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Organisation and Expression of Plant B Chromosomes

by

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A thesis submitted for the degree of
Doctor of Philosophy
in
The University of Adelaide
Department of Genetics
(Faculty of Science)

16th June, 1999

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ABSTRACT

Supernumerary B chromosomes are found in all major taxonomic groups of organisms. Although the B chromosomes significantly increase the nuclear DNA amount, no active genes have yet been unequivocally localised on these chromosomes by genetic or molecular means. However, the presence of B chromosomes is often associated with changes in chromosome behaviour, such as meiotic pairing and recombination, and it is therefore thought that they play a significant biological role. The mechanisms which are responsible for these effects remain to be determined.

Brachycome dichromosomatica is an Australian native ephemeral plant of the arid regions of South Eastern Australia. This species contains only two pairs of chromosomes in the normal complement (A chromosomes) and 0 - 4 B chromosomes in some populations. Sequences that hybridise to ribosomal RNA gene probes are present near the end of the short arm and, to a lesser extent, near the centromere of the B chromosomes of *B. dichromosomatica*.

In Chapter 3, internal transcribed spacer 2 (ITS2) was amplified by PCR from total *B. dichromosomatica* DNA using primers within the conserved regions encoding the 5.8S and 25S stable rRNA species. Comparison of the A chromosome ITS2 sequences, PCR amplified from several individual plants without B chromosomes, with corresponding sequences derived from microdissected B chromosomes revealed two consistent differences between the rDNA of A and B chromosomes. One of these differences produced an *Sfc* I restriction site that was present only in the ITS2 of the B chromosome rDNA. Amplification by PCR of ITS2 from total genomic DNA from plants with and without B chromosomes showed an additive relationship between the amount of PCR product containing the *Sfc* I site and the number of B chromosomes present. Quantitative

analysis indicated that the proportion of total nuclear rDNA present on a single B chromosome varied between 3 and 4% in plants.

In parallel experiments it was found that the rDNA of different chromosome races of *B. lineariloba* and *B. breviscapis* also contained the *Sfc* I restriction site but it was absent from *B. curvicarpa*, *B. dentata*, *B. eriogona*, *B. parvula*, *B. multifida*, *B. segmentosa* and *B. ciliaris*. Further sequence analysis of the ITS2 amplified from *B. lineariloba* (2n=16) identified an A at base 175, similar to that in the B chromosome ITS2, as well as an additional nine bases that differed from both A and B chromosome derived ITS2 sequences.

In Chapter 4, experiments using reverse transcriptase-PCR of the ITS2 region within the 40S precursor rRNA transcript suggested that the B chromosome rDNA was not transcribed in leaf tissue. Similarly, PCR of reverse-transcribed total leaf and flower RNA, using primers specific for the B chromosome ITS2, was unable to detect a transcript from the B chromosome rDNA.

In Chapter 5, molecular cloning of part of an A chromosome rDNA repeat unit enabled the complete sequence of the A chromosome intergenic spacer (IGS) region to be determined and revealed a putative RNA polymerase I transcription initiation site. In addition, three major regions of repeats, envisaged to have an important role in the regulation of rRNA transcription, were identified. Southern hybridisation with IGS-specific probes identified a *Xba* I restriction fragment polymorphism, present in genomic DNA containing B chromosomes and absent in genomic DNA without B chromosomes. This fragment is hypothesised to represent a B-specific IGS. Comparison of the PCR products amplified from genomic DNA with and without B chromosomes and from microdissected B chromosome DNA, with primers specific to the IGS region, identified several putative B

chromosome specific polymorphisms but no clear reason for rRNA gene inactivity on B chromosomes emerged from the sequence data.

In situ hybridisation with IGS-specific probes was not able to confirm the presence of polymorphisms in the region on the B chromosomes, but did corroborate the previous hybridisation of the heterologous cotton rDNA probe to the distal and proximal clusters of rDNA on the B chromosome, indicating that the *B. dichromosomatica* B chromosome contains major and minor loci for this sequence.

In Chapter 6, amplified fragment length polymorphism (AFLP) and random amplification of polymorphic DNA (RAPD) analyses were utilised as approaches to obtain low copy sequences specific to the B chromosomes or sequences common to the A and B chromosomes. AFLP analysis isolated two sequences (2aflp3 and 7aflp3) present in all B chromosome-containing samples examined. In addition, 2aflp3 was amplified from microdissected B chromosome DNA and from one sample without B chromosomes, suggesting that it was a sequence present on the B chromosomes and occasionally on the A chromosomes. 7aflp3 was not able to be amplified from microdissected B chromosome DNA and was postulated to have been isolated because of an A chromosome polymorphism.

RAPD analysis isolated a polymorphic sequence, BdER. Inconclusive evidence suggested that BdER was present on the A and B chromosomes and that it was not ubiquitous to either chromosome type within the population. Sequencing of BdER revealed significant nucleotide identity with retroviral and mitochondrial sequences, suggesting invasion of a subpopulation of A and possibly B chromosomes with mobile elements or organelle-derived DNA.

DECLARATION

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

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

Signed:

Tamzin Donald

16th June, 1999

ACKNOWLEDGEMENTS

Firstly, I wish to thank my supervisor Associate Professor Jeremy Timmis for his advice, never ending encouragement, support and patience.

I am also indebted to Dr Carolyn Leach for sharing her *Brachycome* and B chromosome expertise and for her enthusiasm for my work. Thanks also to Dr Andreas Houben for his help and patience with *in situs* and for Chapter 6.

To everyone in the JT lab, both past and present, it has been a fantastic lab to work in. In particular I would like to thank Tricia Franks for all her assistance and Eun-lee Jeong, for being an inspiration and for always having a smile on her face and something nice to say. Special thanks also to my buddy Sharon Orford, for helping me out with any problem scientific or non-scientific, big or small, for laughing at my jokes!, for all the chats, conferences, muffins, dinners and doozers!!. Thanks Shazzy. To the other current members of the lab – Dawn Verlin, Sarah Harmer and Nigel Percy - thanks for putting up with me and helping to make the lab such an enjoyable place to work.

Thanks also to all the other members of the department who have contributed to this thesis. In particular: Paul Moir for being able to fix anything, for help with computing and just everything really!! Doug Pottrell for being an all round nice guy, for his endless assistance with anything and everything and for his golf lessons; Sharon Kolze for making research so much easier on a day to day basis by being friendly, efficient and always willing to help; Daniella Marshall for tolerating me and the endless hours I have spent in her office; and the regular morning tea gang for scintillating conversations about science, life, and footy.

Sincere thanks to my friend and fellow PhD student, Sue O'Connor, for all the encouragement, lunches, coffees and fun times, and for sharing the last four years with me.

Special thanks to all my family and friends. In particular thanks to Mum and Dad for their encouragement and support throughout this project. Dad thanks for always asking how I was getting on, even when my responses weren't always worth it! To Lu and Scott for looking after me when I needed it most. Sue, Ian, Will and Sarah Leitch for their friendship, support and for making me feel so welcome, especially during the 'Darwin days'. To Prince John Harvey, for being a great friend with impeccable taste in wine!! You're groovy baby!

Last but definitely not least, thanks to Gus Leitch for SO many things, especially his love, encouragement and tolerance over the last six weeks. Ti Amo.

Finally, I would like to dedicate this thesis to Sophie Jackson and Bec Mooney for their undying support, friendship and for all the fun times we have shared. Thanks chicks.

LIST OF ABBREVIATIONS

A	adenine
AFLP	amplified fragment length polymorphism
[α–^{32}P]dATP	alpha-labelled deoxyadenosine triphosphate
B/Bs	B chromosome/B chromosomes
0 B	genomic DNA containing no B chromosomes
+ B	genomic DNA containing B chromosome(s)
1 B	genomic DNA containing one B chromosome
2 B	genomic DNA containing two B chromosomes
3 B	genomic DNA containing three B chromosomes
4 B	genomic DNA containing four B chromosomes
bp, kb	base pairs, kilobase pairs
C	cytosine
°C	degrees Celsius
cDNA	deoxyribonucleic acid complementary to ribonucleic acid
Da, kDa	Dalton(s), kiloDaltons
DAPI	4', 6-Diamidino-2-Phenylindole
dATP	2'-deoxyadenosine-5'-triphosphate
DEPC	diethyl pyrocarbonate

dCTP	2'-deoxycytosine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dH_2O	distilled water
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
dTTP	2'-deoxythymidine-5'-triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
g	force of gravity
gDNA	genomic DNA
(-20)	forward sequencing primer
G	guanine
g, mg, μg, ng	gram(s), milligram(s), microgram(s), nanogram(s)
[γ–^{32}P]dATP	gamma-labelled deoxyadenosine triphosphate
IGS	intergenic spacer
IPTG	isopropyl-β-D-thiogalactoside
ITS1	internal transcribed spacer 1
ITS2	internal transcribed spacer 2
λ	bacteriophage Lambda

L, ml, µl	litre(s), millilitre(s), microlitre(s)
M	moles per litre
mA	milliAmperes
mCi	microCuries
min, hr	minute(s), hour(s)
mRNA	messenger ribonucleic acid
cm, mm, µm, nm	centimetre, millimetre, micrometre, nanometre
NOR	nucleolar organising region
nt	nucleotide(s)
°C	degrees Celcius
OD_{260}	optical density at 260 nm
OD_{600}	optical density at 600 nm
PCR	polymerase chain reaction
PEG	polyethylene glycol
pfu	plaque forming units
PSR	paternal sex ratio
RAPD	random amplification of polymorphic DNA
®	registered
rDNA	ribosomal deoxyribonucleic acid
RNA	ribonucleic acid
RNaseA	ribonuclease A

rpm	revolutions per minute
rRNA	ribosomal RNA
RSP	reverse sequencing primer
SDS	sodium dodecyl sulphate
SSC	saline sodium citrate
T	thymidine
TAE	Tris-acetate-EDTA
Taq	*Thermus aquaticus*
TE	Tris-EDTA
TEMED	NNN'N'-Tetramethylethylendiamine
Tris	Tris[hydroxymethyl]amino methane
U	unit(s) of enzyme
UV	ultraviolet
V	Volts
X-gal	5'-bromo-4-chloro-3-indoyl-β-D-galactopyranoside
% (v/v)	percent volume per volume
% (w/v)	percent weight per volume

CHAPTER 1:

Introduction

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1.1 INTRODUCTION.

Genetic variation is a feature of all populations of sexually reproducing organisms. However, the chromosomes, which act as the vehicles for the transmission of genetic material, are generally constant in form and number in all individuals of a species. This constancy results from the strict requirement for the fidelity of information transfer between generations of cells. In cases where numerical variations occur due to spindle or centromere malfunction, aneuploid or polyploid individuals may be produced. Aneuploidy is frequently associated with deleterious consequences to the individual, but it is estimated that up to 50% of flowering plants are balanced polyploids (Jones, 1995). Another form of numerical variation involves chromosomes, additional to the basic complement, which are not duplicates of any of the standard chromosomes. Several different names have been given to this type of additional chromosome including supernumerary, dispensable, extra, accessory and B chromosome. The term B chromosome, first used by Randolph (1928), will be used in this thesis to distinguish between the supernumerary chromosomes being discussed and the standard or A chromosomes.

1.2 B CHROMOSOMES

Chromosomes that behave as B chromosomes (Bs) were first described by Wilson (1907). B chromosomes have since been found in all major taxonomic groups of organisms and are one of the many causes of the numerical chromosome variation which exists in plant, animal and fungal species (Jones and Rees, 1982; Miao *et al.*, 1991; Covert, 1998). It has been estimated that Bs are likely to be present in 15% of all living species (Beukeboom, 1994), and the most recent published record of B occurrence in plants stated that Bs have been found in 1372 species of flowering plants, leading to an estimate of their occurrence in 10-15% of all flowering plant species (Jones, 1995).

B chromosomes are generally characterised in the following ways (Jones and Rees, 1982):

1) they are dispensable chromosomes found in some, but not all individuals of some populations, and therefore are not necessary for survival, growth or reproduction of the individual organism.

2) they do not pair or recombine at meiosis with any of the A chromosome set.

3) they are morphologically distinguishable from the A chromosomes, often being smaller.

4) they show irregular, non-Mendelian modes of inheritance.

1. 3 EFFECTS.

Although Bs are dispensable, they can affect a variety of cellular and physiological processes suggesting they are not genetically inert (Jones and Rees, 1982). In plants, the presence of Bs is often detrimental; reducing fertility, germination, vegetative growth and flowering. These effects have been demonstrated in several species including *Secale cereale* (Müntzing 1943, 1963; Kishikawa, 1965), *Zea mays* (Randolph, 1941; Carlson, 1986), *Allium shoenoprasum* (Bougourd and Parker, 1979a) and *Aegilops speltoides* (Mendelson and Zohary, 1972). The most prominent phenotypic effect associated with the presence of Bs is in maize (*Zea mays*) where plants with five or more Bs often display longitudinal white leaf stripes and/or narrow leaves (Staub, 1987). In general the effects on phenotype increase with increasing numbers of Bs.

1.3.1 Effects on A Chromosome Behaviour

The most enigmatic effect that B chromosomes have is the control their presence exerts on the behaviour of the A chromosome sets during meiosis.

1.3.1a Chiasma Frequency:

The presence of Bs alters the distribution and frequency of chiasmata in the A chromosomes (John and Hewitt, 1965a, 1965b; Cameron and Rees, 1967; Jones and Rees, 1967; Ayonadu and Rees, 1968; Jones and Rees, 1982; Clough, 1992). In some species there is an increase in chiasma frequency (e.g. *Zea mays*; Ayonadu and Rees, 1968) and in others a decrease (e.g. *Lolium perenne*; Cameron and Rees, 1967) associated with an increasing number of Bs.

It is possible that the effect on the number and position of chiasmata provides a clue to the persistence of these otherwise parasitic chromosomes (Jones and Rees, 1982; Bell and Burt, 1990; Jones, 1995). Firstly, in causing an increase in chiasma frequency, Bs increase the variability within species and may increase genetic variability in populations giving certain individuals a selective advantage. Secondly, Bs are parasitic and may evoke a response in their host organisms. One response could be to increase crossing-over within the A chromosomes, producing a greater variety of offspring and hence a better chance of developing individuals that block the accumulation of the parasitic Bs. Thirdly, as Bs are purely selfish entities, a mechanism that reduces their rate of meiotic loss, by promoting the formation of bivalents between them, might also influence chiasma frequency and/or distribution in A chromosomes. Finally, the effects of Bs on crossing-over in A chromosomes may not have a specific function, but the presence of additional chromosome(s) may result in a general response in the organism. As each additional B increases the total mass of DNA in the nucleus, it is conceivable that some general adjustments need to be made to cope with the extra chromatin and these may include changes in chiasma frequency and/or distribution on the A chromosomes.

In summary, the means by which Bs influence chiasma distribution and frequency in the A chromosome set is not known, but the variation they illicit within and between different species highlights the heterogeneous nature of B chromosome systems.

1.3.1b Pairing in Hybrids

In *Aegilops* and *Lolium* species, Bs have a striking effect on homoeologous chromosome pairing at meiosis. The Bs of some *Aegilops* species have a genetic activity that can restrict homoeologous pairing in hexaploid wheat in a similar manner to the *Ph1* gene present on chromosome 5B (Riley and Chapman, 1958; Sears and Okamoto, 1958). In hybrids of *A. mutica* or *A. speltoides* with polyploid wheat, the presence of B chromosomes, in the absence of the wheat *Ph1* gene, drastically reduces the extent of homoeologous chromosome pairing when compared with hybrids without both the *Ph1* gene and B chromosomes. It therefore appears that the Bs are able to compensate for the absence of effects of the *Ph* gene on the absent 5B chromosome (Dover and Riley, 1972; Ohta, 1991).

In tetraploid hybrids between *Lolium temulentum* and *Lolium perenne*, pairing occurs between both homologous and homoeologous A chromosomes, resulting in multivalents at meiotic metaphase I. However, when B chromosomes from *L. perenne* are present in the hybrids the chromosome pairing behaviour changes dramatically, with bivalents formed between A chromosomes at metaphase I that are comprised strictly of homologous chromosomes (Jenkins, 1986).

It is possible that the restriction to homologous pairing involves a mechanism which transforms multivalents into homologous bivalents and it could be said that this is similar to what occurs in systems where there is no pairing between A and B chromosomes, despite the fact that they are thought to share a high percentage of common DNA sequences (Chilton and McCarthy, 1973; Timmis *et al.*, 1975; John *et al.*, 1991; Tsujimoto and Niwa, 1992; Blunden *et al.*, 1993; Cuardro and Jouve, 1994; López-León *et al.*, 1994;

Jamilena *et al.*, 1994; McQuade *et al.*, 1994; Bougourd *et al.*, 1995; Stark *et al.*, 1996). It is therefore conceivable that there is a universal system associated with B chromosomes that restricts pairing to homologues, inhibiting any possible pairing between Bs and the A chromosomes which would inevitably result in the formation of infertile gametes and subsequent the elimination of the B chromosomes.

1.3.2 Physiological Effects.

Other effects associated with Bs are at the cellular level and include increases in cell size and the duration of the cell cycle, as well as changes in the protein and RNA content of cells and nuclei (Jones and Rees, 1982). In rye, for example, the presence of Bs results in a general reduction in physiological activity throughout the plant including reductions in seed and pollen fertility, vegetative growth and vigour as well as decreases in the levels of total RNA and proteins per cell. These deleterious effects increase in proportion to the number of Bs present and interestingly are much stronger when an odd number of Bs are present compared with an even number of Bs (for review see Bougourd and Jones, 1997). The 'odds-evens' effect was also recorded earlier in several other species (reviewed in Jones and Rees, 1982) but the reason for this difference in effect between odd and even numbers of Bs is not known. However, Bs do pair with each other during meiosis and, in the presence of an odd number of Bs, univalents result. Similarly, in somatic nuclei homologous chromosomes 'associate' with each other and this could also be the case between pairs of Bs, whereas in organisms with an odd number of Bs, single, unassociated Bs will be present. It is possible that the severity of effects associated with odd numbers of Bs, compared with even numbers, is the result of imbalance and/or hemizygous gene expression resulting from the 'unpaired' Bs influencing the general functioning of the cell.

The means by which Bs cause the range of effects discussed are not known. One model for the effects seen is that the increases in nuclear DNA, associated with Bs, affects general cell functioning through increases in total cell mass and cell cycle time, and alterations in protein and RNA content (Jones and Rees, 1982). This hypothesis is supported by the increase in severity of effects associated with increasing B chromosome number. There are likely to be a number of factors, including environmental conditions, intrinsically involved in the range of effects seen and it is probable that a variety of mechanisms exist between the B chromosomes in different organisms.

1.4 B Chromosome Maintenance

There are several mechanisms by which Bs can become established and maintained within populations, despite their often detrimental effects. One such mechanism is abnormal behaviour at cell divisions in specific tissues and cells. Accumulation (drive) of Bs has been observed in mitotic tissue and during meiosis in males and/or females. Furthermore, drive within one sex is sometimes associated with the reverse (drag) in the other. The inheritance of a B chromosome is classified as Mendelian or non-Mendelian on the basis of whether its transmission rate is equal to the Mendelian expectation of 0.5 (Jones and Rees, 1982). Transmission of Bs at frequencies significantly greater than expected on a Mendelian basis results in an increase in the average number of Bs in the progeny compared with the parental generation. This accumulation of B chromosomes is achieved through the processes of non-disjunction and/or directed segregation of the Bs in nuclear divisions before, during, or just after meiosis.

1.4.1 Pre-Meiotic Drive

B chromosomes are generally found to be mitotically stable, as indicated by a constant number of Bs throughout all tissues in the organism. However, in some systems

such as *Aegilops speltoides* and *Crepis capillaris*, non-disjunction occurs in mitotic divisions prior to meiosis and the two daughter chromatids move to the same pole at anaphase. This can result in the accumulation of B chromosomes in one cell lineage and absence from another (Jones and Rees, 1982). In *Aegilops speltoides*, B chromosomes are absent from the roots, but maintained in the remaining tissues (Mendelson and Zohary, 1972), an affect most likely to be due to non-disjunction and chromosome loss in mitotic divisions early in the developing embryo or seedling. Similarly in *Crepis capillaris* the floral components contain a higher average number of Bs than the roots or leaves, preferentially increasing the average number of Bs in the gametes (Rutishauser and Röthlisberger 1966; Parker *et al.*, 1989). As a result of mitotic non-disjunction, the inheritance of B chromosome in *C. capillaris* is greatly enhanced over Mendelian expectations with average transmission of 0.90 through pollen and 0.83 through eggs (Parker *et al.*, 1989).

1.4.2 Meiotic Drive

During meiosis the B chromosomes do not pair or recombine with the As, but they may pair with each other when more than one B chromosome is present. A clear case of B chromosome accumulation due to meiotic drive is found in the grasshopper *Myrmeleotettix maculatus* (Hewitt, 1973). The karyotyping of progeny from single pair crosses revealed B chromosome transmission rates as high as 0.9 in the female in some populations (Hewitt, 1973). Using techniques which allowed the observation of chromosome orientation in the primary oocyte at the onset of anaphase, it was found that the spindle is asymmetrical, with the Bs preferentially distributed towards the egg pole as the primary oocyte entered anaphase of the first meiotic division. B chromosomes were consequently included in the secondary oocyte at an increased frequency over Mendelian expectations and likewise in the gametes and progeny (Hewitt, 1976). In males, however, there is meiotic drag with

transmission rates as low as 0.3. A similar situation was also found in another grasshopper species, *Omocestus burri*, with B chromosomes being included in the secondary oocyte at an increased frequency over Mendelian expectations, but not accumulating in the male germ line (Santos *et al.*, 1993).

In *Lillium callosum* there is also segregation distortion during female meiosis. In the egg mother cell, a univalent B lies outside the metaphase plate and is localised at the micropylar pole of the egg mother cell in about 80% of meiosis (Kayano, 1957).

1.4.3 Post-Meiotic Drive

The most common mode of B chromosome drive is post-meiotic during the mitotic divisions involved in development of the gametophyte. Post-meiotic non-disjunction has been observed in maize (*Z. mays*) where non-disjunction at the second pollen grain mitosis in the male gametophyte results in increased numbers of B chromosomes in the mature gamete (Roman, 1948a; Carlson, 1986). In addition Roman (1948b) found evidence indicating that sperm nuclei containing B chromosomes are then more likely to be involved in fertilisation of the egg. In approximately 60 to 70% of cases, B-containing pollen was shown to preferentially fertilise the egg, increasing the B chromosome frequency within the population (Roman, 1948b; Carlson, 1986). Similarly in *A. mutica* (Ohta, 1996) and *A. speltoides* (Mendelson and Zohary, 1972) B chromosome non-disjunction at first pollen mitosis is followed by their accumulation in the generative nucleus. Crosses in *A. speltoides* revealed that, in the male, the accumulated Bs were disproportionately frequently transmitted to progeny and reciprocal crosses confirmed that no parallel accumulation occurred in the female track (Mendelson and Zohary, 1972).

B chromosome maintenance in *Secale cereale* L. (rye) is unusual in that directed non-disjunction takes place through both the male and female tracks. The B chromosomes undergo non-disjunction at the first mitosis in both the microspore and megaspore and, in

the case of the microspore, then tend to migrate to the pole at which the sperm nucleus will form. The non-disjunction is constant and occurs at a high frequency of 90-95% in all populations studied (Romera *et al.*, 1989).

1.4.4 *Nasonia vitripennis*

Perhaps the most selfish mechanism by which B chromosomes are preferentially transmitted between generations, is seen in the parasitic wasp *Nasonia vitripennis*. In this species, males develop from unfertilised eggs, whereas females develop from fertilised eggs. The male of the species can carry a B chromosome, called the paternal sex ratio (PSR) chromosome, and females that mate with males carrying the PSR chromosome produce all male progeny. After fertilisation by a sperm carrying the PSR chromosome the paternal A chromosome set condenses and is lost from the zygote. The PSR chromosome is itself maintained and subsequently passed on to the next generation (Eikbush *et al.*, 1992).

B chromosomes which undergo the various forms of accumulation discussed can be considered purely selfish, maintained despite the often negative effects on their host's fitness. Interestingly the number of B chromosomes present in any one organism does not generally rise above a certain number and in many plant species, individuals with a larger number of Bs show reduced viability and fertility. In *A. speltoides*, for example, plants with more than three Bs have reduced vigour and fertility, while plants with zero to three B chromosomes show normal growth and fertility (Mendelson and Zohary, 1972). There is also strong evidence that there is selection against organisms with high numbers of Bs in *C. capillaris* (Parker *et al*, 1991), rye (Jones, 1975; Matthews and Jones, 1982) and maize (Carlson, 1986). The strong drive mechanisms associated with Bs therefore appear to be balanced by selection operating against individuals with too many Bs, imposing an upper limit on the numbers tolerated by members of a given species.

