-Roy *et al.* 1996). The mechanisms by which this variation is generated are not yet fully understood (Harr *et al.* 1998), with some loci being more unstable than others (Jeffreys *et al.* 1988). The hypervariable and unstable nature of some SSR loci suggests that they may not be an appropriate source of markers for inferring genetic relatedness in some situations.

The research outlined in the previous chapters has demonstrated that molecular markers linked to both quantitative and qualitative traits can be rapidly identified in special purpose populations developed by breeders. The development of 'partial linkage maps' in combination with bulked segregant analysis will allow major QTLs and disease resistance loci to be quickly identified without the construction of detailed genetic maps. This is particularly useful for species with many chromosomes and low levels of intra-specific polymorphism, such as wheat. The approach used in this study can be applied to other populations for the identification of linked markers associated with other important traits. MAS will never completely replace conventional assays based on phenotype. However, markers are a useful tool for breeders and can be used to increase the efficiency and accuracy of selection.

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