B chromosomes generally occur only in outbreeding plant populations (Jones, 1995) and this is also likely to contribute to the survival of the polymorphism. The existence of Bs is incompatible with inbreeding because selfing would not facilitate the spread and accumulation of B chromosomes in populations.

1.4.5 B Chromosomes Offering an Adaptive Advantage

In the plant species *Lolium perenne* and *Allium shoenoprasum*, the B chromosomes lack drive and there are convincing reports that Bs increase the survival capacity of these plants under certain conditions (Rees and Hutchinson, 1973; Hutchinson, 1975; Holmes and Bougourd, 1989; 1991). In a population of *A. schoenoprasum* found in South Wales (UK), the maintenance of the B chromosome is not dependent on accumulation mechanisms and shows significant loss during transmission in controlled crosses (Bougourd and Parker, 1979; Bougourd and Plowman, 1996). In addition, in the adult plant of *A. schoenoprasum* the presence of B chromosomes has potentially detrimental effects including decreases in plant height, leaf diameter and fertility. However, in the early stages of the plants life cycle, B chromosomes provide a selective advantage to individuals, indicated by a significant difference in the frequency of B-containing individuals between seed and seedling populations (Holmes and Bougourd, 1989). Experimental population studies revealed that the presence of B chromosomes brings forward the onset of germination and enhances the ability of seeds to germinate under drought conditions. The presence of B chromosomes is therefore beneficial in environments where plants carrying them are able to take advantage of minimal rain and germinate promptly. In *A. schoenoprasum* the Bs increase the likelihood of secure establishment before inundation by flooding when ungerminated seeds and non-secure seedlings are washed away (Bougourd *et al.*, 1995). The strong selection of plants with B chromosomes at this stage is therefore sufficient to maintain them in the population despite the absence of drive and the presence

of clear deleterious effects in the adult plant (Holmes and Bougourd, 1989; 1991).

1.5 Further Population Studies

The examination of populations of individuals containing B chromosomes, such as those of *A. schoenoprasum* discussed above, has highlighted a number of important aspects related to the maintenance, distribution, and frequency of B chromosomes.

The distribution and frequency of Bs in natural populations have been closely associated with environmental variables including soil type, altitude and climate (reviewed by Jones and Rees, 1982). In *Brachycome dichromosomatica*, for example, the frequency of +B plants increases relative to 0B plants after successive dry seasons (Smith-White and Carter, 1981).

As well as studying natural populations, experimental populations have been examined to determine environmental factors influencing B chromosome frequency. In populations of rye it was found that the mean frequency of plants with B chromosomes decreases as the density (ie. the number of plants within a certain area) increases (Hutchinson, 1975). Conversely, in similar experiments with *Lolium perenne* it was found that increasing plant density resulted in increased frequencies of +B plants. It was therefore concluded that mortality caused by high plant density directly affects the frequency of plants with B chromosomes within populations and the presence of Bs can be advantageous or disadvantageous, with regard to population density, depending upon the species under consideration (Hutchinson, 1975).

B chromosomes have been reported in German, Swiss, French and British populations of *C. capillaris*, indicating that they are a widespread component of the genetic system of this species over a wide range of environments (Parker *et al.*, 1991). However, surveys of B distribution within forty *C. capillaris* populations from different habitats

within Britain, revealed that Bs were not a consistent feature of all the populations, or of individuals within the populations (Parker *et al.*, 1991). There were generally found to be fewer B chromosomes in populations in the North of Britain compared with the South and B chromosomes reached maximum frequencies at middle altitudes suggesting that in *Crepis*, Bs are excluded from populations growing under suboptimal conditions (Parker *et al.*, 1991). Similarly in *Myrmeleotettix maculatus*, B chromosomes are found more often in populations in the south and east of the UK, zones which are climatically more favourable to grasshoppers (Hewitt, 1973).

From the population studies which have been carried out, it can be concluded that under certain conditions, at certain times of the year or in certain seasons, the selective pressures acting on +B and -B individuals vary, such that the maintenance of Bs does not require specific intrinsic accumulation mechanisms. B chromosomes in some species are found over a wide geographic range, whereas in others they are restricted to a few populations. In the latter case, the distribution of B chromosomes may be attributable to the existence of varying selective pressures in different environments as discussed above, or alternatively to geographic or reproductive isolation preventing the spread of B chromosomes. In the grasshopper *E. plorans*, for example, a broad inland region of the Segura River basin (Northern Africa) which lacks grasshoppers with Bs is not due to selection but to geographical barriers, which separate +B and -B populations, impeding the B's advance and preserving the non-B chromosome region (Cabrero *et al.*, 1997).

1.6 Sequences on the B Chromosomes

Although extensive studies have been made examining the effects and maintenance of B chromosomes, little is known about the specific genes or sequences which reside on B chromosomes and how the genetic material of the B is related to their chromosomal

effects. The wide range of effects associated with the presence of B chromosomes suggests that they are not genetically inert though major gene activity is rare or even absent in B chromosomes (Jones and Rees, 1982).

1.6.1 B chromosome Specific Sequences

Many of the molecular studies of B chromosomes have focussed on isolating B-specific sequences in the hope that they might be involved in the unique mechanisms and effects associated with Bs. These investigations have yielded B-specific repeat sequences in *N. vitripennis* (Nur *et al.*, 1988; Eikbush *et al.*, 1992; Beukeboom and Werren, 1993), rye (Sandery *et al.*, 1990; Blunden *et al.*, 1993), *B. dichromosomatica* (John *et al.*, 1991; Houben *et al.*, 1997), maize (Alfenito and Birchler, 1993), the greater glider (*Petauroides volans*; McQuade *et al.*, 1994), *Drosophila subsilvestris* (Gutknecht *et al.*, 1995), and frog (*Leiopelma hochstetteri*; Sharbel *et al.*, 1998). It remains to be determined what proportion of a B chromosome is repetitive DNA; whether repetitive DNA is a general characteristic of all B chromosomes; whether these repeats are fundamental to the maintenance of B chromosomes from generation to generation; or whether these sequences in any way influence the phenotypes that Bs confer on the individuals carrying them.

1.6.1a *Nasonia vitripennis*

The paternal sex ratio (PSR) chromosome (Section 1.4.4) contains three families of PSR-specific, short, tandemly repeated sequences called *psr* 2, *psr* 18 and *psr* 22 (Nur *et al.*, 1988; Eikbush *et al.*, 1992). In addition a PSR-specific dispersed repeat with identity to long terminal repeat (LTR)-containing retrotransposons has also been isolated (McAllister, 1995).

Experiments aimed at determining the organisation of repeats and location of functional domains which cause the paternal chromosome destruction have shown that when key regions of PSR are deleted the chromosome becomes non-functional. All

complete deletions of the *psr* 22 repeat family are associated with functional loss suggesting that the functional domain of PSR maps closely to *psr* 22. Alternatively normal functioning may be dependent on the non-specific abundance of repeats on the chromosome (Beukeboom and Werren, 1993). Eikbush *et al.*, (1992) proposed two alternative mechanisms for PSR action; (1) the PSR may contain one or a few unique genes that code for a product (i.e. DNA binding protease or methylase) which prevents proper processing of paternal chromosomes; or (2) sequences on PSR may act as binding sites for a product required for paternal chromosome condensation and/or replication.

Despite considerable divergence, the families of PSR-specific repeats share two small palindromic DNA sequences which have been evolutionally conserved. A fourth family, *psr* 79, present on the autosomes and amplified on PSR, does not contain the palindromes (Eikbush *et al.*, 1992). Palindromic sequences have previously been shown to act as binding sites for proteins and the finding that all the PSR-specific repeats contain two highly conserved, palindromic segments suggests that the PSR-specific sequences are the functional domains of the PSR chromosome (Beukeboom and Werren, 1993).

? evolutionarily

Moreover, studies of junctions between *psr* 2 and *psr* 18 showed that all transitions between the repeats occurred near one or other of the palindrome regions, flanked by uniform blocks of each repeat type. These sequences could enhance recombination between repeat units and therefore may have been involved in the expansion of sequences (Reed *et al.*, 1994), rather than being directly involved in the functioning of the PSR chromosome.

1.6.1b *Brachycome dichromosomatica*

A *B. dichromosomatica* B-specific sequence, named Bd49, is a member of a family of high copy, tandemly repeated sequences with striking similarity (John *et al.*, 1991). Quantitative analysis gave an estimate of 1.8×10^5 copies of Bd49-like sequences per B chromosome, corresponding to approximately 10% of the B chromosome complement

(John *et al.*, 1991). Therefore, a significant proportion of the B chromosome of *B. dichromosomatica* consists of a single family of tandemly repeated, B-specific sequences. Junctions of Bd49-like sequence with neighbouring sequences were examined to determine whether particular regions of Bd49 were commonly involved at these sites (Franks *et al.*, 1996). These investigations yielded a result similar to that found in wasp, *N. vitripennis* (Eikbush *et al.*, 1992), in that three junctions were adjacent to an A/T-rich region in Bd49 and a fourth was close to a 25 base pair imperfect dyadic sequence (Franks *et al.*, 1996), suggesting common mechanisms for the expansion of arrays of repeat units in the widely different B chromosomes of plants and insects.

Fluorescence *in situ* hybridisation localised Bd49 to the centromeric region of the B chromosome (Leach *et al.*, 1995) suggesting a possible role for this sequence in centromere function. A search of GenBank DNA databases showed no plant sequences with significant homology to Bd49 (John *et al.*, 1991) and no functional role has been assigned to this sequence.

In addition to the standard B chromosome so far discussed, *B. dichromosomatica* also contains dot-like micro B chromosomes (Section 1.8; Carter and Smith-White, 1972). Members of a tandem repeat family called Bdm29 have been isolated from this micro B chromosome and corresponding sequences were also found in the larger standard B chromosome, in the Bs of other species within the genus, and at lower levels in the A chromosomes of *B. dichromosomatica* (Houben *et al.*, 1997). The existence of this sequence in high copy number on both types of B chromosomes in *B. dichromosomatica*, in addition to Bs in other species of *Brachycome*, suggests a possible role for this sequence in the functioning and/or evolution of the B chromosomes of *Brachycome*.

1.6.1c *Zea mays*

B specific repeat sequences in maize were isolated using differential screening methods and were subsequently localised to the centromere of the maize B chromosome using *in situ* hybridisation (Alfenito and Birchler, 1993). B chromosomes of maize undergo post-meiotic accumulation, via specific non-disjunction at the second pollen mitosis (Roman, 1948a, 1948b; Beckett, 1991; Rushe *et al.*, 1997). Translocation experiments have shown that the non-disjunction is at least partly controlled by the centromere of the B chromosome (Carlson, 1986), suggesting that the B-specific repeat sequences may be functionally involved in non-disjunction (Alfenito and Birchler, 1993; Rushe *et al.*, 1997).

Random amplified polymorphic DNA sequences (RAPDs) are DNA fragments amplified from genomic DNA by PCR using 10-base oligonucleotides as primers (Section 6.3). In maize, RAPD analysis enabled sequences specific to the Bs to be isolated by comparison of amplification products from 0B and +B templates. One B-specific RAPD isolated (pBGBM18.2) showed significant nucleic acid similarity with a maize retroviral element called PREM-1 (Stark *et al.*, 1996). The sequence did not hybridise to genomic DNA without B chromosomes and the levels of hybridisation to genomic DNA with Bs did not increase linearly in relation to the number of Bs, suggesting variation in copy number of the sequence between individual Bs (Stark *et al.*, 1996). PREM-1 elements are long terminal repeats of a family of putative retroelements, primarily transcribed during pollen development (Stark *et al.*, 1996). In maize, Bs are inherited through directed non-disjunction at the second pollen grain mitosis and it may be relevant that the activity of PREM-1 (and possibly the B-specific sequence pBGBM18.2) coincides with the stage at which non-disjunction of the B chromosomes occurs.

1.6.1d Organisation of Repeat Sequences on B Chromosomes

It is interesting that often the B centromere, which behaves differently to the A centromere, contains B-specific sequences (Donald *et al.*, 1995; Alfenito and Birchler, 1993). In general, short tandemly repeated sequences are located at heterochromatic, centromeric and telomeric regions of chromosomes (Lapitan, 1991; Charlesworth *et al.*, 1994). High copy number, tandemly repeated sequences from the B chromosomes of rye (Tsujimoto and Niwa, 1992; Cuadrado and Jouve, 1994; Wilkes *et al.*, 1995), maize (Alfenito and Birchler, 1993), wasp (Beukeboom and Werren, 1993), grasshopper (López-León *et al.*, 1994) and the greater glider (McQuade *et al.*, 1994) have also been assigned to the telomeres or centromeres of their respective B chromosomes.

Generally DNA amplification occurs during periods of genome instability (reviewed in Wintersberger, 1994). The presence of B specific repeats in a range of species suggests that the repetitive DNA may have been amplified during the stabilisation and evolution of the B chromosome, and the sequences may well be responsible for the special characteristics required for survival of the chromosomes. This hypothesis is supported by evidence from *D. subsilvestris* which has dot-like chromosomes as well as Bs (Gutknecht *et al.*, 1995). The repeat sequence, pSsP216, is limited to the centromere of dot-like chromosomes, which behave like normal A chromosomes during meiosis, whereas the B chromosomes are composed entirely of amplified pSsP216 and segregate randomly (Gutknecht *et al.*, 1995). It is therefore possible that the nucleotide sequence of pSsP216 and its tandemly repeated organisation carry information necessary for establishing a B chromosome-like structure.

1.6.2 Ribosomal RNA Genes

Another sequence commonly found on B chromosomes is that of the ribosomal RNA (rRNA) genes (reviewed by Green, 1990, and Jones, 1995; López-León *et al.*, 1991, 1995)

and these genes are probably the only ones of known function associated with these chromosomes.

1.6.2a Structure of rRNA Genes

Ribosomal RNA is a major constituent of the ribosome. The genes which code for the rRNAs are organised into units consisting of the regions encoding the 18S, 5.8S and 25S ribosomal RNAs, separated by internal transcribed spacer one (ITS1) and two (ITS2), and an extended intergenic spacer region (IGS)(Sollner-Webb and Tower, 1986; see Figure 3.1). The rRNA gene repeat units are arranged in tandem (often termed rDNA) and are located at one or a few A chromosome loci which are often cytologically visible as secondary constrictions at mitotic metaphase. The site for rDNA transcription, rRNA processing and ribosome biogenesis is the nucleolus and loci which contain active rRNA genes are referred to as nucleolar organising regions (NORs; Givens and Phillips, 1976). The nucleolus is therefore the cytologically visible consequence of the expression of the rRNA genes.

The 18S-5.8S-25S rRNA is transcribed by RNA polymerase I, the transcription regulatory sequences for which are located in the intergenic spacer between the 3' end of the 25S and the 5' end of the 18S of adjacent repeat units. Transcription of rRNA produces a transient 40S precursor molecule which is processed into the mature 18S, 5.8S and 25S ribosomal subunit RNA species (Sollner-Webb and Tower, 1986). The number of rRNA repeat units present on the A chromosomes varies between different species, and is often considerably greater than that required to sustain ribosome requirements and there is good evidence that many of them are not active (Kavanagh and Timmis, 1986; 1988; Rogers and Bendich, 1987a, 1987b). Greater activity of one NOR compared with another in the same cell is a phenomenon known as nucleolar dominance and is observed in many species containing multiple loci of rRNA genes. Nucleolar dominance in interspecific hybrids or

allopolyploid species is widespread, but the epigenetic mechanisms leading to the formation of a nucleolus around the rRNA genes of only one parent are poorly understood (see Chapter 4).

1.6.2b Ribosomal RNA Genes on B Chromosomes

In situ hybridisation detects rDNA sequences present in multiple clustered repeats whether they are transcribed or not, while silver molecules bind to proteins associated with recently synthesised rRNA (Hubbell, 1985). Visualisation of silver staining is therefore widely accepted to be indicative of the presence of active rRNA genes. These techniques have demonstrated that the B chromosomes of many species have either active nucleolus organising capacity (Green, 1990; Maluszynska and Schweizer, 1989; Friebe, 1989; Mabuchi, 1991) or contain sequences complementary to ribosomal RNA gene probes which are apparently no longer transcribed (Reed, 1993). In addition, it is claimed that some rRNA genes are inactive on the B but maintain the ability to be expressed. In *E. plorans* activity of B-rRNA is restored after fusion to an A chromosome (Cabrero *et al.*, 1987), suggesting that their inactivity on the B may be under similar control mechanisms to those involved in nucleolar dominance.

Fluorescence *in situ* hybridisation with labelled rDNA revealed the presence of an rDNA cluster on both the A and B chromosomes of *B. dichromosomatica* (Donald *et al.*, 1995). The patterns revealed by Southern hybridisation with an rDNA probe appeared identical in 0B and 2B genomic DNA after digestion with a variety of restriction enzymes, indicating that the B chromosome rDNA clusters are indistinguishable from the A chromosome in both restriction fragment length and DNA methylation status (Donald *et al.*, 1995). One interpretation of this result is that the ribosomal DNA cluster on this B chromosome is potentially an active NOR. Another possibility is that the technique was too

insensitive to detect B-specific variants above a large background of A chromosome rDNA.

It is not yet clear whether the rDNA clusters on B chromosomes serve a particular function, but it is possible that having a B chromosome with an active NOR may be selectively advantageous for the +B individual, due to an increase in its ability to meet requirements for ribosomes. However, it is considered that only a small proportion of the rRNA genes on the A chromosomes are transcriptionally active (reviewed in Rogers and Bendich, 1987a) and therefore it is unlikely that having a B with potentially active rDNA would be advantageous. In *E. plorans*, changes in the pattern of NOR activity on the A chromosomes have been associated with the presence of a B chromosome, suggesting that the B chromosome may exert some trans-regulatory effect on the A chromosomes. In the presence of a B chromosome with an active NOR the amount of NOR activity on the As, assessed by the frequency of chromosome associations with active NORs, decreased suggesting that a complex mechanism is involved in the regulation of NOR expression (Cabrero *et al.*, 1987).

Although silver staining is thought to represent nucleolus organising capacity, the presence of transcribed rDNA on B chromosomes has not been confirmed by direct molecular evidence in any species.

1.6.3 Sequences Involved in B chromosome Transmission

The sequences directly involved in the processes controlling non-disjunction and suppression of meiotic loss of the Bs have not been isolated. However, there is strong evidence that B chromosomes contain genetic information which controls their own transmission. Analysis of progeny from crosses between rye genotypes exhibiting different B chromosome transmission rates suggested that non-disjunction in rye is controlled by the B chromosome itself, perhaps by a major gene (Puertas *et al.*, 1993; Puertas *et al.*, 1998).

Evidence to support this suggestion was found when the B chromosomes of rye were transferred to other species, such as *Triticum aestivum* and *Secale vavilovii*, and the same rate of non-disjunction still occurred (Puertas *et al.*, 1993).

Translocation experiments in maize have consistently shown that control of B non-disjunction at the second pollen mitosis is localised to three regions on the B including the centromere, plus a fourth region which is involved in enhancing the frequency of non-disjunction (Carlson, 1986). Furthermore, B chromosomes that are univalent at meiosis often lag at anaphase I, or they may divide "mitotically" and subsequently at anaphase II the single chromatids may lag. Either way they will be excluded from the daughter cell, an event termed meiotic loss (Carlson and Roseman, 1992). Translocation experiments revealed that, in maize, the suppression of B chromosome meiotic loss is genetically controlled and the functional regions were localised to at least one proximal region and one distal region on the B chromosome (Carlson and Roseman, 1992).

However, control of B chromosome transmission cannot be entirely attributed to the B chromosomes in all species, and variation in B chromosome transmission rates has also been associated with differences in background genotypes in *M. maculatus* (Shaw and Hewitt, 1985; Shaw *et al.*, 1985), *Pseudococcus affinis* (Nur and Brett, 1985; 1987; 1988), rye (Romera *et al.*, 1991, Puertas *et al.*, 1993; Puertas *et al.*, 1998), and *A. speltoides* (Cebria *et al.*, 1994). It therefore appears that although B chromosome transmission is often controlled by the Bs themselves, it is also subject to genetic control by the host.

1.7 Origin of B Chromosomes

1.7.1 B chromosomes Originating from the Same Species

The origin of B chromosomes is currently unknown, although many possibilities have been put forward. The most likely source of B chromosomes is widely thought to be

the A chromosomes of the same species. The majority of the B chromosomes which have been examined contain sequences in common with the A chromosomes, supporting the origin of the Bs from the As. However, the common occurrence of highly repetitious sequences that are specific to the Bs does not offer obvious support for this theory of B origin but it is possible that these sequences are present on the A chromosomes in low or unique copies and are amplified during B chromosome development.

In rye, *in situ* hybridisation revealed that B chromosomes mostly contain sequences common to the A chromosome, except for a B-specific region at the telomere of the long arm of the B chromosome. Thus it is believed that at least part of the rye B chromosome may have come from the A chromosomes (Tsujimoto and Niwa, 1992; Blunden *et al.*, 1993). Similar experiments in *C. capillaris* indicated that the B in this species also consists predominantly of DNA sequences that are also present on the standard chromosomes (Jamilena *et al.*, 1994a), suggesting that the B chromosomes also arose from the A chromosomes.

RAPD-PCR (Section 1.6.1c) and genomic *in situ* hybridisation (GISH) have supported the origin of the B chromosome from the A chromosome in maize (Stark *et al.*, 1996). RAPD fragments amplified from genomic DNA with Bs and not from genomic DNA without Bs generally represented sequences on the A and B chromosomes, likely to be derived from dispersed repetitive sequences on the Bs which have not diverged from their A counterparts. In addition RAPD-PCR enabled isolation of a B-specific sequence, which shows significant nucleic acid similarity with a maize retroviral element called PREM-1 (Stark *et al.*, 1996; Section 1.6.1c). The presence of a PREM-1 related sequence on the Bs supports the theory that the B chromosomes originated from within the maize genome (Stark *et al.*, 1996) but the retroviral element could also have invaded the B after the chromosomes initial formation.

Total genomic DNA from 0B maize plants hybridised to almost the entire length of the B except for a small region at one end of the chromosome. This region is thought to be B-specific or to contain highly heterochromatic sequences which may be difficult for the probe to access (Stark *et al.*, 1996). Similarly, in *E. plorans*, fluorescent *in situ* hybridisation revealed that the B chromosome was almost completely composed of rDNA together with a 180 base pair sequence common to the standard and B chromosomes. The order of these sequences on the B, determined by double fluorescence *in situ* hybridisation, coincided with that on the X chromosome and not the As, suggesting that the B originated from the X of the same species (López-León *et al.*, 1994).

In the greater glider, hybridisation of micromanipulated and PCR-amplified B chromosome sequences occurred to both B and non-B chromosome containing genomic DNA, suggesting that a high proportion of the sequences on the Bs were also present on the autosomes in this species (McQuade *et al.*, 1994). Furthermore, *in situ* hybridisation of B chromosome sequences to the autosomes and X chromosome suggested that the chromosomes shared centromeric or pericentromeric sequences (McQuade *et al.*, 1994). The likely origin of these B chromosomes was therefore from within the standard chromosomes of the same species.

1.7.2 B chromosomes Originating from a Different Species

There is also evidence to suggest that the B chromosomes in some species are more closely related to the A chromosomes of a different species than to their "host" genome. Therefore they may have originated prior to divergence of a common ancestor into different species, or through inter-specific hybridisation followed by back crossing with maintenance of a relic chromosome. The presence of B-specific repeat sequences in a number of species (Section 1.6.1) makes this a possible explanation for the origin of B chromosomes.

In *B. dichromosomatica* the presence of the B-specific repeat Bd49 at high copy number in the genomes of other *Brachycome* species including *B. ciliaris* and *B. eriogona* suggests that the one of these species may have been the source of the B chromosome (John *et al.*, 1991; Leach *et al.*, 1995). However the presence of this sequence in low copy number on at least one *B. dichromosomatica* A chromosome allows for the possibility that the B chromosome in fact originated from within the species with which it is currently associated (Leach *et al.*, 1995) by a processes of amplification of low-copy sequences.

To test the hypothesis that the origin of B chromosomes involved inter-specific hybridisation in *Coix* L., Sapre and Despande (1987) constructed hybrids between two allopatric species, *Coix gigantea* and *Coix aquatica*. When one or two chromosomes from the *gigantea* species became incorporated as additional chromosomes into the *aquatica* genome, they showed B chromosome-like behaviour. Through screening *Coix* hybrid derivatives, it was clearly demonstrated that chromosomes which were either homologous, homoeologous and/or non-homologous could flow between species and subsequently establish themselves as B chromosomes. Sequence and hybridisation analysis of the PSR retrotransposon NATE in the wasp *N. vitripennis* (Section 1.4.4) also supported B chromosome origin through interspecific hybridisation, suggesting that the PSR chromosome originated from the closely related genus *Trichomalopsis* (McAllister, 1995; McAllister and Werren, 1997).

Overall strong evidence is available for both an inter- and intra-genomic origin of B chromosomes, and the varying structures and effects of B chromosomes in different organisms suggests that it is likely that the evolution of Bs has been a heterogeneous process.

1.8 *BRACHYCOME DICHROMOSOMATICA*

Brachycome dichromosomatica is an outbreeding, ephemeral native to Australia and belongs to the Compositae family (tribe Asteraceae; Figure 1.1A). It has two pairs of chromosomes (2n = 4; Figure 1.1B; Carter and Smith-White, 1972). The genus *Brachycome* is divided into two subgenera, and contains approximately 70 species. *B. dichromosomatica* is in the subgenus *Eubrachycome*, and was formerly known as Race A of *B. lineariloba* (Carter, 1978a). The species is not internally homogeneous and is made up of four cytodemes A_1, A_2, A_3 and A_4 (Smith-White and Carter, 1970) which may also be grouped as var. *alba* (A_3) and var. dichromosomatica (A_1, A_2 and A_4) based on their geographical distribution (Carter, 1978b). The cytodemes are distinguished from one another by chromosome interchanges, differences in chromatin condensation patterns at meiotic first prophase, and suppression or loss of the nucleolar organiser (Watanabe *et al.*, 1975). The following thesis will focus on cytodeme A_1 of *B. dichromosomatica.*

The number of submetacentric Bs naturally existing within *B. dichromosomatica* cytodeme A_1 varies from 0 to 3 (Figure 1.1B), but 4B plants have resulted from controlled crosses in greenhouse conditions. The B chromosomes in cytodeme A_1 are characteristically early condensing, contain a near terminal secondary constriction on the short arm and are mitotically stable as indicated by a constant number of Bs in root, shoot, and floral tissue (Carter, 1978a). The B chromosomes in this species have no exophenotypically obvious effects, but it has been shown that they affect chiasma frequency when present in increasing numbers (Clough, 1992).

Depending on the number of Bs present, meiotic behaviour in the first anaphase of meiosis in pollen mother cells can be irregular. When one B is present, a small proportion of the univalent Bs are lost. When two Bs are present, meiosis is usually regular. The two Bs pair and divide normally, with each of the four microspores per pollen mother cells,

Figure 1.1:

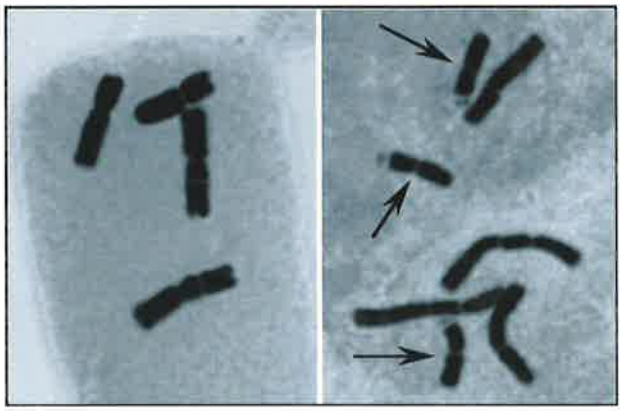
A. *B. dichromosomatica* an ephemeral annual, Australian native daisy.

B. Metaphase spreads of *B. dichromosomatica* root tips.

The karyotypes of the individual spreads are indicated and the B chromosomes are arrowed.

A

B



2n=4 2n=4+3B

receiving one B. When three Bs are present, it is thought that two of the Bs pair and that the remaining univalent is regularly lost, supported by a lack of plants with more than three Bs in the population (Smith-White and Carter, 1981).

First pollen grain division, which results in the formation of the generative and vegetative nuclei, involves a high percentage of non-disjunction and directed segregation of B chromosomes. A microspore with one B yields a generative nuclei with two B chromosomes. This nucleus then undergoes a second division to yield the male gamete nuclei (Smith-White and Carter, 1981). In embryo sacs there is no non-disjunction or preferential segregation of the B chromosomes, indicated in megaspores with one B yielding egg nuclei with one B.

In addition to the large, standard B chromosomes (4 μm in length) described above, dot-like micro B chromosomes which are less that 1 μm in length, are present in *B. dichromosomatica* (Carter and Smith-White, 1972). In contrast to the larger Bs, the micro Bs are mitotically unstable, varying in number in somatic tissues of an individual plant. Furthermore, the micro Bs do not pair with each other or with the As or the standard B chromosomes (Carter and Smith-White, 1972) and are visibly very condensed at all stages of the cell cycle and able to form double micro B chromosomes or chain-like formations at mitotic metaphase (Houben *et al.*, 1997b).

The micro B shares a number of DNA sequences with the A chromosomes, including telomere, rDNA sequences and other sequences common to the A and B chromosomes (Houben *et al.*, 1997b). In addition the micro Bs contain a highly repeated sequence (Bdm29) present also in lower copy number on the larger B chromosome, in other Bs within the genus and at very low levels on the A chromosomes (Houben *et al.*, 1997b; Section 1.6.1b). As both the micro Bs and the larger B chromosomes contain Bdm29 and

rDNA sequences it is possible that both types were derived from a common ancestral chromosome. Alternatively the micro B may represent a forerunner of the larger B into which it evolved by the amplification and addition of more DNA (Houben *et al.*, 1997b).

The work presented in this project concentrates on the standard B chromosomes of *B. dichromosomatica* cytodeme A_1.

1.9 Summary and Aims of Project

In diploid organisms, the gain or loss of chromosome regions, or complete chromosomes generally has severe, often lethal effects. The existence of additional DNA at high frequency in diploid organisms and its maintenance through generations, makes B chromosomes unique and of significant interest. Increased knowledge of the genomic quality of B chromosomes and the relationship of their DNA sequences to those on the A chromosomes will contribute in determining how B chromosomes are tolerated and maintained, and may shed light on the possible origin of these little-understood chromosomes.

B. dichromosomatica has many advantages as material for the study of B chromosomes. In addition to the strong background of information and the variety of plant material available, they have a low A chromosome number making them amenable to both cytological and molecular analysis.

The specific aims of this project were to use molecular techniques to determine whether DNA sequences present on the B chromosomes in *B. dichromosomatica* are transcribed. This goal was approached by characterising much of the rDNA repeat unit from the A chromosome and comparing the structure and sequence of this region with homologous sequences on the B chromosomes. Random amplification of polymorphic DNA (RAPD) PCR and amplified fragment length polymorphism (AFLP) were also used

in an attempt to isolate non-repetitive sequences specific to the B chromosomes of *B. dichromosomatica*.

Chapter 2:

Materials and Methods

2.1 MATERIALS

2.1.1 *B. dichromosomatica* Plant Material, DNA Samples and Genomic Library

Brachycome dichromosomatica seeds (2n=4 +1-3B) were provided by Associate Professor Jeremy Timmis and Dr. Carolyn Leach from stocks which have been collected from wild populations in three areas near Port Augusta in South Australia each year since 1989. In addition, some lineages from crosses between suitable chromosome types have been maintained in glasshouse conditions and the plants with 4 B chromosomes were obtained from these stocks. Seed from *Brachycome lineariloba* (race C) were provided by Dr. Carolyn Leach.

B. dichromosomatica genomic DNA samples used for AFLP and RAPD analysis were supplied by Dr. Andreas Houben. Genomic DNA samples from *B. lineariloba* race E and B, *B. breviscapis, B. tetrapterocarpa, B. curvicarpa*, *B. dentata*, *B. eriogona*, *B. parvula*, *B. multifida*, *B. segmentosa* and *B. ciliaris* were supplied by Dr. Carolyn Leach.

The *B. dichromosomatica* 3B genomic DNA library was constructed in λGEM-II™ (Promega) by Dr. Tricia Franks.

2.1.2 Chemical Reagents

General laboratory chemicals were of analytical research grade and were purchased from a variety of manufacturers including Aldrich Chemical Co, BDH Chemicals Australia Pty Ltd, Oxoid Pty Ltd and Sigma Chemical Company. Specialist reagents used in this study are listed below:

"Amberlite" monobed resin	BDH Chemicals, Australia
BSA	Boehringer Mannheim, Germany
β-mercaptoethanol	BDH Chemicals, Australia
Caesium chloride	BDH Chemicals, Australia
Chemicals for oligonucleotide synthesis	Beckman Instruments, Palo Alto, CA

DAPI	Sigma Chemical Co, St Louis, MO
DEPC	Sigma Chemical Co, St Louis, MO
Dextran sulphate	Progen Industries Ltd, Australia
Fluorescein streptavidin	Vector Laboratories, Inc. Burlingame, CA
Guanidine thiocyanate	Sigma Chemical Co, St Louis, MO
PEG 8000	Sigma Chemical Co, St Louis, MO
p-phenylenediamine	Sigma Chemical Co, St Louis, MO
Spermidine	Sigma Chemical Co, St Louis, MO
TEMED	BDH Chemicals, Australia
TRIzol® Reagent	Gibco BRL, Gaithersburg, MD
Urea	BDH Chemicals, Australia

2.1.3 Stains

Bromophenol blue	Sigma Chemical Co, St Louis, MO
Ethidium bromide	Sigma Chemical Co, St Louis, MO
Fuschin (basic)	BDH Chemicals, Australia

2.1.4 Enzymes

Restriction endonucleases (and 10 x restriction buffers) were obtained from Promega, New England Biolabs and Boehringer Mannheim. The remaining enzymes used in this study were obtained from the following manufacturers:

Calf Intestinal Phosphatase	Boehringer Mannheim, Germany
Cellulase "Onozuka R10"	Serva, Germany
DNase I (grade 1)	Boehringer Mannheim, Germany
DNase I (RNase free)	Boehringer Mannheim, Germany
Lysozyme	Sigma Chemical Co, St Louis, MO
M-MLV reverse transcriptase	Promega, Madison, WI
Pectolyase Y-23	Sigma Chemical Co, St Louis, MO
RNaseA	Sigma Chemical Co, St Louis, MO
RNasin®	Promega, Madison, WI
T_4 DNA ligase	New England Biolabs, Beverly, MA
T_4 polynucleotide kinase	Boehringer Mannheim, Germany
Taq DNA polymerase	Bresatec, Adelaide, Australia

2.1.5 Radioactive Isotopes

[α–^{32}P]dATP (3000 Ci/mmole; ethanolic)	Bresatec, Adelaide, Australia
[α–^{32}P]dATP (3000 Ci/mmole; Redivue)	Radiochemical Centre, Amersham, UK
[γ–^{32}P]dATP (4000 Ci/mmole; aqueous)	Bresatec, Adelaide, Australia

2.1.6 Nucleic Acids

A number of plasmid clones and oligonucleotides were used in this study and are presented in Table 2.1 and Table 2.4 respectively. In Table 2.4b recognition sites for the restriction enzymes *Eco* RI (GAATTC) and *Xba* I (TCTAGA) within the primers are underlined. In Table 2.4c selective nucleotides incorporated into AFLP primers are underlined. Primers used for RAPD analysis were provided by Dr. Andreas Houben. Molecular weight markers and other nucleic acids were purchased from the following suppliers:

DNA ladder, 100 bp	Promega, Madison, WI
DNA ladder, 100 bp	Bresatec, Adelaide, Australia
Lambda DNA restricted with *Hin*d III	Progen Industries Ltd, Australia
Salmon sperm DNA	Sigma Chemical Co, St Louis, MO
Unlabelled deoxyribonucleotides	Boehringer-Mannheim, Germany

2.1.7 Antibiotics and Indicators

Ampicillin	Boehringer-Mannheim, Germany
IPTG	Boehringer-Mannheim, Germany
Kanamycin	Sigma Chemical Co, St Louis, MO
Tetracycline	Sigma Chemical Co, St Louis, MO
X-gal	Progen Industries Ltd, Australia

2.1.8 Cloning Vectors

The plasmid and bacteriophage vectors used in this study are presented in Table 2.2.

2.1.9 Bacterial Strains

The bacterial strains used in this study are presented in Table 2.3.

Table 2.1: Plasmid clones

Plasmid	Insert	Vector	Location	Reference
pCU18	A 10 kb fragment of cucumber *Cucumis sativus* rDNA, including 25S, 18S genes and transcribed spacers	pACYC184	Chapter 5	Kavanagh and Timmis, 1986
pGhR1	Complete rDNA repeat unit of cotton *G. hirsutum* L.	pBluescript^{R}SK(-)	Chapter 3, 5	Orford, 1996

Table 2.2: Plasmid and bacteriophage vectors

Vector	Source	Use	Reference
Plasmids			
pGEM^{R}-T	Promega, Madison, WI	Cloning of PCR products	Robles and Doers, 1994
pGEM^{R}-T Easy	Promega, Madison, WI	Cloning of PCR products	Robles and Doers, 1994
Phagemids			
pBluescript^{R}SK(-)	Stratagene GmbH, Heidelburg, Germany (excision from λZAP^{R}II)	General cloning and subcloning vector	Short *et al.*, 1988
Phage			
λZAP^{R}II	λZAP^{R}II digested with *Eco*RI and dephosphorylaetd; Stratagene GmbH, Heidelburg, Germany	Cloning of rDNA from *B. dichromosomatica.*	Short *et al.*, 1988
ExAssist™ helper filamentous phage	Stratagene GmbH, Heidelburg, Germany	Rescue of phagemids from λZAP^{R}II vector	Hay and Short, 1992

Table 2.3: Bacterial strains (*Escherichia coli*)

E. coli Strain	Source	Use	Genotype and Reference
DH-5αF'	Laboratory stock	Host for cloning into pGEM®-T and general cloning	*recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17 (r_K^-, m_K^+), *supE*44, *relA*1, *deoR*, Δ(*lacZYA-argF*)U169 [F', F 80d*lacZ*ΔM15]; Hanahan, 1983
KW251	Promega, Madison, WI	Host for propagation of λGEM-II™ *B. dichromosomatica* 3B genomic library	*supE*44, *galK*2, *galT*22, *metB*1, *hsdR*2, *mcrB*1, *mcrA*, [*arg*A81:Tn10], *recD*1014
SOLR™	Stratagene GmbH, Heidelburg, Germany	Host for excision of phagemid from λZAP®II vector	e14⁻(*mcr*A), Δ(*mcrCB-hsdSMR-mrr*)171, *sbcC*, *recB*, *recJ*, *umuC*::Tn*5*(kan^r), *uvrC*, *lac*, *gyrA96*, *relA1*, *thi-1*, *endA1*, λ^R, [F' *proAB*, *lacI*^{q}ZΔ*M15*]Su⁻; Hay and Short, 1992
XL1-Blue	Stratagene GmbH, Heidelburg, Germany	Host for propagation of λZAP®II and general cloning	*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*, *supE44*, *relA1*, *lac*, [F' *proAB*, *lacI*^{q}Z Δ*M15*, Tn*10*(tet^r)]; Bullock *et al.*, 1987

Table 2.4a: Oligonucleotide primers to plasmid vector sequences

Primer	Sequence (5'→3')	Use	Location
T3	ATT AAC CCT CAC TAA AG	PCR primer for amplification of inserts in pBluescript® clones	Chapter 4, 5
T7	AAT ACG ACT CAC TAT AG	PCR primer for amplification of inserts in pBluescript®, pGEM®-T clones	Chapter 4, 5
SP6	TAT TTA GGT GAC ACT ATA G	PCR primer for amplification of pGEM®-T inserts	Chapter 6
RSP	AAC AGC TAT GAC CAT G	Sequencing of pBluescript® inserts	Chapters 3, 4, 5, 6
(-20)	GTA AAA CGA CGG CCA GT	Sequencing of pBluescript® inserts	Chapters 3, 4, 5, 6

Table 2.4b: Oligonucleotide primers designed from insert sequences

Primer	Sequence (5'→3')	Use	Location
Primers for Amplification Across ITS Regions			
XF18S	GCTCTAGAGGAAGGAGAACTCG	Amplification of ITS1 from the 3' end of 18S	Chapter 3, 4
ER58S	CTGAATTCGAGATATCCGTTGCC	Amplification of ITS1 from the 5' end of 58S	Chapter 3, 4
XF58S	CTTCTAGAGCCTGGGCGTCACG	Amplification of ITS2 from the 3' end of 58S	Chapter 3, 4
ER25S	CGGAATTCTGACCTGGGGTCGC	Amplification of ITS2 from the 5' end of 25S	Chapter 3, 4
BITS2	GCGACACACGACACGATTCT	Amplification from Sfc I site of ITS2 towards the 3' end of the 58S.	Chapter 4
Primers for Sequencing IGS			
Pd26r1	GGTCGTTTCCGAGCAAAAG	Sequencing of IGS within BdrD(2.5)	Chapter 5
Pd26r3	GTGTCACGACACCGCAAAAG	Sequencing of IGS within BdrD(2.5)	Chapter 5
Pd26f1	TGAACGCGTGGCCGTGCCTGC	Sequencing of IGS within BdrD(2.5)	Chapter 5
Pd26f3	GGGCACCACTCGGATGGGC	Sequencing of IGS within BdrD(2.5)	Chapter 5
Pd26f2	CTCGGGAGGGACTACCAAGC	Sequencing of IGS within BdrD(2.5)	Chapter 5
Primers for PCR Analysis of IGS			
PIGS1	TTCAGCCCTTTGTCGCTCCGATCC	Amplification of section of IGS from 3' end of 25S	Chapter 5
PIGS2	TTCCAACTCGAGCGTTGGGTTTCG	Amplification of section of IGS from within IGS towards 25S	Chapter 5
P18S	GCATATGTCTACTGGCAGG	Amplification of section of IGS from 5' end of 18S	Chapter 5
PIGS3	GACCCGAAAACCAACGCTCG	Amplification of section of IGS from within IGS towards 18S	Chapter 5

Table 2.4c: Oligonucleotide used in AFLP Analysis

Primer	Sequence (5'→3')	Use	Location
Adaptor Oligonucleotides			
*Pst*IA	CTCGTAGACTGCGTACATGCA	Oligonucleotide used to construct *Pst* I adaptor	Chapter 6
*Pst*IB	TGTACGCAGTCTAC	Oligonucleotide used to construct *Pst* I adaptor	Chapter 6
*Mse*IA	GACGATGAGTCCTGAG	Oligonucleotide used to construct *Mse* I adaptor	Chapter 6
*Mse*IB	TACTCAGGACTCAT	Oligonucleotide used to construct *Mse* I adaptor	Chapter 6
Primers for AFLP Preamplification			
*Mse*I+C	GATGAGTCCTGAGTAAC	Primer with one selective nucleotide used in AFLP pre-amplification	Chapter 6
*Pst*I+A	GACTGCGTACATGCAGA	Primer with one selective nucleotide used in AFLP pre-amplification	Chapter 6
Primers for AFLP Amplification			
*Mse*I+CAA	GATGAGTCCTGAGTAACAA	Primer with three selective nucleotides used in AFLP amplification	Chapter 6
*Mse*I+CAG	GATGAGTCCTGAGTAACAG	Primer with three selective nucleotides used in AFLP amplification	Chapter 6
*Mse*I+CAT	GATGAGTCCTGAGTAACAT	Primer with three selective nucleotides used in AFLP amplification	Chapter 6
*Mse*I+CCA	GATGAGTCCTGAGTAACCA	Primer with three selective nucleotides used in AFLP amplification	Chapter 6
*Mse*I+CCT	GATGAGTCCTGAGTAACCT	Primer with three selective nucleotides used in AFLP amplification	Chapter 6
*Mse*I+CGA	GATGAGTCCTGAGTAACGA	Primer with three selective nucleotides used in AFLP amplification	Chapter 6
*Pst*I+AA	GACTGCGTACATGCAGAA	Primer with two selective nucleotides used in AFLP amplification	Chapter 6
*Pst*I+AG	GACTGCGTACATGCAGAG	Primer with two selective nucleotides used in AFLP amplification	Chapter 6
Primers for Amplification of AFLP Clones			
P2aflp3-1	GCTTAACGATAGTGTGGAAAGGG	Amplification of 2aflp3 from p2aflp3 and genomic DNA	Chapter 6
P2aflp3-2	CTGCAGAATTCCCAAGTCGCGGAG	Amplification of 2aflp3 from p2aflp3 and genomic DNA	Chapter 6
P7aflp3-1	CGTTAACGATGAAGTAAATGATCGGGC	Amplification of 7aflp3 from p7aflp3 and genomic DNA	Chapter 6
P7aflp3-2	CTGCAGAAGTGTCGTTTATAGAAGGG	Amplification of 7aflp3 from p7aflp3 and genomic DNA	Chapter 6

2.1.10 Kits and Miscellaneous Materials

Kits and other materials used in this study were obtained from the following manufacturers:

ABI Prism Dye Terminator Ready reaction Mix.	Applied Biosystems-Perkin Elmer.
Anti-DIG-rhodamine Fab fragments	Boehringer-Mannheim, Germany
Bio-Gel P60 (50-100 mesh)	Bio-Rad Laboratories, Richmond, CA
Bio-Gel P60 (100-200 mesh)	Bio-Rad Laboratories, Richmond, CA
Biotinylated anti-streptavidin	Vector Laboratories
Bioprobe® Nick Translation DNA Labelling System	Sigma Chemical Co, St Louis, MO
DIG-Nick Translation Mix	Boehringer-Mannheim, Germany
DNA UltraFast Cleavage and Deprotection Kit	Beckman Instruments, Palo Alto, CA
Exassist™/SOLR™ System	Stratagene, La Jolla, CA
GIGAprime DNA Labelling Kit	Bresatec, Adelaide, Australia
Glass beads, 150-212 μm	Sigma Chemical Co, St Louis, MO
Hybond™-N+ nylon membrane	Amersham, UK
Packagene® Lambda DNA	Promega, Madison, WI
Packaging System	
pGEM®-T Vector System	Promega, Madison, WI
pGEM®-T Easy Vector System	Promega, Madison, WI
Rhodamine anti-sheep IgG	Jackson ImmunoResearch Laboratories Inc.
Sepharose CL-6B	Pharmacia, Uppsala, Sweden
Sequenase Version 2.0 DNA	United States Biochemical, Cleveland, OH
Sequencing Kit	
Spin-X® Centrifuge	Corning Incorporated, Corning, NY
Tube Filter, 0.45 μm	
Ultrafree®-MC 0.45 μm filter unit	Millipore, Bedford, MA
X-ray film	Fuji RX Medical X-ray Film, Fuji Photo Film Co, Ltd

2.2 Solutions, Buffers and Media

2.2.1 Solutions and Buffers

All solutions and buffers were prepared using millipore water and autoclaved where possible, otherwise were sterilised by passage through a 0.2 μm filter. Solutions for RNA work were treated with 0.1% (v/v) DEPC as per Sambrook *et al.* (1989) prior to autoclaving. All restriction endonucleases and most other enzymes were provided with incubation buffers. Solutions and all other buffers routinely used in this study were as follows:

Acrylamide stock	38% (w/v) acrylamide and 2% (w/v) bis-acrylamide, deionised with monobed resin
Denaturing solution	1.5 M NaCl, 0.5 M NaOH
1 x Denhardt's	0.02% (w/v) Ficoll®, 0.02% (w/v) PVP, 0.02% (w/v) gelatin
DNase I Reaction Buffer	0.1 M NaAc, 5 mM $MgSO_4$, pH 5.0
6 x DNA loading buffer	0.25% (w/v) bromophenol blue, 40% (w/v) sucrose
Feulgen Stain	0.4% (w/v) Fuschin (basic), 0.15 M HCl, 100 mM NaS_2O
Hybridisation buffer	5 x SSPE, 5 x Denhardt's, 0.5% (w/v) SDS, 100 mg/ml denatured, sonicated salmon sperm DNA
Lysing solution	10 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA, 4 mg/ml lysozyme
Neutralising solution	1.5 M NaCl, 1 M Tris-HCl pH 7.2, 1 mM EDTA
Phenol/Chloroform	50% (v/v) phenol, 48% (v/v) chloroform, 2% (v/v) isoamylalcohol, buffered with an equal volume of Tris-HCl pH 8.0, 0.2% (v/v) β-mercaptoethanol
SM buffer	100 mM NaCl, 10 mM $MgSO_4$, 50 mM Tris-HCl pH 7.5, 2% (w/v) gelatin

1 x SSC	0.15 M NaCl, 0.015 M Na_3-citrate, adjusted to pH 7.2 with NaOH
1 x SSPE	0.18 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA pH 7.2
10 x STE	100 mM Tris-HCl pH 8.0, 1 M NaCl, 10 mM EDTA
Stop buffer	10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0, 0.2% (w/v) SDS
1 x TAE	40 mM Tris-HCl, 20 mM Na-acetate, 2 mM EDTA, adjusted to pH 7.8 with glacial acetic acid
1 x TBE	0.9 M Tris-borate, 0.002 M EDTA pH 8.0
1 x TE	10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0

2.2.2 Media

All liquid and solid media were prepared using millipore water and sterilised immediately by autoclaving. The compositions of various media were as follows:

Luria broth (L-broth)	1% (w/v) tryptone, 1% (w/v) NaCl, 0.5% (w/v) yeast extract, adjusted to pH 7.2 with 1 M NaOH
Luria agar (L-agar)	Luria broth with the addition of 1% (w/v) bacteriological agar No. 1
NZY agar	1% (w/v) NZ amine (casein hydrolysate), 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, 1% (w/v) bacteriological agar No. 1
NZY top agar/agarose	1% (w/v) NZ amine (casein hydrolysate), 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, 10 mM $MgSO_4$, 0.7% (w/v) bacteriological agar No. 1 or agarose
TB agar	1% (w/v) tryptone, 0.5% (w/v) NaCl, 1% (w/v) bacteriological agar No. 1
TB top agar/agarose	1% (w/v) tryptone, 0.5% (w/v) NaCl, 0.01 M $MgSO_4$, 0.7% (w/v) bacteriological agar No. 1 or agarose

2.3 METHODS

2.3.1 Plant Growth Conditions

Brachycome dichromsomatica seeds were soaked in H_2O overnight. Using a dissecting microscope the embryo was separated intact from the hard coating and the surrounding integument.

Dissected embryos were stored on absorbent sponge soaked in water to germinate. After germination seedlings were transferred to potting compost in seedling trays, covered with a small vial, and placed in a controlled temperature growth cabinet. In a further 4 - 6 weeks seedlings were large enough for the collection of root tips and were transferred to larger pots in the glasshouse.

2.3.2 Karyotyping techniques

Root tips were cut from plants and placed in ice cold water overnight, then fixed in 3:1 ethanol:glacial acetic acid.

Fixed root tips were washed in H_2O for five mins and treated with HCl at 60°C for 10 mins. They were then rinsed in H_2O and stained with Feulgen Stain for at least 20 minutes. In order to prepare mitotic chromosomes, the root tips were placed between a slide and a coverslip in a drop of 45% acetic acid and squashed. The different cytodemes (A_1, A_2, A_3 and A_4) of *B. dichromosomatica* were distinguished on the basis of their chromosome morphology as described by Watanabe *et al.* (1975).

2.3.3 General Techniques

2.3.3a Restriction Enzyme Digests

All reactions involving restriction endonucleases were performed under conditions as recommended by the manufacturers. In general, five units of enzyme were added per µg of DNA to a maximum enzyme volume of 20% of the total reaction volume and the reaction

was allowed to proceed for 2-3 hr. RNaseA was added to a final concentration of 0.5 μg/μl to all restrictions. In genomic DNA digestions spermidine was also included to a final concentration of 3 mM. Samples of plant genomic DNA were restricted with 10 units of enzyme per μg of DNA for at least 16 hr.

2.3.3b Electrophoretic Separation of DNA on Agarose Gels

DNA was size fractionated by electrophoresis on 0.7% (w/v) agarose gels in submarine gel tanks with 1 x TAE as the running buffer. Amplified DNA samples were analysed by electrophoresis on 1.2-1.5% (w/v) agarose gels. Products resulting from *Sfc* I restriction of ITS2-PCR products were analysed by electrophoresis on 2% (w/v) agarose gels. Samples were loaded with 1 x loading buffer and electrophoresed at 60-80 V until the bromophenol blue marker dye had migrated a sufficient distance to allow adequate separation of the DNA fragments. Following electrophoresis, the DNA was stained in 5 μg/ml of ethidium bromide and viewed under UV light. Genomic DNA gels to be Southern blotted were electrophoresed at 30 V for at least 16 hr. Where applicable, sizes of DNA fragments were estimated using the Fragrap computer program (Duggleby *et al.*, 1981).

2.3.3c Recovery of DNA from Agarose Gels

DNA was eluted from agarose gels using the freeze-squeeze method adapted from Thuring *et al.* (1975). The frozen gel slice was squeezed through a 26G syringe needle into the upper compartment of an Ultrafree®-MC or Spin-X® Centrifuge Tube 0.45 μm filter and washed through with 200 μl of 1 x TAE buffer. The DNA was allowed to elute from the gel at room temperature for 15 min. Centrifugation for 2-3 min at 7000 x g yielded a solution of DNA in the lower tube compartment and the DNA was recovered by precipitation with ethanol.

2.3.3d Precipitation of DNA

Method 1 : Precipitation by Ethanol

DNA was precipitated by the addition of one tenth of the volume of 3M Na-acetate pH 5.2 followed by 2-2.5 volumes of cold redistilled ethanol. The DNA was left to precipitate at -20°C for a period of 2-16 hr, depending on its molecular weight. The DNA was recovered by centrifugation at 12000 x g for at least 15 min at 4°C. The pellet was washed with cold 70% (v/v) ethanol, dried under vacuum and resuspended in a minimal volume of 1 x TE.

Method 2 : Precipitation by Isopropanol

In some cases, DNA was precipitated by addition of one tenth of the volume of 3M Na-acetate pH 5.2 followed by an equal volume of cold isopropanol. The DNA was left to precipitate at room temperature for 5 min before microcentrifugation at 12000 x g for at least 15 min. The resulting DNA pellet was washed with cold 70% (v/v) ethanol, dried under vacuum and resuspended in a minimal volume of 1 x TE.

2.3.3e Purification of DNA Using Phenol/Chloroform Extraction

DNA was purified from proteins by the addition of an equal volume of phenol/chloroform and vigorous mixing. The emulsion was centrifuged at 12000 x g for 2 min and the aqueous layer transferred to a new tube before the extraction was repeated. Contaminating phenol was removed by the addition of an equal volume of chloroform, mixing and centrifugation as above, and the DNA solution transferred to a new tube.

2.3.3f Determination of DNA and RNA Concentration

The concentration of DNA and RNA in solution was determined by UV spectrophotometry at a wavelength of 260 nm or, in the case of freeze-squeezed restriction

fragments, by gel electrophoresis of a sample in conjunction with the molecular size markers lambda bacteriophage DNA restricted with *Hin*d III.

2.3.4 Preparation of DNA Samples

2.3.4a Isolation of Genomic DNA from Plants

Total genomic DNA from the leaves of *Brachycome dichromosomatica* was isolated by the method of Wienand and Feix (1980).

2.3.4b Small-scale Plasmid Preparation

Method 1 : Alkaline Lysis Plasmid Minipreps

Plasmid DNA for general applications was prepared using the scaled-up version of the alkaline lysis method (for 10 ml of overnight bacterial culture) as per Sambrook *et al.*(1989).

Method 2 : Boiling Lysis

Plasmid DNA for manual sequencing was prepared using the boiling lysis method of Holmes and Quigley (1981), scaled down for 1.5 ml of a 10 ml overnight bacterial culture.

2.3.5 Cloning Strategies

2.3.5a Preparation of Insert DNA and Plasmid Vector

DNA fragments for ligation were generated in one of two ways. DNA was restricted to completion with the appropriate restriction enzyme and then recovered from agarose gels using the freeze-squeeze method or purified by phenol/chloroform extraction and precipitation with ethanol.

Vector DNA for use in ligations was digested with the appropriate restriction enzyme, purified by extraction with phenol/chloroform and precipitated with ethanol. Completely digested plasmid DNA, analysed on an agarose gel, was treated with calf intestinal phosphatase (CIP) essentially as per Sambrook *et al.* (1989) in order to prevent

self-ligation. The 50 µl reaction was incubated at 37°C for 30 min, after which 10 µl 10 x STE, 5 µl 10% (v/v) SDS and 40 µl dH_2O were added and the mixture incubated at 68°C for 15 min to inactivate the enzyme. A phenol/chloroform extraction was carried out and the DNA precipitated by the addition of ethanol. The final concentration of the vector was 50 ng/µl.

2.3.5b Cloning of Products Generated by PCR

ITS1 and ITS2 PCR products were purified by centrifugation through a Sepharose CL-6B chromatography column (Murphy and Kavanagh, 1988) prior to restriction with *Eco*RI and *Xba* I, purification by phenol/chloroform extraction, precipitation with ethanol and cloning into pBluescript®SK(-). Other PCR products were cloned directly into pGEM®-T or pGEM®-T Easy following centrifugation through a Sepharose CL-6B chromatography column (Murphy and Kavanagh, 1988).

2.3.5c Ligations

Ligations were performed using 50-100 ng of vector DNA and an equimolar amount of insert DNA, catalysed by 1 Weiss unit of T_4 DNA ligase in a 10 µl reaction. All ligations were placed in a 7 L insulated waterbath at 16°C and left overnight in a 4°C coldroom, except for ligations involving pGEM®-T vectors, which were carried out as per manufacturer's instructions.

2.3.5d Preparation and Transformation of Competent *E. coli*

Preparation of DH5αF' and XLI-Blue competent cells for transformation was performed using the calcium chloride method as per Sambrook *et al.* (1989). Transformation of competent *E. coli* cells with ligation mixtures was achieved using heat shock at 42°C as per Sambrook *et al.* (1989). The cells were pelleted at 7000 x g for 30 sec, resuspended in 400 µl L-broth and plated onto L-agar plates supplemented with 100 µg/ml

ampicillin. Colonies containing recombinant plasmids were selected by plating onto L-agar medium containing 100 μg/ml ampicillin with an overlayer of L-agar plus 100 μg/ml ampicillin, 200 μg/ml X-gal and 160 μg/ml IPTG.

2.3.6 Preparation of RNA Samples

Total RNA was prepared from the leaf tissue of *B. dichromosomatica* and *B. lineariloba*, using the guanidine thiocyanate-CsCl method (Chirgwin *et al.*, 1979) or TRIzol® Reagent. Total RNA was prepared from the floral tissue of *B. dichromosomatica* and *B. lineariloba* using TRIzol® Reagent, as per manufacturer's instructions.

2.3.7 Phage Manipulation

2.3.7a Preparation of Plating Cells for Phage

Bacterial cells for plating of phage were prepared as per Stratagene instructions for the Predigested λZAP®II/*Eco*RI Cloning Kit and diluted to OD_{600} = 0.5 with 10 mM $MgSO_4$ before use.

2.3.7b Plating and Picking Phage

Phage and host cells were mixed and incubated at 37°C for 15-20 min prior to plating on the appropriate medium. Phage for the screening of libraries were plated on 150 mm plates using 600 μl of plating cells and 8 ml top agarose, melted to 48°C. Phage for secondary and tertiary screening were plated on 85 mm plates using 200 μl of plating cells and 3 ml top agar or agarose, melted to 48°C. Plates were incubated at 37°C for between 8 and 16 hr.

Positive plaques were aligned to the agar plates. Plaques were plugged into 0.5 ml SM buffer and 20 μl chloroform using the large end of a sterile pasteur pipette and the phage allowed to elute at room temperature for 2 hr or at 4°C overnight. Phage were plated

at low density on an 85 mm plate for secondary screening and the procedure repeated for a tertiary round of purification.

Following tertiary screening, recombinant pBluescript®SK(-) was rescued from λZAP®II using the ExAssist™/SOLR™ System, following manufacturer's instructions.

2.3.8 Transfer of DNA to Membranes

2.3.8a Southern Blotting

Following electrophoresis, DNA was transferred and alkali-fixed onto a Hybond™-N+ nylon membrane, following the instructions of the manufacturer.

2.3.8b Colony Hybridisation

Colony hybridisation was achieved using the method described by Grunstein and Hogness (1975). Recombinant bacterial colonies were patch plated onto an L-agar plate supplemented with 100 μg/ml ampicillin and replica plated onto a Hybond-N™+ membrane which overlayed another such plate. Following growth of bacterial colonies overnight at 37°C, the nylon filter was placed colony side up on a pad of three pieces of Whatman® 3MM paper soaked in lysing solution for 20 min, with blotting onto paper towel every 5 min. The filter was transferred to a fresh pad of 3MM paper soaked in denaturing solution plus 1% (w/v) SDS for a total of 20 min with blotting every 7 min. This procedure was repeated with 3MM soaked in neutralising solution before the filter was partially air-dried. Bacterial debris was removed from the filter using absorbent cotton wool and washing in 2 x SSC. The DNA was alkali-fixed onto the filters as per Amersham instructions.

2.3.9 Radiolabelling of DNA probes

2.3.9a Oligolabelling Using Random Oligonucleotides

DNA fragments requiring radioactive labelling were purified by size fractionation on an agarose gel and freeze-squeezing as previously described. Labelling of 50-200 ng of

double-stranded DNA was performed by primer extension of random oligonucleotides (Feinberg and Vogelstein, 1983) using the GIGAprime DNA labelling kit and [α-^{32}P]dATP.

2.3.9b Oligolabelling Using PCR

Reamplified AFLP products were labelled by PCR. Approximately 5 µl of PCR product was incubated with 3 µCi of [α-^{32}P]dATP using reaction conditions outlined in Section 2.3.15e for 15 cycles. The probe was denatured by incubation at 94°C for 5 mins before cooling on ice and addition to a 0.5 ml hybridisation volume.

2.3.9c RNA labelling

Prior to labelling, 2 µg of total RNA was incubated at 100°C for 3 mins then cooled on ice. The total RNA was end-labelled in 1 x kinase buffer in the presence of 50 µCi of [γ-^{32}P]dATP and 15 units of T4 polynucleotide kinase, in a total volume of 40 µl. The reaction was incubated at 37°C for 60 mins then the probe denatured at 90°C for 1 min before being added to a 0.5 ml hybridisation volume.

2.3.9d Removal of Unincorporated Nucleotides

Unincorporated nucleotides were removed from oligolabelled probes by spun column chromatography through Bio-Gel P60 resin columns made from 100 µl G50-100 beads and 400 µl G100-200 beads. Columns were packed and equilibrated with 100 µl stop buffer by centrifugation at 170 x g for 2 min. Labelled DNA fragments were eluted by adding the labelling reaction plus stop buffer (to a total volume of 100 µl) to the top of the column and recentrifugation at 170 x g for 2 min.

2.3.10 Prehybridisation, Hybridisation and Washing

2.3.10a Prehybridisation Conditions

All filters were prehybridised, hybridised and washed under the same conditions except that plaque lifts were separated by nylon grids and prehybridised for at least 16 hr. Filters were prehybridised in a solution of 5 x SSPE, 5 x Denhardt's, 0.5% (w/v) SDS and 100 μg/ml denatured sonicated salmon sperm DNA at 65°C for a minimum of 2 hr. The volume of prehybridisation solution was approximately 0.1 ml/cm^2 of filter.

2.3.10b Probe Denaturation and Hybridisation

Following addition of 300 μg sonicated salmon sperm DNA, the radiolabelled probe was denatured by the addition of 35 μl 1 M NaOH and incubation at room temperature for 10 min. The solution was neutralised by adding 35 μl 1 M HCl and the denatured probe stored on ice. Filters were hybridised by labelled probe at a concentration of approximately 1 x 10^6 cpm per ml of hybridisation solution (to a maximum volume of 6 ml) for a minimum of 16 hr at 65°C.

2.3.10c Washing of Filters

Filters were washed in a solution of 2 x SSC, 0.1% SDS at 65°C for 30 min, 1 x SSC, 0.1% SDS at 65°C for 30 min and 0.5 x SSC, 0.1% SDS at 65°C for a further 30 min. Southern blots subject to a high stringency wash were incubated in 0.1 x SSC, 0.1% SDS at 65°C for 2 hr.

2.3.10d Autoradiography, Phosphorimagery and Stripping of Filters

Filters were covered in plastic wrap to prevent drying out and exposed to X-ray film at -70°C for an appropriate period of time, backed by an intensifying screen (DuPont Hi-Plus). Filters probed with AFLP and RAPDs were often exposed to Fuji Imaging Plates (Fuji Photo Film, Co., Ltd) for periods of time ranging from 30 mins to overnight and

analysed using the MacBAS Version 2.X computer program (Fuji Photo Film, Co., Ltd). Quantification of the radioactive signals emitted from ITS2 PCR products before and after restriction with *Sfc* I, was achieved using MacBAS Version 2.X computer program (Fuji Photo Film, Co., Ltd).

Southern blots and plaque lifts were stripped of probe by incubating for 10 min in 0.1 x SSC, 1% SDS at 90°C. Stripped filters were rinsed in 2 x SSC and stored in plastic wrap at 4°C for rehybridisation.

2.3.11 Design and Synthesis of DNA Oligonucleotides

Oligonucleotide primers were synthesised on 30 nM columns using an Oligo100 DNA Synthesiser (Beckman) and purified by the method of Sawadogo and Van Dyke (1991) using a DNA Ultrafast Cleavage and Deprotection kit. Alternatively oligonucleotides were synthesised commercially (Bresatec). All oligonucleotides were adjusted to a concentration of 100 ng/μl for PCR or 10 μM for automated sequencing. Primers used for manual sequencing were further diluted to 1 μM. Annealing temperatures were calculated according to the formula $T_A = 2(A+T)+4(G+C)-5$ °C.

2.3.11a Primers for Sequencing

Oligonucleotide primers for sequencing were designed to unambiguous previously sequenced regions, priming towards a region of unknown sequence. They were generally synthesised as 20-mers at spacings of approximately 200 to 400 bp and contained a GC proportion of at least 50%.

2.3.11b Primers for PCR

Oligonucleotide primers for PCR were designed to contain the same proportion of purine and pyrimidine bases as that found in the target sequence. Efficient priming at high temperatures was maximised by the inclusion of two G or C residues at the 3' end of the

primers. In addition, primer pairs were checked at their 3' ends for the possibility of primer-dimer formation and intramolecular bonding using the Pcfold (Zuker, unpublished) and Molecule (Thompson, unpublished) computer programs.

2.3.12 Sequencing of Double-stranded DNA Templates

2.3.12a Preparation of Template DNA for Manual Sequencing

A 3 μg sample of the plasmid DNA to be sequenced was made up to 18 μl with dH_2O and 2 μl of 10 mg/ml RNaseA added before incubation at 37°C for 15 min. Following addition of 5 μl 1 M NaOH, 1 mM EDTA the solution was incubated for a further 15 min at 37°C. The denatured DNA was purified by centrifugation through a Sepharose CL-6B column (Murphy and Kavanagh, 1988), yielding 25 μl of single-stranded template DNA which was then stored on ice.

2.3.12b Preparation of Template DNA for Automated Sequencing

Plasmid DNA prepared using the alkaline lysis method was resuspended in 250 μl 1 x TE and incubated at room temperature for 10 mins with 1 μl of 10 mg/ml RNaseA. DNA was precipitated by the addition of 125 μl 30% PEG_{800}/1.5 M NaCl and the mixture incubated on ice for 30 mins. The DNA was pelleted by centrifugation at 12,000 x g at 4°C for 30 mins and washed with 95% ethanol before being drained, desiccated briefly and resuspended in 300 μl of 1 x TE.

The DNA was extracted sequentially with phenol, phenol/chloroform and chloroform and precipitated with one half the volume of 7.5 M NaAc and 2 volumes of 100% ethanol. The pellet was washed with 70% ethanol, vacuum dried and resuspended in 20 μl of 1 x TE.

2.3.12c Manual Sequencing Reactions

The dideoxy chain termination reaction (Sanger *et al.*, 1977) was performed using the Sequenase Version 2.0 kit and [α^{32}P]dATP, as described by the manufacturer of the kit. Where possible, sequence was confirmed by sequencing of both DNA strands.

2.3.12d Automated Sequencing Reactions

Automated sequencing reactions were performed using dye-terminator cycle sequencing reactions (Applied Biosystems-Perkin Elmer), according to the manufacturer's instructions. All automated sequencing (ABI Prism Model 377) was done at the Institute of Medical and Veterinary Science, Adelaide, South Australia.

2.3.12e Preparation of Sequencing Gels

A 4% (w/v) acrylamide, 8 M urea, 1 x TBE gel solution was made up to 100 ml with water. Following addition of 78 mg ammonium persulphate and 60 µl TEMED, the gel was mixed and poured between glass plates.

2.3.12f Denaturing Gel Electrophoresis

Manual sequencing reactions were run on a Model S2 sequencing gel electrophoresis system (BRL). Using 1 x TBE as the running buffer, the gel was pre-electrophoresed at 50 mA for 15 min. Following denaturation for 2 min at 90°C, 3 µl of each DNA sequencing reaction was loaded into the gel wells and electrophoresis continued until the tracking dye had reached the bottom of the gel plates. The gel was run between 30 mA and 50 mA but the voltage not allowed to exceed 1700 V. The loading process was repeated for medium- and short-length runs. Following electrophoresis, the sequencing gel was fixed in 20% (v/v) methanol and 10% (v/v) glacial acetic acid for 30 min to remove the urea. Finally, the gel was dried at 65°C for at least 3 hr and autoradiographed at room temperature.

2.3.12g Sequence Analysis Software

The compilation and comparison of multiple nucleotide sequences was performed using the facilities provided by the Australian National Genomic Information Service (ANGIS). Multiple sequence alignments were done using the Eclustalw program (Thompson *et al.*, 1994). FASTA was used to search the nucleotide databases (Altschul *et al.*, 1990; Gish and States, 1993). The repeat structure of the IGS sequences was determined using the DotPlot program (Maizel and Lenk, 1981)

2.3.13 Polymerase Chain Reaction (PCR)

Reaction mixtures (25 µl) for PCR contained 0.4 mM each of dATP, dCTP, dGTP and dTTP, 1 x PCR reaction buffer, 3 mM $MgCl_2$, 100 ng of each primer, 1.5 U of *Taq* DNA polymerase and 100 ng of template DNA. DNA was amplified in a programmable temperature controller (ARN Electronics) or a PTC-200 DNA engine (MJ Research, Inc.) generally programmed as follows: initial template denaturation was at 94°C for 2 min followed by 25 PCR cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min and extension at 72°C for 1 min. If the product was to be cloned, an additional cycle was carried out with an extension time of 7 min.

Annealing temperature varied depending on the primer pairs but was generally 45-55°C. Amplifications using BITS2 required annealing temperature of 70°C. The extension time also varied between different amplification reactions, according to the length of the fragment to be amplified (based on a synthesis rate of 1 kb per min).

2.3.14 Reverse Transcription (RT)

Prior to reverse transcription, 10 µg of total RNA was treated with DNase I (RNase free) to remove any contaminating DNA. Specifically, precipitated RNA was incubated in

1 x DNase I reaction buffer and 20 U of DNase I (RNase-free) for 30 mins at 25°C, and the reaction terminated by incubating at 95°C for 5 mins.

Reaction mixtures (30 µl) for reverse transcription contained 0.5 mM each of dATP, dCTP, dGTP and dTTP, 1 x reaction mix , 3.3 µM of primer ER25S, 13.3 U/µl M-MLV reverse transcriptase, RNasin® to 1 U/µl and approximately 10 µg of DNAse-treated total RNA. The reaction was incubated at 42°C for 60 mins, and terminated at 75°C for 10 mins. For RT-PCR 3 µl of the reverse transcription reaction was used and the reactions set up as for PCR from DNA templates.

2.3.15 Amplified Fragment Length Polymorphisms (AFLP)

2.3.15a Preparation of AFLP DNA Samples

Initially 2 µg of genomic DNA samples was digested with 30 U of both *Pst* I and *Mse* I in 1 x restriction-ligation buffer (100 mM Tris HAc, 100 mM MgAc, 500 mM KAc, 50 mM DTT, pH 7.5).

2.3.15b Preparation of Adaptors for AFLP

Oligonucleotides *Mse* IA (50 µM) and *Mse* IB (50 µM) were combined in a total volume of 20 µl, and *Pst* IA (5 µM) and *Pst* IB (5 µM) were combined in a total volume of 40 µl. To instigate annealing the oligonucleotide combinations were heated at 90°C for 3 mins and then allowed to anneal at room temperature for at least 30 mins.

2.3.15c Ligation of Adaptors to DNA

Adaptors were ligated with 1 µg of restricted DNA in 1 x restriction-ligation buffer with 0.2 mM ATP, 1 µl of *Mse*-adaptor mix, 1 µl of *Pst*-adaptor mix and catalysed by 1 Weiss unit of T_4 DNA ligase in 60 µl reaction mix for 3 hours at 37°C, then overnight at 4°C. The DNA was subsequently precipitated with ethanol and resuspended in 60 µl of 0.1 x TE.

2.3.15d AFLP Preamplification

Initial AFLP reactions were carried out with *Pst* I and *Mse* I adaptor-specific primers with one selective nucleotide (*Pst* I+A; *Mse* I+C). Pre-amplification reaction mixtures (25 µl) contained 0.2 mM each of dATP, dCTP, dGTP and dTTP, 1 x PCR reaction buffer, 1.5 mM $MgCl_2$, 50 ng of each primer, 1.5 U of *Taq* DNA polymerase and 4 µl of template DNA. DNA was amplified in a programmable temperature controller (ARN Electronics) programmed as follows: initial template denaturation was at 94°C for 2 min followed by 20 cycles of denaturation at 94°C for 30 secs, primer annealing at 56°C for 1 min and extension at 72°C for 1 min. Following amplification 100 µl of dH_2O was added to the reaction tubes and this was used as the template DNA for the amplification step following.

2.3.15e AFLP Amplification

AFLP amplifications employed two oligonucleotide primers one corresponding to the *Pst* I ends with two selective nucleotides (*Pst* I+AA or *Pst* I+AG) and one corresponding to the *Mse* I ends with three selective nucleotides (*Mse* I+CAA, *Mse* I+CAG, *Mse* I+CAT, *Mse* I+CCA, *Mse* I+CCT, or *Mse* I+CGA). Initially 50 ng of the *Pst* I primer was radioactively labelled with 10 µCi of [γ–^{32}P]dATP in 1 x kinase buffer and the reaction catalysed by 10 U of polynucleotide kinase at 37°C for 60 mins.

AFLP reactions (20 µl) contained 0.2 mM each of dATP, dCTP, dGTP and dTTP, 1 x *Taq* reaction buffer, 1.5 mM $MgCl_2$, 30 ng of *Mse* I+three nucleotides primer, 25 ng of *Pst* I+two nucleotides primer, 5 ng of *Pst* I+two nucleotides primer labelled with [γ–^{32}P]dATP, 1 U of *Taq* DNA polymerase and 2 µl of template DNA. DNA was amplified in a programmable temperature controller (ARN Electronics) programmed as follows: initial template denaturation was at 94°C for 2 min followed by one cycle of denaturation at 94°C for 30 secs, primer annealing at 65°C for 30 secs and extension at 72°C for 1 min. This cycle is followed by 9 cycles over which the annealing temperature is decreased by 1°C per

cycle, followed by 25 cycles of denaturation at 94°C for 30 secs, primer annealing at 56°C for 30 secs and extension at 72°C for 1 min.

AFLP reactions were run on 6% acrylamide gels prepared and run as for sequencing gels described in Section 2.3.12. Following electrophoresis, the gel was fixed and dried as for sequencing gels, and autoradiographed overnight at room temperature. Bands of interest were rehydrated with 1 x TE, excised from the gel and the DNA eluted by placing the gel piece in 10 µl of 1 x TE.

2.3.15f Reamplification of AFLP Fragments

AFLP fragments eluted from the gel were reamplified using the same PCR conditions as for the amplification step outlined above, with 1 µl of eluted DNA and unlabelled *Pst* I primer.

2.3.16 Random Amplification of Polymorphic DNA

RAPD reactions were performed according to the method of Williams *et al.*, (1990) with some modifications. Reaction volumes were 25 µl and contained of 2 units of *Taq* polymerase, 2.5 µl of 10 x reaction buffer (1 M Tris HCl pH 8.3, 1 M KCl, 1 M $MgCl_2$, 1 mg/ml gelatine), 0.8 mM each of dATP, dCTP, dGTP and dTTP, 1 µl of primer and approximately 50 ng of genomic DNA.

DNA was amplified in a PTC-200 DNA Engine (MJ Research, Inc.) generally programmed as follows: initial template denaturation was at 94°C for 2 min followed by 45 PCR cycles of denaturation at 94°C for 30 secs, primer annealing at 30°C for 30 secs and extension at 72°C for 2 mins. An additional cycle was included with an extension time of 7 mins. RAPDs of interest were extracted from the gel by freeze-squeezing and cloned directly in pGEM®-T Easy.

2.3.17 *In situ* hybridisation:

2.3.17a Preparation of probes

The probe BdrD(0.6) was labelled with biotin-dUTP using the Bioprobe Nick Translation DNA Labelling System as per manufacturer's instructions. BdrD(2.5) was labelled with DIG-dUTP using the DIG-Nick Translation Mix according the manufacturer's instructions.

For each chromosome sample to be hybridised, approximately 50 ng of the labelled probe was combined with 10 µg of sonicated salmon sperm DNA and made up to a volume of 20 µl with H_20. The DNA mix was then ethanol precipitated at -70°C for 30 mins.

2.3.17b Preparation of slides

Root tips were collected and fixed as for karyotyping techniques. Fixed root tips were then washed in H_2O at room temperature for 20 mins and the cell walls digested for 20 minutes at 37°C, using an enzyme mixture of 2.5% (w/v) pectolyase Y-23 plus 2.5% (w/v) Cellulase "Onozuka R10" in 75 mM KCl and 7.5 mM EDTA, pH 4.0 (Kakeda *et al.*, 1991). After washing with H_2O, single root tips were placed on a microscope slide in a drop of 45% acetic acid. A coverslip was then placed over the root meristem and tapped with a dissecting needle. Preparations were viewed using phase-contrast microscopy (Olympus).

2.3.17c Fixing of Slides

Slides of appropriate quality were frozen in liquid nitrogen and then the coverslip removed. Slides were air dried and dehydrated by washing in 70%, 95% and 100% ethanol each for 30 minutes at room temperature. Slides were stored at 4°C and used within one week of preparation.

2.3.17d RNase Treatment

Slides were treated with RNase A (100 μg/ml in 2 x SSC) in a 2 x SSC saturated atmosphere at 37°C for 40 mins, washed in 2 x SSC pH 7.0 two times at room temperature for five mins and dehydrated in 70%, 95% and 100% ethanol before air drying.

2.3.17e Denaturation

The chromosome DNA was denatured for 2 minutes in denaturation mix (70% deionized formamide, 2 x SSC pH 7.0) at 70°C, then re-dehydrated in 70% then 100% ethanol for 5 mins each at -20°C and air dried.

2.3.17f Hybridisation

The precipitated, dried DNA probe was resuspended in 6 μl of formamide per slide to be hybridised, shaking at room temperature for 15 mins, followed by the addition of an equal volume of 4 x SSC, 20 x dextran sulphate and shaking for a further 15 mins. The probe was then denatured at 95°C for 10 mins. Following chilling on ice, 12 μl of probe was added to each slide and hybridised overnight at 37°C, in a 2 x SSC saturated atmosphere.

Slides were then washed in 50% deionised formamide in 2 x SSC at 42°C (2 x 10 mins), and 2 x SSC pH 7.0 at 42°C (3 x 5 mins).

2.3.17g Signal Detection

Slides were pre-incubated by the addition of 100 μl of blocking solution containing 5% BSA, 0.2% Tween 20, 4 x SSC for 30 mins. Detection of hybridised biotin and DIG-labelled probes was achieved by incubating slides with fluorescein streptavidin (5 μg/ml) and anti-DIG-rhodamine Fab fragments (20 μg/ml) respectively in 5%BSA, 0.2% Tween 20, 4 x SSC for 60 mins at room temperature. For signal amplification the slides were washed in 0.2% Tween 20, 4 x SSC (3 x 5 mins), and treated with Biotinylated anti-

streptavidin (1 μg/ml) and Rhodamine anti-sheep IgG (0.015 mg/ml) in 1% BSA, 4 x SSC for 30 minutes then washed in 0.05% Tween 20, 4 x SSC (2 x 5 mins). The FITC signals were reamplified with fluorescein streptavidin (5 μg/ml) and the slides were washed in 4 x SSC, 0.1% Tween 20 (3 x 5 mins).

Chromosomes were counter stained by the addition of DAPI in antifade (10 mg/ml p-phenylenediamine, 90% (v/v) glycerol), and a coverslip applied. Preparations were examined by epi-fluorescence microscopy using an excitation wavelength of 450 - 490 nm. Photographs were taken using a CCD camera (Olympus) and manipulated using Adobe Photoshop™.

CHAPTER 3:

Sequence Analysis of Ribosomal DNA Internal Transcribed Spacers 1 and 2

3.1 INTRODUCTION

Previously, in *Brachycome dichromosomatica*, fluorescent *in situ* hybridisation revealed the presence of rRNA genes at a major cluster near the satellited end of the short arm of the B chromosomes and, to a lesser extent, proximally on the same arm (Donald *et al.*, 1995; Houben *et al.*, 1998). The apparent regular occurrence of rRNA genes on B chromosomes in many species (Section 1.6.2b; reviewed by Green, 1990; Jones, 1995), along with the fact that the terminal region of the B in *B. dichromosomatica* was observed to be associated with a nucleolus at mitotic prophase in some cells and frequently so associated at diplotene of meiosis (Donald *et al.*, 1995), suggested that the rRNA genes of the *B. dichromosomatica* B chromosome may be transcriptionally active (Donald *et al.*, 1995).

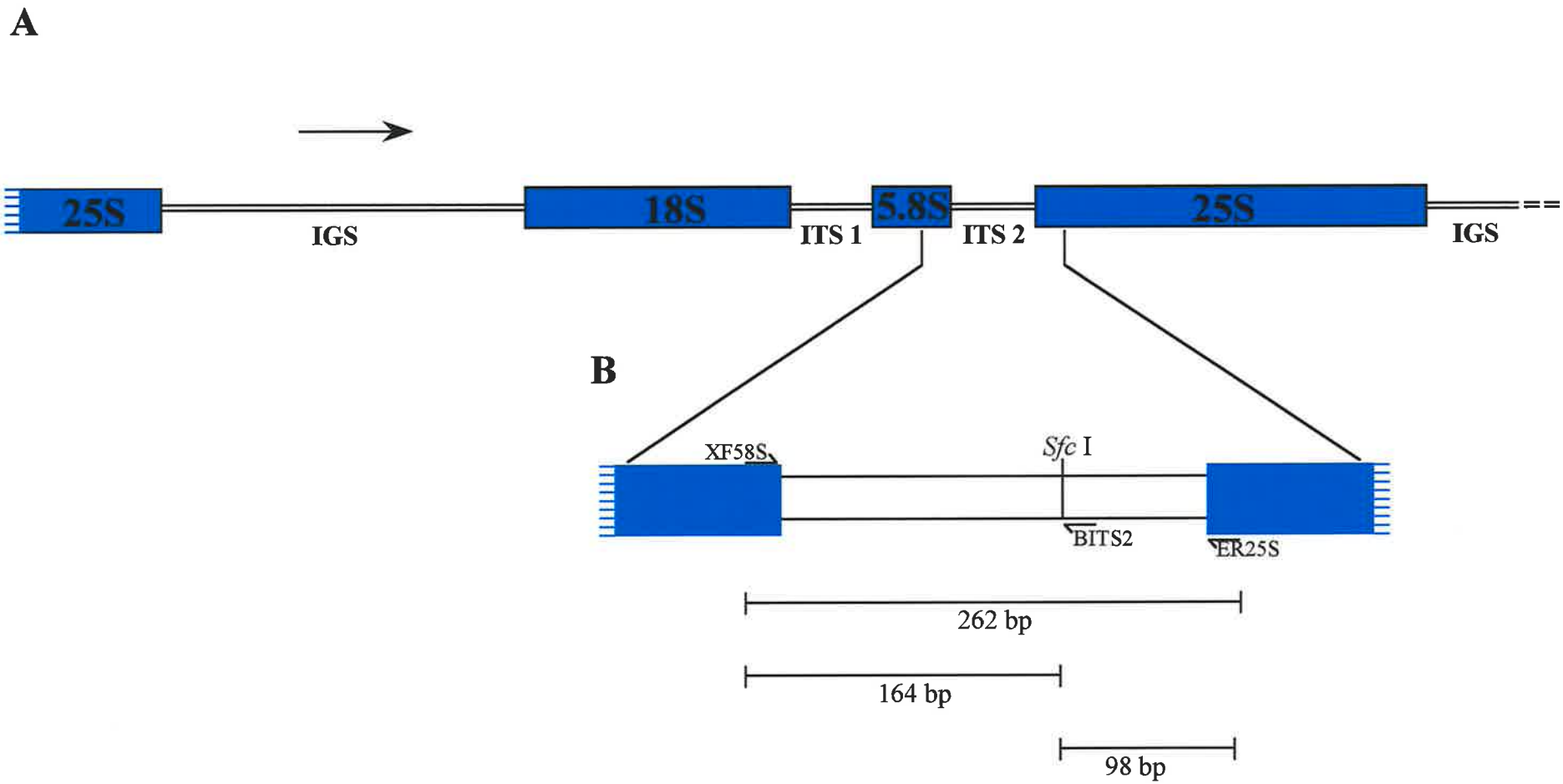
Individual rRNA gene repeat units consist of three regions which encode the mature 18S, 5.8S and 25S rRNAs. These regions are separated by internal transcribed spacer 1 (ITS1) and internal transcribed spacer 2 (ITS2). Included in each repeat unit, upstream of the 18S region, is an intergenic spacer (IGS) region that contains the RNA polymerase I promoter (Figure 3.1A, Section 1.6.2a).

The regions of the genes that encode the stable rRNA species of the ribosome are among the most conserved genomic sequences in evolution, whereas the transcribed and untranscribed spacers show high levels of sequence variation between different organisms, between closely related species (Kavanagh and Timmis, 1988; Arnheim, 1993; Polanco and de la Vega, 1994; Hershkovitz and Zimmer, 1996) and even within individuals of the same species (Kavanagh and Timmis, 1986; Ganley and Scott, 1998). Intragenomic rDNA diversity is however generally low and this low diversity reflects concerted evolution within and between ribosomal loci. Concerted evolution is the non-independent evolution of repetitive DNA sequences resulting in a sequence similarity of repeating units that is

Figure 3.1:

A. Organisation of the ribosomal RNA gene repeat unit (adapted from Kavanagh and Timmis, 1988). The 18S, 5.8S and 25S coding regions are indicated, separated by internal transcribed spacer 1 (ITS1) and internal transcribed spacer 2 (ITS2), and an extended intergenic spacer region (IGS). The arrow indicates the direction and approximate start point of rRNA transcription.

B. Detail of the ITS2 region with arrows showing position and orientation of primers XF58S, BITS2 and ER25S. The *Sfc* I site specific to B chromosome ITS2 sequences is indicated as well as the size of the PCR (262 bp) and *Sfc* I restriction products (164 bp and 98 bp).


A
25S
IGS
18S
ITS 1
5.8S
ITS 2
25S
IGS
B
XF58S
Sfc I
BITS2
ER25S
262 bp
164 bp
98 bp

greater within than among species, implying a homogenising mechanism which results in the evolution of such sequences in concert (Dover, 1982).

Due to concerted evolution there are no substantial differences in sequence between the several hundred to several thousand copies of the rDNA spacers within each species. However, comparisons between species show differences indicating that they are not under strong selection but free to diverge. There are also suggestions that concerted evolution can act between non-homologous chromosomes when there are loci for a multigene family on more than one pair of chromosomes within a species. In humans, for example, identity in rDNA sequences is maintained in five NOR regions positioned on non-homologous chromosome pairs (Jorgenson *et al.*, 1992; Dover, 1993).

Alignment of the ITS sequences from different angiosperm families indicated that plant sequences within ITS1 and ITS2 regions have diverged very rapidly at the nucleotide level compared with regions encoding the mature rRNA species (Baldwin *et al.*, 1995). The ITS regions have consequently been used extensively in phylogenetic studies of the most recent evolutionary events including inter- and intra-species comparisons (Baldwin *et al.*, 1995; Hershkovitz and Zimmer, 1996). The use of the rDNA ITS regions in phylogenetic studies has the advantage that within a species they are highly homogenised through concerted evolution but between species they show enough variation to enable resolution of species relationships. Therefore, despite the possibility of a homogenising mechanism, it was thought feasible that the internal transcribed spacer regions on the B chromosome rDNA could differ in sequence from those on the A chromosomes. Differences between the A and B chromosome rDNA could be attributed either to the B chromosome differing in sequence prior to its introduction into the *B. dichromosomatica* complement, or the independent divergence of ITS sequences on A and B chromosomes over time associated with a different selection on these regions and a separate or absent

concerted evolution mechanism for the B chromosome rDNA clusters. Described in this chapter is the sequence analysis undertaken in order to identify any variation between the internal transcribed spacer regions on the A and B chromosomes of *B. dichromosomatica.*

3.2 Analysis of the ITS 1 And ITS2 on the A and B Chromosomes.

A PCR approach was used to amplify ITS1 and ITS2 from both A and B chromosome rDNA, with primers designed using the melon ribosomal RNA sequence (Figure 3.2; Kavanagh and Timmis, 1988). Forward primers XF18S and XF58S (Table 2.4b) were designed specifically to the 3' ends of the 18S- and 5.8S-encoding regions respectively (Figure 3.1B, 3.2). The reverse primers ER58S and ER25S (Table 2.4b) were designed to amplify from the 5' ends of the 5.8S- and 25S-encoding regions respectively (Figure 3.1B, 3.2). Internal *Xba* I (XF58S, XF18S) and *Eco* RI (ER25S, ER58S) restriction sites were included in the primers (Table 2.4b) to facilitate cloning of the PCR products.

3.2.1 ITS Sequences Amplified from the A Chromosomes

Using the genomic DNA from *B. dichromosomatica* 0B plants as a template, XF18S and ER58S (Figure 3.1B, 3.2) amplified a fragment of approximately 340 bp in size, including primers, representing ITS1 (result not shown). The positive control template was a clone of the entire cotton ribosomal repeat unit (pGhR1; Orford, 1996), from which a fragment of approximately 400 bp was amplified (result not shown). The *B. dichromosomatica* products obtained were restricted with *Eco* RI and *Xba* I and cloned into pBluescript®SK(-). Clones were selected by hybridisation with the original ITS1 PCR product (Figure 3.3A, A'). Sequencing of positive ITS1 clones (Figure 3.3A') confirmed that the PCR products were significantly similar to the ITS1 of a closely related species of the Asteraceae, *Diplostephium rupestre*, which showed 93% identity at the nucleotide level (GenBank and EMBL, 1995).

```
AAGTCCACTG AACCTTATCA TTTAGAGGAA GGAGAAGTCG TAACAAGGTT TCCGTAGGTG
                                XF18S                                  18S

AACTGCGGAA GGATCATTGt cgatgcctaa acatcaaacg acccgcgaac gcgtttaaaa

acaaactgtt cgcgttaggg gcgggggaa gcatgctctt tggctgcctc ctccccttcc
                                                                       ITS1
aacgcgttta aacaaaaccc cgcgcaggtc gcgccaagga acttgaaatg aattcgcctg

tcccctgccc cggcctcggc gtgcggggga tggagcattc tagtcgtatt actaaCAACG

ACTCTCGGCA ACGGATATCT CGGCTCTCGC ATCGATGAAG AACGTAGCGA AATGCGATAC
        ER58S                                                          5.8S
TTGGTGTGAA TTGCAGGATC CCGCGAACCA CCGAGTCTTT GAACGCAAGT TGCGCCCGGA

GCCTTCTGGC CGAGGGCACG TCTGCCTGGG CGTCACGCat cgctgccccc accacacaac
                                XF58S
tctccccatg cggggtcgtt gtgaaggcag ggacacacac tggcctcccg tacgcaccgt

cgtgcggatg gcttaaattt gagtcctcga tgctcgtcgt cgcgacacta cggtggttga
                                                                       ITS2
ttcaactcgg tgacgcgtct cgacctcgac gtcgacttca cggactcctt cacgaccctt

cgaacgccgc cccttaaaag gacgacgctc tcgacGCGAC CCCAGGTCAG GCGGGACTAC
                                        ER25S                          25S
CCGTGAGAGT TTAAGCATAT CAATAAGCGG AGGAAAAGAA ACTTACAAGG ATTCCCCTAG
```

Figure 3.2:

Nucleotide sequence of melon 18S-25S region adapted from Kavanagh and Timmis (1988). The internal transcribed spacer regions 1 (ITS1) and 2 (ITS2) are shown in lower case and indicated on the right of the figure. The regions to which the XF18S, ER58S, XF58S and ER25S primers were designed are underlined. The 5' - 3' orientation of each primer is indicated by the direction of the arrows.

Figure 3.3:

A. Agarose gel electrophoresis of putative ITS1 clones derived from 0B genomic DNA (pBdITS1A1-9).

Each lane contains approximately 2 μg of plasmid DNA restricted with *Eco* RI and *Xba* I. The numbers on the left of the gel represent the molecular sizes (kb) of lambda bacteriophage DNA restricted with *Hin*d III. The identity of the clone in each lane is indicated.

A'. Hybridisation of a Southern blot of the gel in A with the original ITS1 PCR product.

B. Agarose gel electrophoresis of putative ITS2 clones derived from 0B genomic DNA (pBdITS2A1-12).

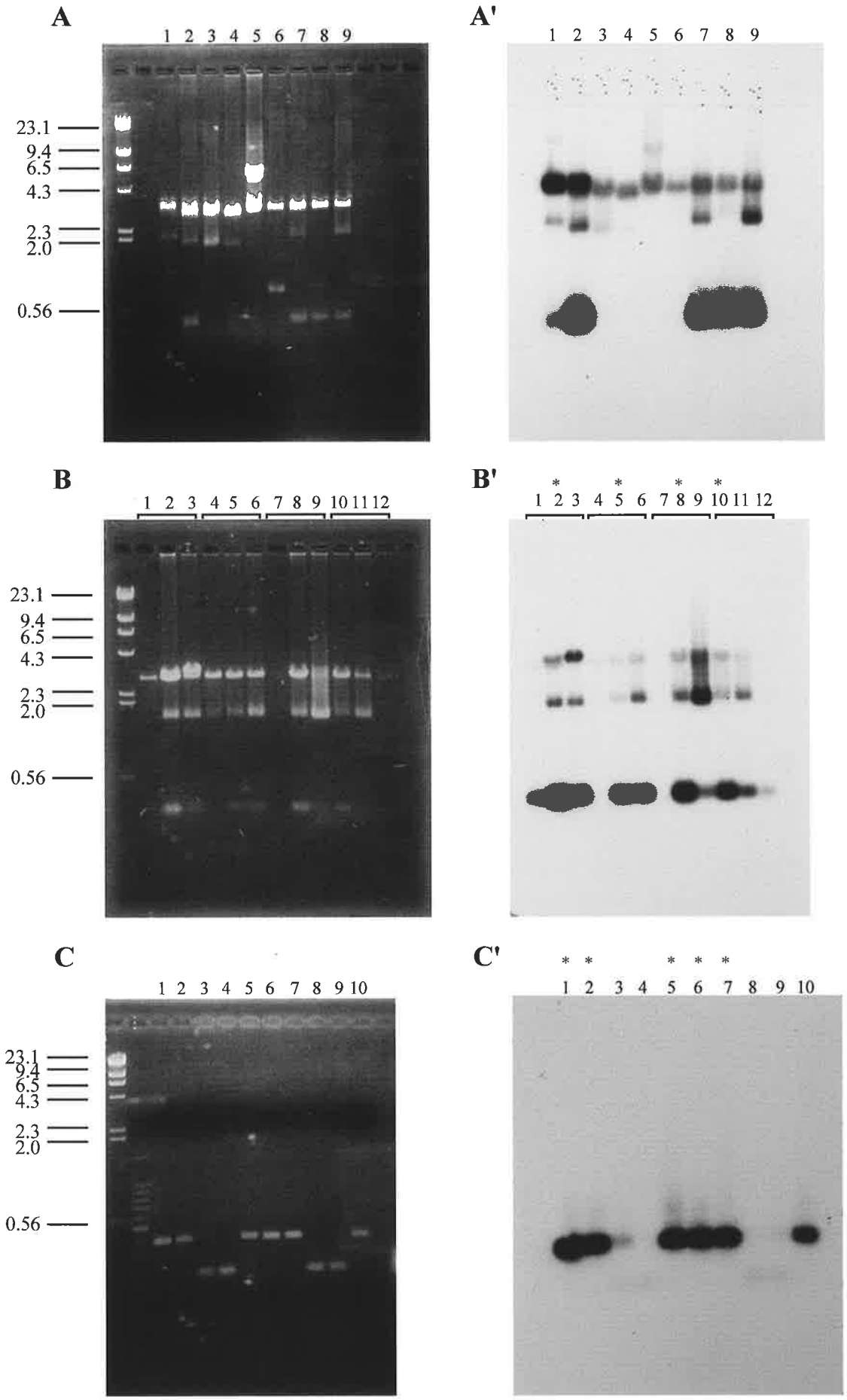
Each lane contains approximately 2μg of recombinant plasmid DNA restricted with *Eco* RI and *Xba* I . The numbers on the left of the gel represent the molecular sizes (kb) of lambda bacteriophage DNA restricted with *Hin*d III. The identity of the clone in each lane is indicated.

B'. Hybridisation of a Southern blot of the gel in B with the original ITS2 PCR product amplified from genomic DNA. The asterisks indicate the clones from which the inserts were sequenced (Figure 3.4).

C. Agarose gel electrophoresis of PCR products resulting from amplification across the insert of individual putative ITS2 clones derived from DOP-PCR-amplified microdissected B chromosome DNA (pBdITS2B1-10).

Each lane was loaded with 10 µl of a 25 µl reaction volume. The numbers on the left of the gel represent the molecular sizes (kb) of lambda bacteriophage DNA restricted with *Hin*d III and the second lane contains molecular size markers (100bp DNA ladder) consisting of fragments that range in size from 100 bp to 1000 bp in 100 bp increments, plus an additional 1500 bp fragment. The identity of the template clone in each lane is indicated.

C'. Hybridisation of a Southern blot of the gel in C with the ITS2 PCR product from DOP-PCR amplified microdissected B chromosome DNA. The asterisks indicate the clones from which the inserts were sequenced (Figure 3.4).


A
1 2 3 4 5 6 7 8 9
23.1
9.4
6.5
4.3
2.3
2.0
0.56
A'
1 2 3 4 5 6 7 8 9
B
1 2 3 4 5 6 7 8 9 10 11 12
23.1
9.4
6.5
4.3
2.3
2.0
0.56
B'
* * * *
1 2 3 4 5 6 7 8 9 10 11 12
C
1 2 3 4 5 6 7 8 9 10
23.1
9.4
6.5
4.3
2.3
2.0
0.56
C'
* * * * *
1 2 3 4 5 6 7 8 9 10

Primers XF58S and ER25S (Figure 3.1B, 3.2) amplified a product of approximately 280 bp from the cotton rDNA clone pGhR1 and a 260 bp product from DNA prepared from a range of *B. dichromosomatica* plants without B chromosomes (result not shown). The putative ITS2 PCR products from the different 0B *B. dichromosomatica* plants were cloned and recombinant clones selected by hybridisation with the original ITS2 PCR product (Figure 3.3B, B'). Sequence of the positive clone pBdITS2A2 (Figure 3.3B, B'; lane 2) confirmed that the product obtained was the ITS2 of *B. dichromosomatica*. The most closely related sequences in the database was found within another member of the Asteraceae, *Colotis dentex*, and showed 91% nucleotide identity across the amplified region (GenBank and EMBL, 1996).

Alignment of the nucleotide sequences obtained from clones derived from genomic DNA of four distinct 0B plants (Figure 3.3B, B'; lanes 2, 5, 8 and 10), and therefore representative of the A chromosome ITS2 sequences, revealed a low level of sequence heterogeneity between the individual 0B clones. Of the total 218 base pairs five bases differed in one or more individual clones (Figure 3.4), representing 2.3% variation. The sequence discrepancies probably represent genuine polymorphisms in the A chromosome ITS2, or alternatively some or all may be due to PCR errors.

3.2.2 ITS Sequences Amplified from the B Chromosomes

The availability of B chromosomes only within an A chromosome background makes it difficult to specifically analyse sequences on the B chromosomes, especially when they are similar in sequence to counterparts on the A chromosomes, as is the case for rDNA. There was therefore a requirement for a pure B chromosome DNA source and this instigated the use of microdissection to isolate individual B chromosomes which were subsequently amplified according to Pich *et al.* (1994), using the degenerate

Figure 3.4:

Alignment of ITS2 nucleotide sequences from BdITS2A clones (A2, A5, A8 and A10) with BdITS2B clones (B1, B2, B5, B6 and B7) and a single *B. lineariloba* clone (*Bl*). Consistent differences between the ITS2 of A and B chromosome derived clones are boxed. The *Sfc* I site (CTACAG) present in the BdITS2B and *B. lineariloba* sequences is highlighted and polymorphisms specific to individual clones are indicated.

```
                                                                    50
A2    ATCGCGTCGC CCCCCACCGT TCTTTCCTTC GGGATGATT- GGCTGGGGGC
A5    ATCGCGTCGC CCCCCACCAT TCTTTCCTTC GGGATGATT- GGCTGGGGGC
A8    ATCGCGTCGC CCCCCACCAT TCTTTCCTTC GGGATGATT- GGCTGGGGGC
A10   ATCGCGTCGC CCCCCACCAT TCTTTCCTTC GGGATGATT- GGCTGGGGGC
B1    ATCGCGTCGC CCCCCACCAT TCTTTCCTTC GGGATGATT- GGCTGGGGGC
B2    ATCGCGTCGC CCCCCACCAT TCTTTCCTTC GGGATGATT- GGCTGGGGGC
B5    ATCGCGTCGC CCCCCACCAT TCTTTCCTTC GGGATGATTA GGCTGGGGGC
B6    ATCGCGTCGC CCCCCACCAT TCTTTCCTTC GGGATGATT- GGCTGGGGGC
B7    ATCGCGTCGC CCCCCACCAT TCTTTCCTTC GGGATGATT- GGCTGGGGGC
Bl    ATCGCGTCGC TCCCCACCAT TCAATCCGTC AGGATGATT- GGCCGGGGGC

                                                                   100
A2    GGATACTGGT CTCCCGTTCC TCACCGAGCG GTTGGCCAAA ACAAGAGTCC
A5    GGATACTGGT CTCCCGTTCC TCACCGAGCG GTTGGCCAAA ACAAGAGTCC
A8    GGATACTGGT CTCCCGTTCC TCACCGAGCG GTTGGCCAAA ACAAGAGTCC
A10   GGATACTGGT CTCCCGTTCC TCACCGAGCG GTTGGCCAAA ACAAGAGTCC
B1    GGATACTGGT CTCCCGTTCC TCACCGAGCG GTTGGCCAAA ACAAGAGTCC
B2    GGATACTGGT CTCCCGTTCC TCACCGAGCG GTTGGCCAAA ACAAGAGTCC
B5    GGATACTGGT CTCCCGTTCC TCACCGAGCG GTTGGCCAAA ACAAGAGTCC
B6    GGATACTGGT CTCCCGTTCC TCACCGAGCG GTTGGCCAAA ACAAGAGTCC
B7    GGATACTGGT CTCCCGTTCC TCACCGAGCG GTTGGCCAAA ACAAGAGTCC
Bl    GGATACTGGT CTCCCGTTCC TCACCGAGCG GTTGGCCAAA ACAAGAGTCC

                                                                   150
A2    CCTTTGAAGG ATGCACGACT AGTGGTGGTT GACAAAACTA CGGAATCGTG
A5    CTTTTGAAGG ATGCACGACT AGTGGTGGTT GACAAAACTA CGGAATCGTG
A8    CTTTTGAAGG ATGCACGACT AGTGGTGGTT GACAAAACTA CGGAATCGTG
A10   CTTTTGAAGG ATGCACGACT AGTGGTGGTT GACAAAACTA CGGAATCGTG
B1    CTTTTGAAGG ATGCACGACT AGTGGTGGTT GACAAGACTA CAGAATCGTG
B2    CTTTTGAAGG ATGCACGACT AGTGGTGGTT GACAAAACTA CAGAATCGTG
B5    CTTTTGAAGG ATGCACGACT AGTGGTGGTT GACAAAACTA CAGAATCGTG
B6    CTTTTGAAGG ATGCACGACT AGTGGTGGTT GACAAAACTA CAGAATCGTG
B7    CTTTTGAAGG ATGCACGACT AGTGGTGGTT GACAAAACTA CAGAATCGTG
Bl    CTTTTGAAGG ATGCACGACT AGTGGTGGTT GACAAAACTA CAGAATCGTG

                                                                   200
A2    TCGTGTGTCT TTTCAAAAAG GTGCTTCTTA ATAGACCCAA CGTGTTGTCA
A5    TCGTGCGTCT TTTCAAAAAG GTGCTTCTTA ATAGACCCAA CGTGTTGTCA
A8    TCGTGTGTCT CTTCAAAAAG GTGCTTCTTA ATAGACCCAA TGTGTTGTCA
A10   TCGTGCGTCT CTTCAAAAAG GTGCTTCTTA ATAGACCCAA TGTGTTGTCA
B1    TCGTGTGTCT TTTCAAAAAG GTGCATCTTA ATAGACCCAA CGTGTTGTCA
B2    TCGTGTGTCT TTTCAAAAAG GTGCATCTTA ATAGACCCAA CGTGTTGTCA
B5    TCGTGTGTCT TTTCAAAAAG GTGCATCTTA ATAGACCCAA CGTGTTGTCA
B6    TCGTGTGTCT TTTCAAAAAG GTGCATCTTA ATAGACCCAA CGTGTTGTCA
B7    TCGTGTGTCT TTTCAAAAAG GTGCATCTTA ATAGACCCAA CGTGTTGTCA
Bl    TCGCGTGTCT TTTATAAAAG GTGCATCTTA ATAGACCCAA CGTGTTGTCG

                         218
A2    TTTGACAACG CTTCGACC
A5    TTTGACAACG CTTCGACC
A8    TTTGACAACG CTTCGACC
A10   TTTGACAACG CTTCGACC
B1    TTTGACAACG CTTCGACC
B2    TTTGACAACG CTTCGACC
B5    TTTGACAACG CTTCGACC
B6    TTTGACAACG CTTCGACC
B7    TTTGACAACG CTTCGACC
Bl    TTTGACAACG CTTCGACC
```

oligonucleotide primer (DOP) MW-6 technique (Telenius *et al.*, 1992). Attempts to amplify the ITS1 directly from microdissected B chromosome DNA using primers XF18S and ER58S were unsuccessful suggesting that this region was either not complete or unrepresented in the DOP-PCR-amplified DNA, possibly due to its large size. Alternatively it may have been unrepresented in the template because of poor amplification by the DOP primers. However, a similar PCR approach, using the ITS2 specific primers XF58S and ER25S, successfully amplified a product from the DOP-PCR-microdissected B chromosome DNA which was the same size as that obtained from 0B DNA. Products resulting from several individual PCRs were cloned, as described previously, and recombinant clones selected by hybridisation with the ITS2 PCR product amplified from DOP-PCR amplified microdissected B chromosome DNA (Figure 3.3C, C').

A comparison of the A chromosome ITS2 sequences with five individual inserts from microdissected, DOP-PCR-amplified B chromosome clones (Figure 3.3C, C') revealed an additional three sequence variants, two of which were consistently different between the A and B chromosome sequences (Figure 3.4). At base 142, an A was present in each B chromosome sequence whereas a G was present in all A chromosome ITS2 clones. In addition, at base 175 an A was present in B chromosome-derived ITS2 clones compared with a T in those from the A chromosomes (Figure 3.4). Fortuitously the substitution at base 142 introduced a restriction site for the enzyme *Sfc* I (CTACAG) in the B chromosome ITS2 sequence which was not present on the corresponding A chromosome sequences (Figure 3.4).

3.3 Analysis of the ITS2 in 0B and +B Genomic DNA

To determine whether the *Sfc* I polymorphism was a regular difference between the A and B chromosome rDNA clusters, and not a cloning or PCR-based artifact, PCR of the

ITS2 using XF58S and ER25S primers was performed using genomic DNA from two different 0B as well as individual 1B, 2B and 3B plants. The positive controls in this experiment were provided by the inclusion of template DNA from a B chromosome-derived ITS2 clone of known sequence (pBdITS2B1, Figure 3.4) and microdissected-B chromosome DNA, which were amplified in parallel. The negative controls were an A chromosome-derived clone of known sequence (pBdITS2A2, Figure 3.4) and a reaction lacking template included to check for contamination of the components of the PCR reaction. A 260 bp product, representing the ITS2 region, was amplified from each genomic DNA sample and from the microdissected-B chromosome templates, as well as from ITS2 clones of A and B chromosome derivation (Figure 3.5). The absence of a PCR product in the control reaction (Figure 3.5) confirmed that the experimental reaction was specific and contained no contaminant DNA. The PCR products obtained were restricted with *Sfc* I and visualised on 1.8% agarose gels alongside their unrestricted counterparts (Figure 3.5).

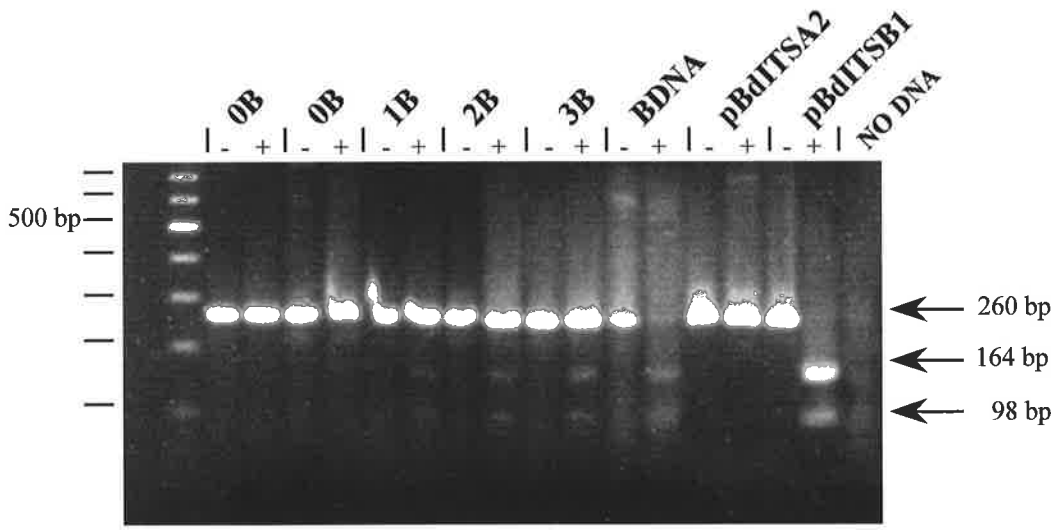
A proportion of the PCR products amplified from 1B, 2B and 3B plants was digested with *Sfc* I (Figure 3.5) and the amount of 164 and 98 bp restriction products appeared to increase additively with the number of B chromosomes present in the sample. The 260 bp amplicon from 0B samples showed no cleavage upon digestion with *Sfc* I (Figure 3.5), supporting the hypothesis that ITS2 regions containing an *Sfc* I site were found exclusively on the B chromosome rDNA cluster.

Upon restriction with *Sfc* I, both the pBdITS2B1 and microdissected B-derived PCR products were reduced in their entirety to the two expected fragments, whereas the pBdITS2A2-derived PCR product showed no restriction (Figure 3.5). The implication of the positive controls was that the *Sfc* I enzyme was working to maximum efficiency because the PCR product derived from the B chromosome clone pBdITS2B1 (known to

Figure 3.5:

Agarose gel electrophoresis of ITS2-PCR products resulting from amplification of *B. dichromosomatica* genomic DNA using primers XF58S and ER25S.

Each lane is loaded with a 10 μl sample from a reaction volume of 25 μl, except for the first lane which contains molecular size marker (100 bp ladder with visible fragments ranging from 100bp to 700bp in 100bp increments). Lanes marked with "-" and "+" contain PCR product before and after digestion with *Sfc* I respectively. The sizes of PCR and restriction fragments are marked on the right hand side of the gel. The *B. dichromosomatica* chromosome constitutions, microdissected B-DNA (BDNA) and ITS2 clones from which DNA templates were derived are indicated.


0B
0B
1B
2B
3B
BDNA
pBdITSA2
pBdITSB1
NO DNA
- + - + - + - + - + - + - + - +
500 bp
260 bp
164 bp
98 bp

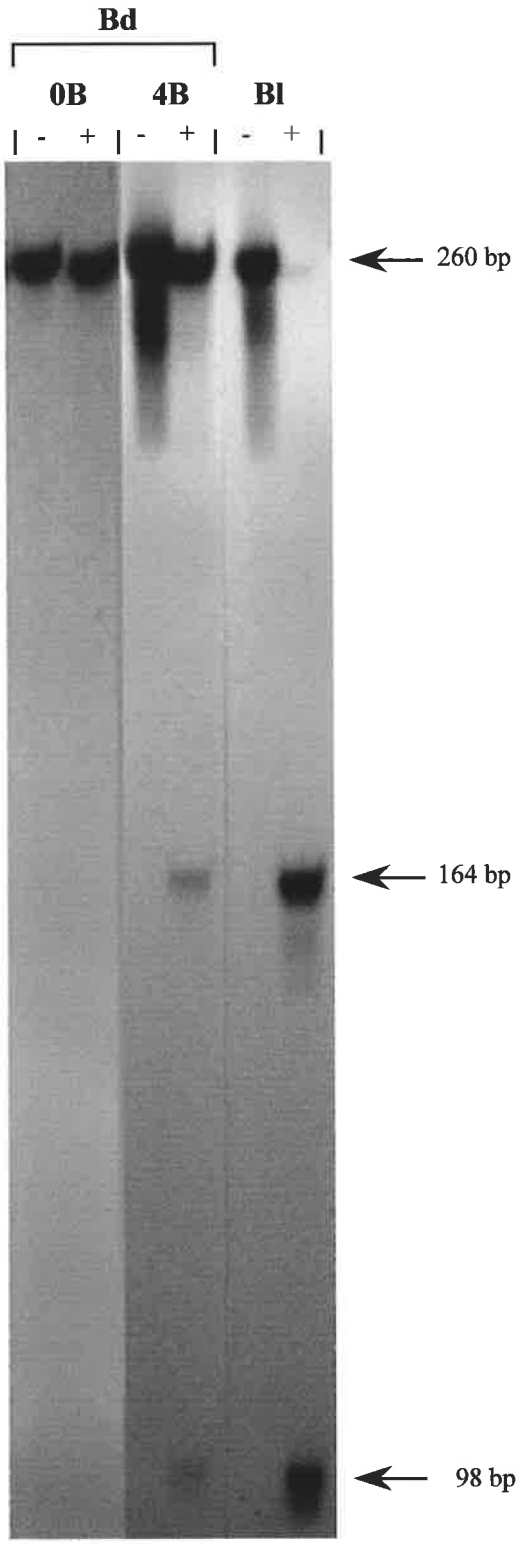
contain the *Sfc* I site, Figure 3.4) was completely reduced to its two component fragments. Furthermore, the observation that all ITS2 regions cloned from the DOP-PCR products obtained from microdissected B chromosomes contained the *Sfc* I site (Figure 3.4), together with the demonstration of complete digestion of sequences amplified directly from microdissected DNA (Figure 3.5), suggested that all the B chromosome rDNA repeats have an A at position 142 of the ITS2 region whereas those on the A chromosomes all have a G at this position. However, the possibility that DOP-PCR preferentially amplified the A_{142}-substituted DNA and the presence of G_{142} on some ITS2 sequences of the B chromosome could not be ruled out.

In parallel with the above experiments, the ITS2 region was amplified from a closely related species of *Brachycome*, *B. lineariloba* race C (Section 3.4). The resulting PCR product, of approximately the same size as that amplified from *B. dichromosomatica*, was cloned and sequenced. Alignment of the *B. lineariloba* ITS2 sequence (Figure 3.4, *Bl*) with those of the A and B chromosomes of *B. dichromosomatica* revealed a high level of similarity (Figure 3.4). The *B. lineariloba* ITS2 also had an A at base 142 within a *Sfc* I site similar to the *B. dichromosomatica* B chromosome rDNA. *B. lineariloba* race C has a chromosome complement of 2n=16 and does not have any B chromosomes (Carter and Smith-White, 1972). The presence of an A at base 142 in *B. lineariloba* race C suggested that the *Sfc* I site would be found in all the ITS2 sequences in this species because of concerted evolution (Section 3.1). The ITS2 regions from 0B and 4B *B. dichromosomatica* and *B. lineariloba* template DNA were amplified in the presence of [α-^{32}P]dATP, the products were digested with *Sfc* I and the fragments resolved by electrophoresis on a 6% polyacrylamide gel (Figure 3.6). The ITS2 product of *B. lineariloba* was restricted in its entirety in to two products of 164 and 98 bp (Figure 3.6, Bl), confirming that *B. lineariloba* contained only ITS2 sequences containing the *Sfc* I site. In addition, a proportion of the

Figure 3.6:

Polyacrylamide gel electrophoresis of ITS2-PCR products resulting from amplification of genomic DNA with primers XF58S and ER25S in the presence of [α-^{32}P]dATP.

Each lane is loaded with a 1 µl sample from a reaction volume of 25 µl. Lanes marked with "-" and "+" contain PCR product before and after digestion with *Sfc* I respectively. The sizes of PCR and restriction fragments are marked on the right hand side of the gel. The chromosome constitutions and species of origin of the plants from which the leaf DNA templates were derived are indicated (Bd = *B. dichromosomatica*, Bl = *B. lineariloba*).


Bd
0B
4B
Bl
-
+
-
+
-
+
260 bp
164 bp
98 bp

ITS2 product amplified from DNA from *B. dichromosomatica* plants with 4B chromosomes was restricted with *Sfc* I and there was no evidence of any restriction of the product from 0B DNA template (Figure 3.6). *B. lineariloba* template nucleic acids were therefore incorporated in the following experiments to monitor the efficiency of restriction enzyme digestion.

To further test whether the A_{142} base was representative of the B chromosome rDNA and not of the A chromosome rDNA, [α-^{32}P]dATP was incorporated during PCR amplification from total DNA of 0B, 1B, 2B, 3B and 4B *B. dichromosomatica* plants and from *B. lineariloba*. The products were digested with *Sfc* I, as before, and fragments resolved on 6% polyacrylamide gels (Figure 3.7). The radioactively labelled products amplified from *B. lineariloba* and a proportion of products from 1B, 2B, 3B and 4B genomic DNA were cleaved by *Sfc* I into the two expected fragments (Figure 3.7). Again there was no digestion with *Sfc* I of the PCR products originating from any 0B genomic DNA template (Figure 3.7).

Despite previously being totally restricted with *Sfc* I (Figure 3.6), the *B. lineariloba* ITS2 product in Figure 3.7 did not show complete digestion. This suboptimal enzyme efficiency was not considered likely to be present in other samples where the amount of DNA was clearly less (Figure 3.7). The prominent band with mobility between the 164 and 98 bp products was caused by the presence of non-denatured PCR products.

The proportion of the ITS2 PCR product that was digested with *Sfc* I consistently correlated with the number of Bs present (Figure 3.7). Phosphorimage analysis of the proportion of the original ITS2 product that was restricted by *Sfc* I (Appendix 1), confirmed this direct correlation and indicated that 3-4% of the ribosomal genes in a 1B plant contain the *Sfc* I site. An average of 6% of the ITS2 product from a 2B DNA template was restricted with *Sfc* I, 9-10% from 3B and 13% from a 4B sample (Figure 3.8). The

Figure 3.7:

Polyacrylamide gel electrophoresis of ITS2-PCR products resulting from amplification of genomic DNA with primers XF58S and ER25S in the presence of [α-^{32}P]dATP.

Each lane is loaded with a 1 μl sample from a reaction volume of 25 μl. Lanes marked with "-" and "+" contain PCR product before and after digestion with *Sfc* I respectively. The sizes of PCR and restriction fragments are marked on the right hand side of the gel. The chromosome constitutions and species of origin of the plants from which the leaf DNA templates were derived are indicated (Bd = *B. dichromosomatica*, Bl = *B. lineariloba*).

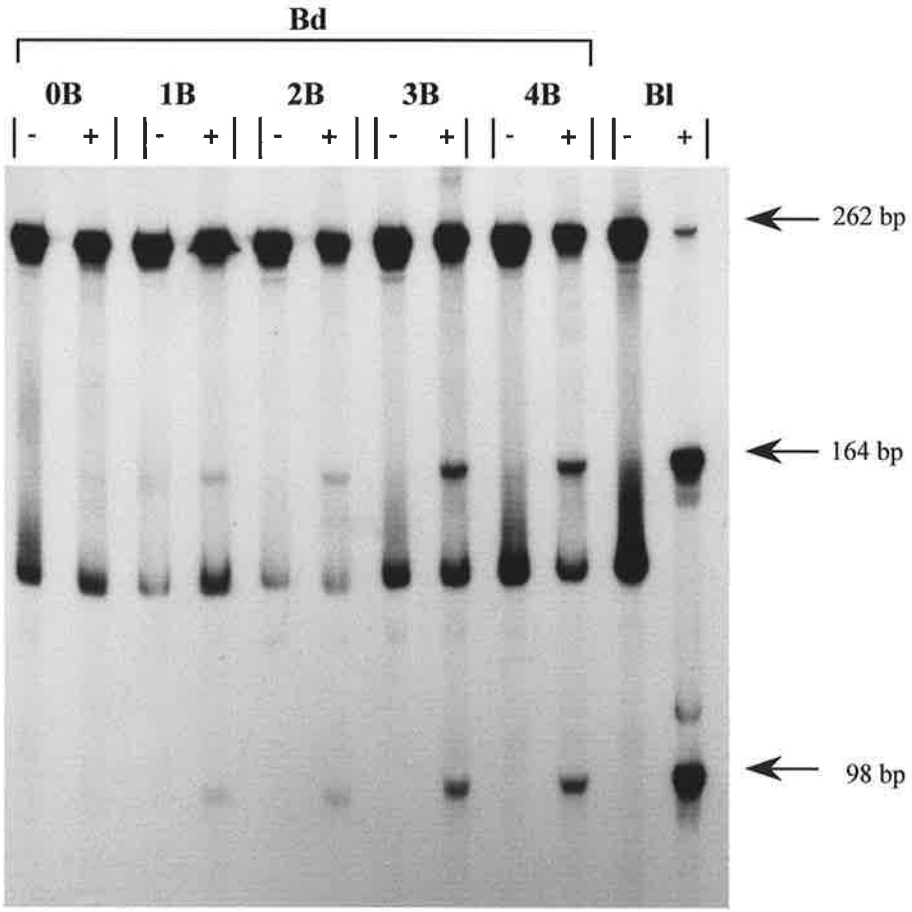
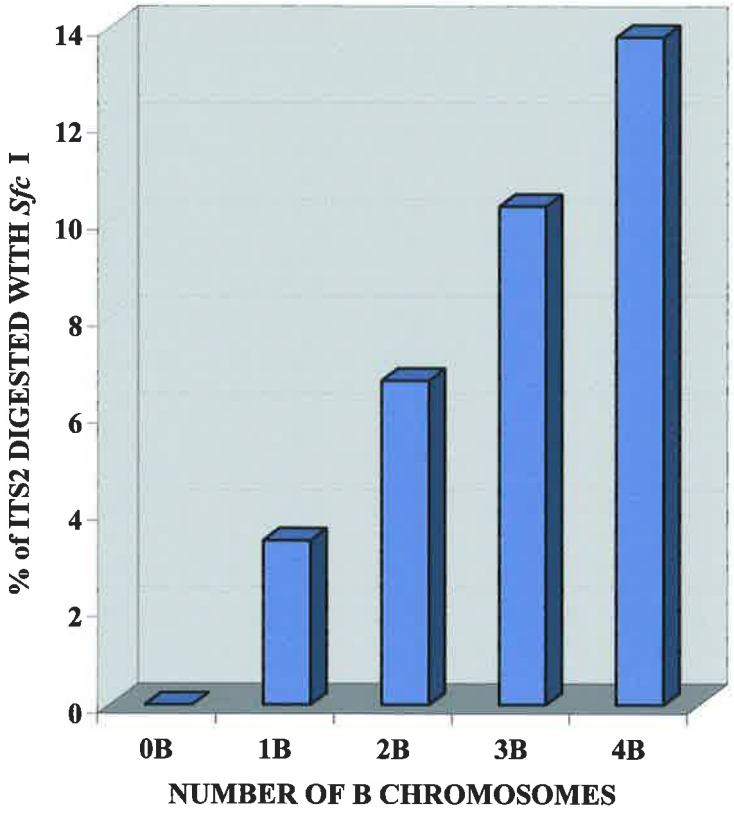

Bd
0B
1B
2B
3B
4B
Bl
- + - + - + - + - + - +
262 bp
164 bp
98 bp

Figure 3.8:

The relationship between the amount of the ITS2-PCR product which restricted with *Sfc* I and the number of B chromosomes present in template DNA samples.


% of ITS2 DIGESTED WITH *Sfc* I
14
12
10
8
6
4
2
0
0B
1B
2B
3B
4B
NUMBER OF B CHROMOSOMES

variation noted in different estimates (Appendix 1) might reflect real variation in the rRNA gene number on the A and B chromosomes in different genotypes, as well as technical errors. The level of enzyme efficiency was monitored throughout these experiments by inclusion of the *B. lineariloba*, which contains only *Sfc* I containing ITS2s. It was concluded from these experiments that the ITS2 in the B chromosome rDNA cluster of *B. dichromosomatica* is homogeneous for an A at base 142 which differs from the G present in the A chromosome ITS2. The difference creates a *Sfc* I restriction site specific for the B chromosome rDNA which enables the ITS2 of B and A chromosomes to be readily distinguished. Quantitative estimates indicate that 3-4% of the total rDNA population is contributed by a single B chromosome.

3.4 Analysis of other Species of *Brachycome*.

The origin of the B chromosomes in *B. dichromosomatica* is unknown, but it is possible they originated through a hybridisation event between *B. dichromosomatica* and another *Brachycome* species (Section 1.7.2; John *et al.*, 1991; Leach *et al.*, 1995).

B. lineariloba was originally divided into five races (A-E) which differed in chromosome number. Races A and D were later identified as a different species (*B. dichromosomatica* and *B. breviscapis* respectively; Carter, 1978b). The presence of a B-specific restriction site in the ITS2 region of the B chromosomes of *B. dichromosomatica*, which is also present in the A chromosomes of *B. lineariloba* race C, suggested that a screen for this site in the A chromosome ITS2 regions of other species may provide information about the genomic origin of the B chromosomes.

Amplification of the A chromosome ITS2 region from a range of different *Brachycome* species, followed by restriction with *Sfc* I and visualisation on agarose gels (Figure 3.9) indicated that, in addition to *B. lineariloba* race C (2n=16), the ITS2 region of

two other chromosome races of *B. lineariloba* race E and B (2n=10 and 2n=12) as well as *B. breviscapis* (formerly *B. lineariloba* race D, 2n=8) also contained the *Sfc* I restriction site. However, the site was absent from the A chromosome rDNA of *B. tetrapterocarpa, B. curvicarpa*, *B. dentata*, *B. eriogona*, *B. parvula*, *B. multifida*, *B. segmentosa* and *B. ciliaris* (Figure 3.9).

Previous comparisons of the karyotypes of races E (2n=10), B (2n=12), C (2n=16) and *B. breviscapis* (formerly *B. lineariloba* race D, 2n=8) suggested that these races have a basic set of four chromosome pairs in common and the higher chromosome types are related to the former race D by successive chromosome additions (Kyhos *et al.*, 1977; Watanabe and Smith-White, 1987). It was therefore not unexpected that the ITS2 sequences of races B, D and E are similar to race C (Section 3.3) with respect to digestion with *Sfc* I. The relationship of the A chromosomes of *B. dichromosomatica* (formerly race A of *B. lineariloba*) with *B. breviscapis* and the *B. lineariloba* races is not known but is thought to be more distantly related (Watanabe and Smith-White, 1987).

3.5 CONCLUSION

Sequence comparisons of the ITS2 region from the A and B chromosomes of *B. dichromosomatica* and the A chromosomes of *B. lineariloba* provided some clues to the possible origin and evolution of the B chromosome rDNA. Superficially the presence of the A_{142} and A_{175} nucleotides in *B. lineariloba* ITS2 suggests an origin of the B chromosome rDNA in the *B. lineariloba* species. However, full sequence comparison of the ITS2 derived from the A chromosomes of *B. lineariloba* (race C) and *B. dichromosomatica* with the B chromosomes of *B. dichromosomatica* indicated that the entire B chromosome ITS2 sequence was more similar to those of the A chromosome rDNA cluster of its "host" genome than that of *B. lineariloba.* The ITS2 of *B. lineariloba*

Figure 3.9:

Agarose gels showing ITS2-PCR products resulting from amplification of genomic DNA from a range of *Brachycome* species using primers XF58S and ER25S.

Each lane is loaded with a 10 μl sample from a reaction volume of 25 μl, except for the first lane which contains molecular size marker (100 bp ladder) with visible fragments ranging from 100 bp to 700 bp in 100 bp increments. Lanes marked with "-" and "+" contain PCR product before and after digestion with *Sfc* I respectively. The size of PCR and restriction products are marked on the right hand side of the gel.

The template DNA was derived from the following *Brachycome* species:

A. *B. lineariloba* Race C (2n=16)

B. *B. lineariloba* Race E (2n=10)

C. *B. lineariloba* Race B (2n=12)

D. *B. breviscapis* (formerly Race D, 2n=8)

E. *B. tetrapterocarpa*

F. *B. curvicarpa*

G. *B. dentata*

H. *B. eriogona*

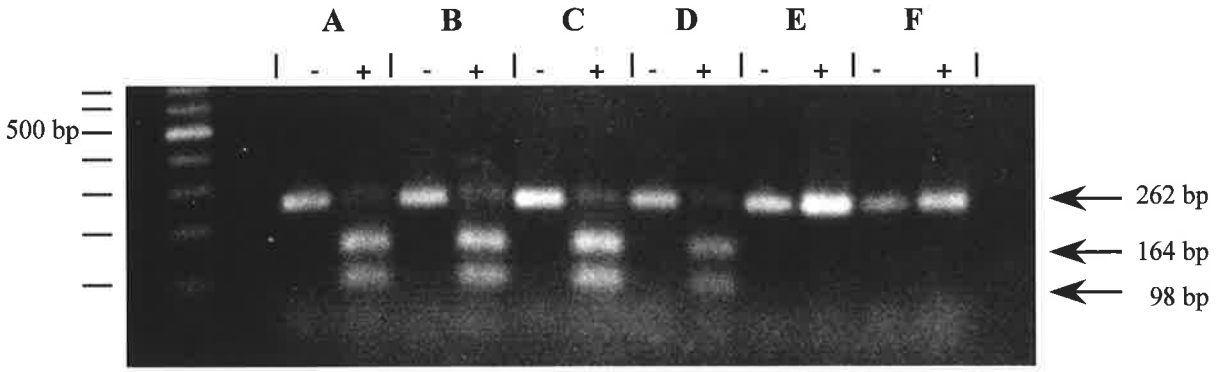
I. *B. parvula*

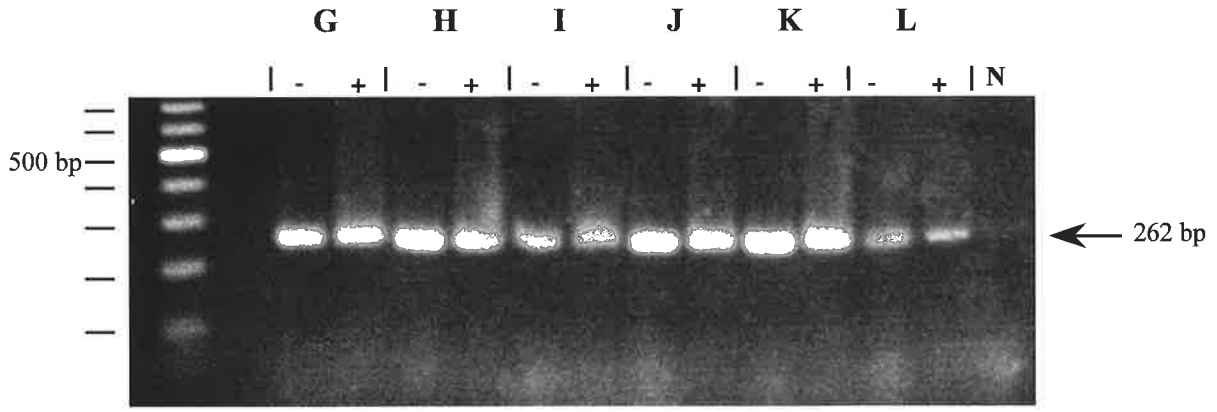
J. *B. multifida*

K. *B. segmentosa*

L. *B. ciliaris*

N. no DNA template (-ve control).


A
B
C
D
E
F
| - + | - + | - + | - + | - + | - + |
500 bp
262 bp
164 bp
98 bp


G
H
I
J
K
L
| - + | - + | - + | - + | - + | - + | N
500 bp
262 bp

race C, in addition to the *Sfc* I site and A/T substitution at position 175, also differed from any of the *B. dichromosomatica* ITS2 B or A chromosome sequences at nine additional sites (Figure 3.4). Alignment of angiosperm ITS2 sequences identified six regions that were conserved in all sampled angiosperms and six alternating variable regions (Hershkovitz and Zimmer, 1996). With one exception, the polymorphisms observed between *B. dichromosomatica* and *B. lineariloba* were confined to the variable regions as would be expected for variation within a genus (Hershkovitz and Zimmer, 1996).

It is possible that the A_{142} and A_{175} nucleotide substitutions in the *B. dichromosomatica* B chromosomes and *B. lineariloba* A chromosomes did not occur independently. However, the presence of nine additional nucleotide differences between ITS2 of *B. dichromosomatica* Bs and *B. lineariloba* indicated that the B chromosomes in *B. dichromosomatica* did not evolve directly from *B. lineariloba*. Instead they may have shared a common ancestor, prior to the 'introduction' of the B chromosomes into *B. dichromosomatica*. *B. lineariloba* would subsequently have evolved independently of the *B. dichromosomatica* A and B chromosome rDNA sequences (Figure 3.10a), which explains the variation between the ITS2 sequences of the two species (Figure 3.4). If the B chromosome evolved from an ancestor common with *B. lineariloba*, as suggested in Figure 3.10A, the B chromosome ITS2 has been separated from the A chromosome of *B. dichromosomatica* for longer than it has been separated from *B. lineariloba*. Therefore, more differences would be expected between A and B chromosome sequences. As this is not the case one must invoke a mechanism that homogenises, to an extent, while tolerating a degree of variation.

The low level of variation between the ITS2 sequences derived from the B and A chromosomes of *B. dichromosomatica* implies that it is more likely that the B chromosome rDNA originated from the A chromosomes of *B. dichromosomatica* and that the sequences

have since diverged (Figure 3.10b). The fact that the *B. dichromosomatica* B chromosomes and *B. lineariloba* A chromosomes have in common the A_{142} and A_{175} sequences would therefore be fortuitous and this is possible considering these nucleotides are present in regions of the ITS2 which commonly show variation between species (Hershkovitz and Zimmer, 1996). The presence of the A_{142} and A_{175} sequences in all the ITS2 regions amplified from the B chromosomes suggests that they evolved in concert with each other and not with those of the A chromosomes of *B. dichromosomatica*.

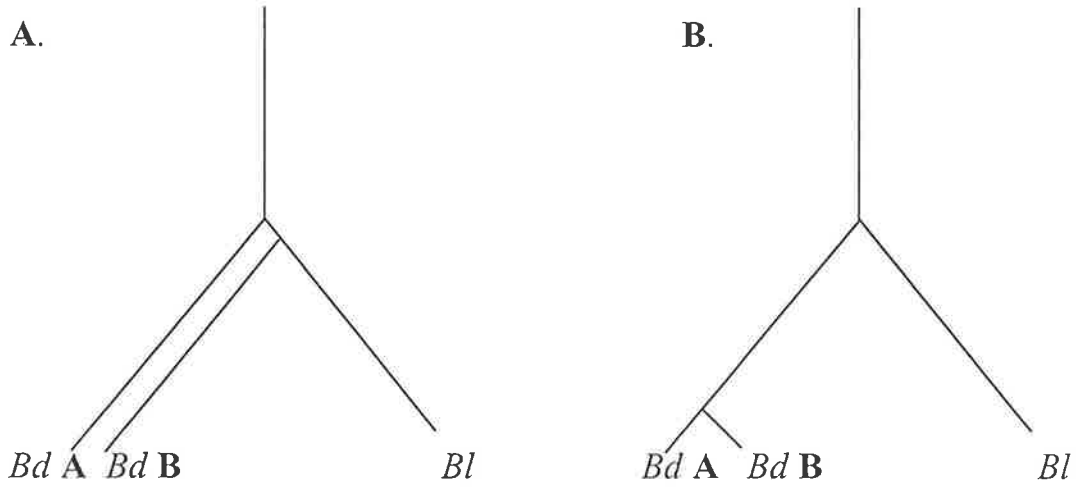


Figure 3.10:

Possible pathways for the evolution of the *B. dichromosomatica* B chromosome (*Bd* **B**) ITS2;

A. from the *B. lineariloba* A chromosome (*Bl*) ITS2, before B chromosome 'introduction' into *B. dichromosomatica*.

B. from the *B. dichromosomatica* A chromosome (*Bd* A).

CHAPTER 4:

Transcriptional Analysis of Ribosomal RNA Internal Transcribed Spacer 2

4.1 INTRODUCTION

This chapter describes experiments to determine if the ribosomal RNA genes on the B chromosome of *B. dichromosomatica* are transcribed.

The initial transcript of a ribosomal RNA gene is a nominal 40S precursor RNA from which the regions corresponding to ITS1, ITS2 and the RNA flanking the 18S and 25S regions are removed during processing to yield the three structural RNAs of the mature ribosomal RNA species (Section 1.6.2a; Sollner-Webb and Tower, 1986; Kavanagh and Timmis, 1988). Within total RNA the transitory 40S RNA species is present for a period of time following rRNA transcription and then during the processing of the 40S pre-rRNA the ITS regions are removed (Sollner-Webb and Tower, 1986). Therefore, if the B chromosome rRNA genes of *B. dichromosomatica* are transcribed, then within the total RNA of a +B plant, B chromosome ITS2 sequences would be expected in proportion to the number of B chromosomes present (Chapter 3).

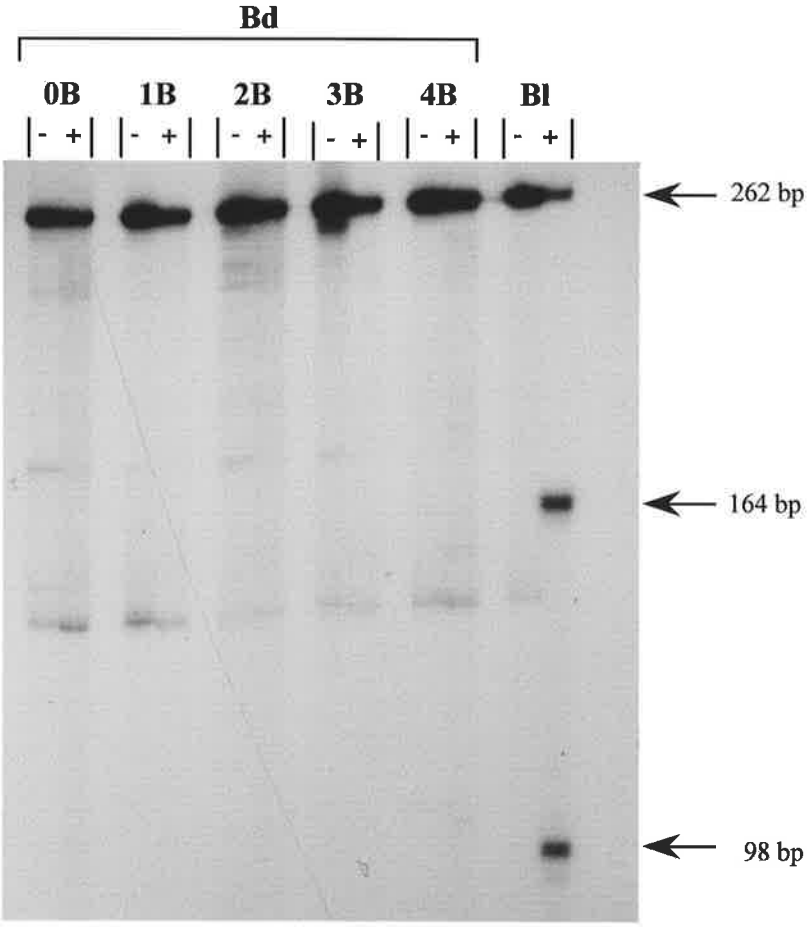
4.2 TRANSCRIPTIONAL ANALYSIS OF THE ITS2 ON THE B CHROMOSOMES IN LEAF TISSUE.

Total RNA was prepared from leaf material from the same 0B, 1B, 2B, 3B and 4B reverse transcribed using the ER25S primer (Figure 3.1B, 3.2; Table 2.4b) and the single stranded DNA products PCR-amplified using the XF58S and ER25S primers. The RT-PCR products were restricted with *Sfc* I and resolved alongside their unrestricted counterparts on a 6% polyacrylamide gel (Figure 4.1).

As expected, RT-PCR from the 0B plant showed no restriction of the 262 bp product with *Sfc* I (Figure 4.1) indicating that none of the rRNA transcribed in 0B plants contained the *Sfc* I site. This supported the previous conclusion that the ITS2 regions of the rRNA

Figure 4.1:

Polyacrylamide gel electrophoresis of RT-PCR products resulting from amplification of total leaf RNA with primers XF58S and ER25S in the presence of [α-^{32}P]dATP. Each lane is loaded with 1 μl of sample from a reaction volume of 25 μl. Lanes marked with "-" and "+" contain RT-PCR products before and after digestion with *Sfc* I respectively. The sizes of RT-PCR and restriction products are marked on the right hand side of the gel. The chromosome constitutions and species of origin of the plants from which the leaf DNA templates were derived are indicated (Bd = *B. dichromosomatica*, Bl = *B. lineariloba*).


Bd
0B
1B
2B
3B
4B
Bl
- +
- +
- +
- +
- +
- +
262 bp
164 bp
98 bp

genes on A chromosomes did not contain any *Sfc* I sites (Chapter 3). In contrast, in *B. lineariloba*, where all copies of the rRNA gene contain an *Sfc* I site in the ITS2 (Chapter 3), the RT-PCR products were efficiently and fully restricted by *Sfc* I (Figure 4.1). However, in *B. dichromosomatica* plants containing B chromosomes, where a proportion of the rRNA genes were demonstrated to contain the *Sfc* I site (Figure 3.7), there was no evidence that any of the RT-PCR products were derived from these genes (Figure 4.1).

From the above results it was apparent that the rDNA located on the B chromosome was not transcribed in proportion to its abundance in the nuclei of B chromosome-containing individuals. However, low levels of transcription could not be ruled out.

4.2.1 Detection of Low Levels of B Chromosome rRNA Transcription.

An experiment designed to detect low levels of transcription of the B chromosome rDNA utilised the specific primer BITS2 (Figure 3.1B, 4.2; Table 2.4b) and this approach was first tested on DNA templates. The B-specific primer contained a diagnostic base at the 3' end which was designed to amplify exclusively from *Sfc* I-containing ITS2 regions (Figure 3.1B, 4.2). At a PCR annealing temperature of 70°C the BITS2 and XF58S primers (Figure 3.1B, 3.2) gave no product from 0B genomic DNA (Figure 4.3). However, they were efficient in amplifying the 164 bp product from 4B genomic DNA of a *B. dichromosomatica* plant as well as *B. lineariloba* DNA (Figure 4.3), supporting the previous conclusion that only the *B. dichromosomatica* B chromosome and the *B. lineariloba* ITS2 regions have an A at base 142 of their sequence.

To explore the sequence of the 40S pre-rRNA in the RNA population, cDNA was first synthesised from total RNA of 0B and +B plants using ER25S as a primer and the single stranded molecules produced were then amplified with BITS2 and XF58S. No BITS2-specific products were evident in samples originating from either 0B or 4B

5.8S← **→25S**

```
120 AGTGGTGGTTGACAAAACTACGGAATCGTGTCGTGTGTCT 160 pBdITS2A2 (A chromosome)
120 AGTGGTGGTTGACAAAACTACAGAATCGTGTCGTGTGTCT 160 pBdITS2B1 (B chromosome)
                                3' TCTTAGCACAGCACACAGCG 5' BITS2 primer
```

Figure 4.2:

Region of the ITS2 to which the BITS2 primer was designed.

Bases 120 - 160 of the ITS2 sequence of pBdITS2A2 and pBdITS2B1, and their orientation in relation to the 5.8S and 25S coding regions are shown. Within the pBdITS2B1 sequence the *Sfc* I site is in bold and the differential A at base 142 is in red. The sequence of BITS2 primer is shown with the *Sfc* I site-specific T in red.

Figure 4.3:

Agarose gel showing leaf PCR and RT-PCR products amplified using BITS2 and XF58S primers.

Each lane is loaded with a 10 μl sample from a reaction volume of 25 μl, except for the first lane which contains molecular size markers (100 bp DNA ladder) with visible fragments ranging from 100 bp to 500 bp in 100 bp increments. The chromosome constitutions and species of origin of the plants from which the DNA and RNA templates were prepared are indicated. ND and NR represent the no DNA template and no RNA template, negative controls.

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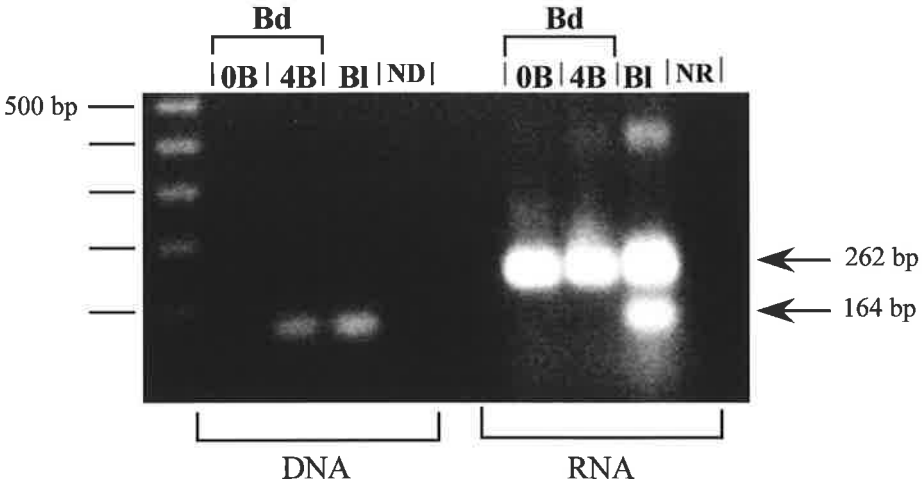


B. dichromosomatica plants (Figure 4.3) but the expected 164 bp product was present in amplifications of RNA from *B. lineariloba* (Figure 4.3). The RT-PCR products of 262 bp in the cDNA lanes (Figure 4.3) represent the entire ITS2 which was amplified due to residual ER25S primer being carried over from the reverse transcription reactions and the presence of this product was useful as an indication that the PCR was efficient in individual reactions.

The results obtained using the potentially more sensitive BITS2 primer assay strongly supported the previous results obtained. It was clear, therefore, that the A_{142}-type ITS2 sequences, which are considered to be a component of all of the rRNA genes of the B chromosome, do not give rise to a 40S transcript in leaf tissue and therefore these genes do not contribute to the 80S ribosomes in the cytoplasm of a plant with B chromosomes.

It was apparent from the preceding experiments that the rRNA genes on the A chromosomes are preferentially expressed in leaf tissue, indicating that the B chromosome rDNA is inactive or that some form of nucleolar dominance is occurring. Nucleolar dominance is an epigenetic phenomenon that occurs when one set of ribosomal RNA genes is expressed to the exclusion of another in species hybrids or allopolyploids. Suppression by mechanisms related to those involved in nucleolar dominance may occur where the rDNA cluster on the A chromosomes is preferentially expressed, at the expense of those on the B, but this is impossible to test because material which contains only B chromosome RNA is not available.

A feature of nucleolar dominance in *Brassica* hybrids is that sets of rRNA genes which are inactive in leaf tissues are expressed in all floral organs (Chen and Pikaard, 1997). It was therefore possible that the B chromosomes of *B. dichromosomatica*, though inactive in leaf tissue, may be active in floral tissues. This idea of differential activity in different tissues is supported by the previously-reported regular association of B chromosomes with

nucleoli during meiosis (Donald *et al.*, 1995). In addition, during the first pollen grain division, which results in the generative and vegetative nuclei, the B chromosomes in *B. dichromosomatica* often undergo non-disjunction and directed segregation into the generative nucleus which then goes on to form the male gamete (Section 1.8; Smith-White and Carter, 1981). It is through this process that the B chromosomes in this species are maintained and if the B chromosomes are transcriptionally active at any stage of development, it may be during this important stage.

4.3 Transcriptional Analysis of the ITS2 on the B Chromosomes in Floral Tissue

Total RNA prepared from flower buds, with anthers at various stages of pollen development, was reverse transcribed with ER25S as previously described and PCR-amplified with BITS2 and XF58S primers. A 164 bp product, representing ITS2 sequences which contain an A at base 142 (Figure 3.4, 4.2), was amplified from *B. lineariloba* and not from any of the 0B, 1B, 2B, 3B or 4B floral RNA templates included in the experiment (Figure 4.4). There was therefore no evidence for any transcription of *Sfc* I-containing ITS2 regions in the floral tissues. The presence of the 262 bp RT-PCR product in all samples was due to the carry over of ER25S primer from the reverse transcription reaction and showed that RT- PCR was efficient in each reaction. It was therefore concluded that there was no transcription from the B chromosome rDNA in floral tissue, which included pollen mother cells at various stages of meiosis and pollen grains at a range of stages of maturity.

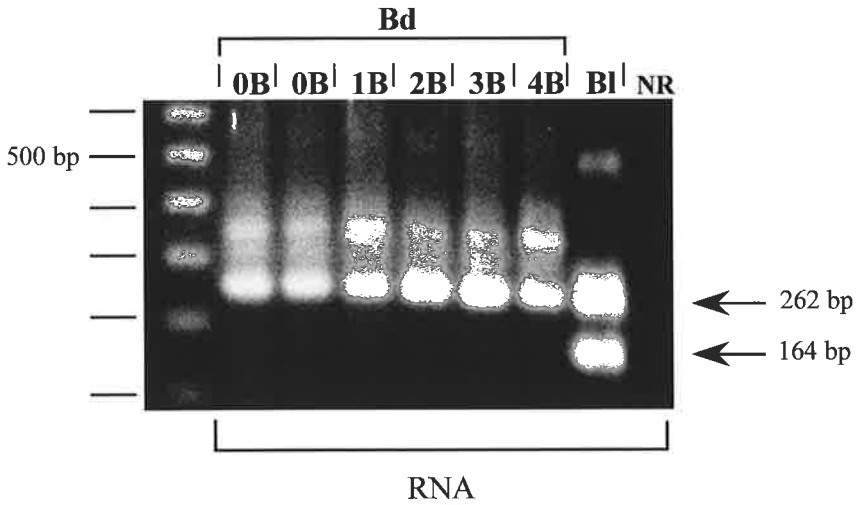
4.4 Summary and Discussion

In summary, results from these experiments indicate that the rRNA genes on the B chromosomes in *B. dichromosomatica* are consistently maintained in an inactive state in

Figure 4.4:

Agarose gel showing flower RT-PCR products, from RNA of flower tissue, amplified using BITS2 and XF58S primers.

Each lane is loaded with a 10 μl sample from a reaction volume of 25 μl, except for the first lane which contains molecular size markers (100 bp DNA ladder) with visible fragments ranging from 100 bp to 500 bp in 100 bp increments. The chromosome constitutions and species of origin of the plants from which the RNA templates were prepared are indicated. NR represents the no RNA template, negative control.


Bd
0B
0B
1B
2B
3B
4B
Bl
NR
500 bp
262 bp
164 bp
RNA

leaf and floral tissues and that they do not contribute to the ribosomes present in a plant with B chromosomes. The level of sensitivity of the experiments discussed, including those with the use of the B-specific primer BITS2, is not certain but it is likely to be high. It is clear that the B chromosome sequences are not transcribed in proportion to the amount of rDNA present on the Bs, as estimated by phosphorimage analysis (Section 3.3), and it is probable that they are completely inactive.

Plants are known to carry from a few hundred to 31 000 rRNA gene copies per diploid cell (Ingle *et al.*, 1975) and evidence suggests that superfluous rDNA exists in some plants that may comprise 90% or more of the total (reviewed in Rogers and Bendich, 1987a). No association has been found between rRNA gene number and phenotype, vigour or rRNA content (Buescher *et al.*, 1984) and it is not understood why plants maintain such a high number of rDNA repeats which are inactive. However, it is possible that the inactive B chromosome rDNA is under the control of similar repression mechanisms. Alternatively B chromosome rDNA activity may be suppressed by nucleolar dominance (Section 1.6.2a) imposed by the A chromosome genes. The mechanisms behind the control of nucleolar dominance are unknown but it is often associated with variation between the IGS lengths of the different sets of rRNA genes. It is unlikely that spacer structure alone is responsible for nucleolar dominance and several epigenetic phenomena, including DNA methylation and chromatin modification, may have a major influence in cases where changes in gene expression are mitotically or meiotically stable, as seems to be the case for the *B. dichromosomatica* B chromosome rDNA.

4.4.1 Intergenic Spacer Variation in the Regulation of rRNA Gene Expression

Variation in the length of the IGS is a common feature of rRNA genes in a range of plant species and it is thought that increased length of the intergenic region of a rRNA gene relative to that of other rRNA genes in the cell, may play a role in determining the potential

activity of the gene (Sardana *et al.*, 1993). In plants and animals, most spacer length variation is due to differences in the number of tandemly repeated IGS subrepeats thought to include RNA polymerase I enhancers (Sardana *et al.*, 1993; Flavell *et al.*, 1986; Hemleben *et al.*, 1988). An appealing hypothesis to explain nucleolar dominance has been that the dominant rRNA genes have more transcriptional enhancers which enables them to outcompete other genes for transcription factors because of higher binding affinities to transcription factors present in rate limiting quantities. In hexaploid wheat, for example, the longest IGS region is consistently associated with the highest levels of rRNA gene activity (Sardana *et al.*, 1993). Furthermore the NOR of the diploid, *Aegilops umbellulata*, which has rRNA genes with even longer spacers suppresses all native wheat NORs (Martini *et al.*, 1982). However, in hybrids of *Brassica* spp., the rRNA genes with the shorter IGS region are expressed to the exclusion of those with longer IGS regions (Chen and Pikaard, 1997), indicating that nucleolar dominance is not always associated with length variation. Moreover, in *Arabidopsis*, switches in dominance between different sets of rRNA genes imply that nucleolar dominance is not due to simple intrinsic numerical affinity for transcription factors (Chen *et al.*, 1998).

4.4.2 DNA Methylation in the Regulation of rRNA Gene Expression

Extensive methylation of CpG and CXG sequences is common in plant DNA and rDNA is on average more heavily methylated than the bulk of nuclear DNA (Ellis *et al.*, 1983; Steele-Scott *et al.*, 1984; von Kalm *et al.*, 1986; Flavell *et al.*, 1988). In plants with a relatively large number of rRNA gene repeats, a high percentage of rDNA is not transcriptionally active and in these species, it has been found that the cytosine methylation levels are related to rRNA gene expression (Flavell *et al.* 1988; Jupe and Zimmer, 1993; Torres-Ruiz and Hemleben, 1994). The link between methylation and gene inactivation is not understood, but there is a strong and well established correlation between reduced

cytosine methylation of the rRNA promoter region and enhanced NOR activity (Flavell *et al.*, 1988; Jupe and Zimmer, 1993). It is not known whether demethylation in rDNA is the result or the cause of gene activation. The treatment of wheat × rye interspecific hybrids with 5-azacytidine, which inhibits cytosine methylation, stably reactivated the suppressed rRNA genes of rye origin (Neves *et al.*, 1995), suggesting that methylation has a direct role in rDNA suppression.

Despite the fact that it has been shown that the methylation of rDNA is indistinguishable between 0B and +B *B. dichromosomatica* plants using Southern analysis (Donald *et al.*, 1995), the possibility that the B chromosome rRNA promoter is highly methylated in relation to the active A chromosome rRNA promoter, cannot be ruled out. In *B. dichromosomatica* there is a high copy number of rRNA genes on the A chromosomes, of which a majority are methylated (Donald *et al.*, 1995). It is likely that the presence of B chromosome rDNA methylation would be difficult to detect above the background of that are present on the A chromosomes and the requirement for a means of distinguishing between A and B chromosome IGS regions becomes apparent.

It is possible that B chromosome rRNA genes are inactivated by mechanisms other than methylation and that methylation is secondary to inactivation rather than its primary cause. In the grasshopper *E. plorans* embryos, for example, the B-rRNA genes are inactive irrespective of their methylation status, suggesting the involvement of other regulatory/silencing factors (López-León *et al.*, 1995). In this species, as assessed by silver staining, the rRNA genes can be activated by translocation to the A chromosomes, indicating that the chromosomal environment of the rRNA genes influences whether or not they are active (Cabrero *et al.*, 1987).

4.4.3 Chromatin Modifications in the Regulation of Gene Expression

In addition to methylation, there is evidence that chromatin structure also has a role in regulating rDNA activity. In maize hybrids, for example, in which one parental NOR is dominant, a proportion of the rDNA is highly methylated, DNase I-resistant and exhibits low levels of transcription. In contrast, the expressed rDNA repeats are undermethylated and DNase I-sensitive. DNase I sensitivity indicates that the rDNA is in chromatin with an open conformation and therefore accessible to DNase I digestion. Therefore, in maize increased susceptibility of rDNA to DNase I digestion, together with undermethylation, is associated with rRNA gene activity (Thompson and Flavell, 1988; Jupe and Zimmer, 1993).

The packaging of DNA within chromatin is seen increasingly as having an essential role in the regulation of nuclear processes. The levels of histone H4 acetylation in mitotic chromosomes reflects, and possibly determines, potential transcriptional activity (Jeppesen and Turner, 1993; Houben *et al.*, 1997a). It is also thought that cytosine methylation may directly affect the structure of the chromatin and this may be involved in the inheritance of repressed chromatin states. Evidence suggests that histone deacetylases are guided to specific chromatin domains by underlying genomic methylation patterns and that methylation-dependent transcriptional silencing relies on histone acetylation (Bestor, 1998).

Differences in the level of histone H4 acetylation and timing of DNA replication between A and B chromosomes of *B. dichromosomatica* have been demonstrated (Houben *et al.*, 1997a). The core histones are very basic proteins, the N-terminal tails of which contain four lysine residues (5, 8, 12 and 16). These lysine residues are acetylated, or deacetylated, in various patterns correlated with gene activity which they may regulate by modifying nucleosome assembly or interaction with other proteins, including RNA

polymerases and transcription factors. The B chromosomes of *B. dichromosomatica* label weakly with antibodies against H4Ac5 and H4Ac8, in comparison to that seen for the A chromosomes. In contrast, the distributions of H4 acetylated forms of lysine 12 and 16 were indistinguishable between chromosome types. The B chromosomes were also found to be late replicating in comparison to the A chromosomes which is characteristic of inactive chromatin (Houben *et al.*, 1997a). The mechanism by which H4 acetylation and late replication might control the transcriptional inactivity of chromatin of B chromosomes remains to be established, but it is possible that the Bs may be in a chromatin conformation which is inaccessible to RNA polymerases and transcription factors.

Chapter 5:

Cloning of the Ribosomal RNA Repeat Unit

5.1 INTRODUCTION

Sensitive experiments described in the previous chapter indicated that the ribosomal RNA genes present on the B chromosomes of *B. dichromosomatica* are not transcribed. Previously, Southern hybridisation of 0B and +B genomic DNA, restricted with a variety of enzymes and probed with the cucumber ribosomal repeat unit, revealed no differential hybridisation patterns, suggesting the rDNA is identical on the A and B chromosomes (Donald *et al.*, 1995). The use of the heterologous cucumber probe in these experiments would be expected to detect restriction fragments containing the highly conserved coding regions 25S, 5.8S and 18S genes of the rDNA repeat. However, fragments containing only the divergent spacer regions would not be expected to hybridise (Section 3.1). In particular the intergenic spacer (IGS), located between the 3' end of the 25S and the 5' end of the 18S coding regions (see Figure 3.1A), is of interest as it contains the RNA polymerase I promoter and other important regulatory regions which control transcription. Although the mechanism by which the rDNA of B chromosomes is maintained in an inactive state is speculative it is possible that differences between A and B chromosome RNA polymerase I promoter region may account for the inactivity of the B chromosome rDNA.

Due to the low level of sequence conservation of the IGS region across species (Hemleben *et al.*, 1988; Delcasso-Tramousaygue *et al.*, 1988; Marilley and Pasero, 1996; Wu and Fallon, 1998), a heterologous probe would not be expected to detect this region using Southern analysis. It was therefore considered necessary to clone the rRNA gene from *B. dichromosomatica* 0B DNA to allow a search for differences in this region between the A and B chromosomes and enable them to be examined as single entities. This chapter discusses the cloning of the *B. dichromosomatica* rDNA repeat unit and the analysis of the A and B chromosome rRNA clusters which followed.

5.2 Cloning of the *B. dichromosomatica* Ribosomal RNA Repeat Unit

An analysis of the basic structure of the *B. dichromosomatica* rDNA repeat unit was initiated by hybridising restricted 0B genomic DNA with the cloned rRNA gene (pGhR1) from cotton (Orford, 1996). In general, the restriction endonucleases cut the genomic *B. dichromosomatica* DNA into several rDNA-hybridising fragments (Figure 5.1) with the exception of *Eco* RV which produced a single band (Figure 5.1, lane 10). This result suggested that the *B. dichromosomatica* rDNA repeat unit contained a single *Eco* RV site and that the size of the repeat unit was approximately 9.5 kb in length. The inability of the enzymes *Xho* I, *Pst* I, *Not* I, *Cla* I and *Sma* I to restrict the genomic DNA, indicated by hybridisation of GhR1 to high molecular weight DNA (Figure 5.1, lanes 3, 5, 6, 9 and 12), was thought to be due to the high levels of rDNA methylation (Steele-Scott *et al.*, 1984) or to the lack of sites for these enzymes on the repeat units. It was unlikely to be due to partial digestion because measures, including high enzyme concentrations and increased reaction times, were taken to ensure that the DNA was totally digested.

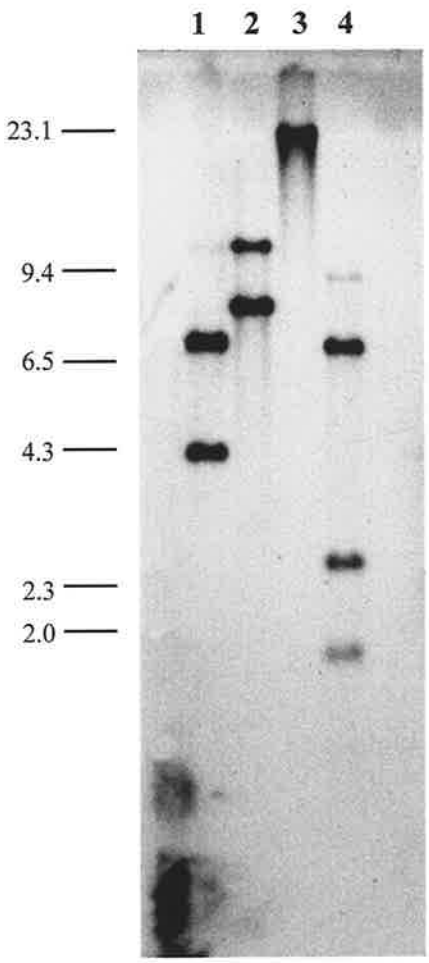
B. dichromosomatica genomic 0B DNA restricted with *Eco* RV was run on an agarose gel. The region containing 9 to 10 kb DNA fragments was excised from the gel and the DNA eluted using the freeze-squeeze method (Section 2.3.3c). The resulting product was ligated into *Eco* RV-restricted pBluescript®SK(-) and recombinant clones selected using blue-white colour selection. Despite screening of approximately 1500 recombinant colonies with GhR1, no ribosomal clones were obtained (result not shown).

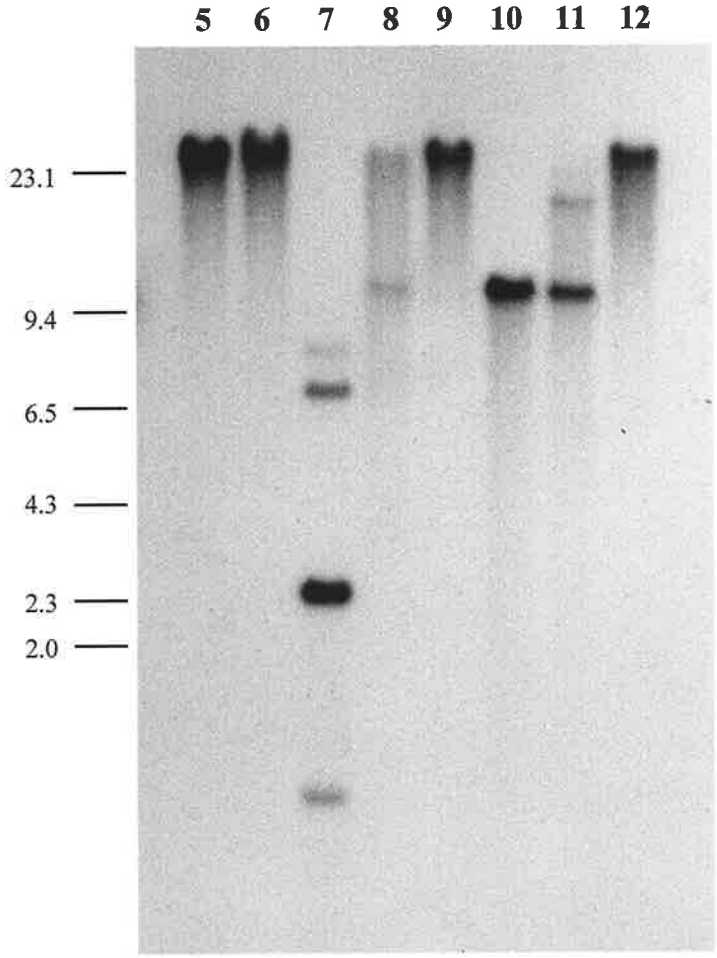
The inability to clone the complete ribosomal repeat unit using the endonuclease *Eco* RV, led to an attempt to clone it using other restriction enzymes. Genomic 0B DNA restricted with *Eco* RI and Southern-hybridised with the cotton rDNA probe showed two hybridising fragments of approximately 4 kb and 6 kb in size (Figure 5.1, lane 1).

Figure 5.1:

Southern hybridisation of *B. dichromosomatica* DNA probed with the insert from the cotton rDNA clone pGhR1.

Each lane was loaded with 2 μg of total genomic DNA from a *B. dichromosomatica* plant without B chromosomes digested with the following restriction endonucleases: 1. *Eco* RI; 2. *Xba* I; 3. *Xho* I; 4. *Sac* I; 5. *Pst* I; 6. *Not* I; 7. *Bam* HI; 8. *Hin*d III; 9. *Cla* I; 10. *Eco* RV; 11. *Spe* I and 12. *Sma* I. The numbers on the left represent the molecular sizes (kb) of lambda bacteriophage DNA restricted with *Hin*d III.


1 2 3 4
23.1
9.4
6.5
4.3
2.3
2.0


5 6 7 8 9 10 11 12
23.1
9.4
6.5
4.3
2.3
2.0

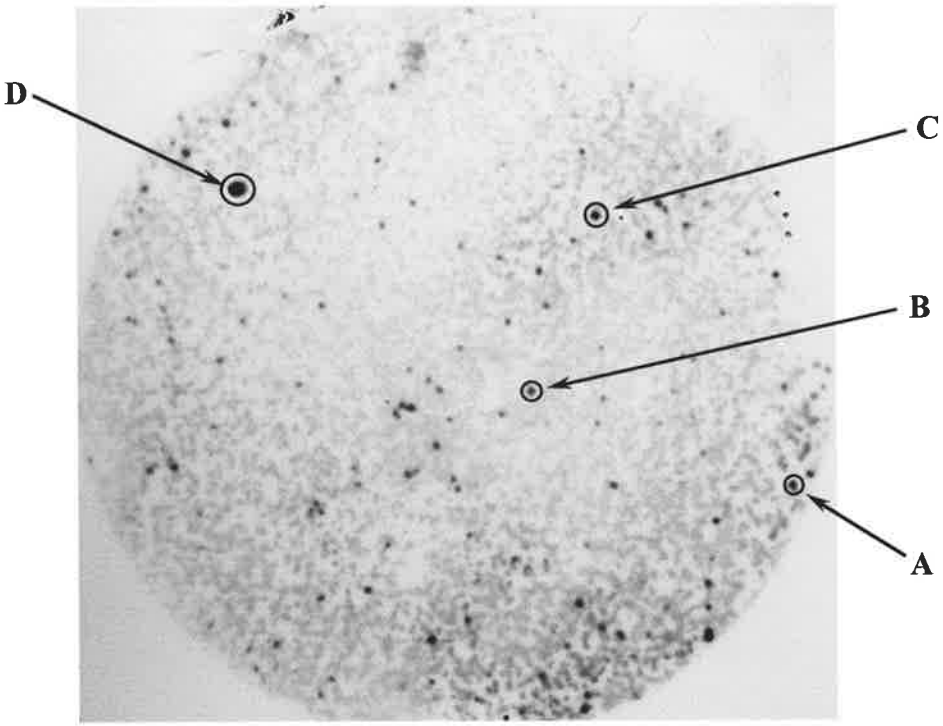
Genomic 0B DNA was restricted with *Eco* RI and ligated directly into *Eco* RI-digested λ Zap®II. Screening of approximately 50,000 plaques, using GhR1 as a probe, detected many putative ribosomal DNA clones (Figure 5.2). There was a distinct variation in the levels of plaque hybridisation to GhR1 and examples of the strongest hybridisers (λBdrC, λBdrD and λBdrE) plus some weaker hybridisers (λBdrA, λBdrB, λBdrF and λBdrG) were selected for further analysis (Figure 5.2).

Secondary and tertiary screening recovered five (λBdrA, λBdrB, λBdrC, λBdrD and λBdrF), and the inserts were rescued from these λZap®II clones. Duplicate *Eco* RI-digested plasmid DNA preparations of the clones were electrophoresed on agarose gels, Southern blotted and hybridised with GhR1 (Figure 5.3A, A'). Clones pBdrA, pBdrB and pBdrF had *Eco* RI inserts of less than 0.5 kb in length which showed no significant hybridisation to GhR1 (Figure 5.3A, A'). It was concluded that they did not contain ribosomal sequences, consistent with the lower levels of GhR1 hybridisation seen on the initial screen (see Figure 5.2). In contrast, pBdrC and pBdrD had inserts of approximately 0.7 kb and 6 kb respectively, both of which strongly hybridised GhR1 (Figure 5.3A, A'). The insert of pBdrD closely corresponded in size to the 6 kb *Eco* RI genomic DNA fragment which hybridised GhR1 and was therefore tentatively assigned to this segment of the ribosomal repeat unit. In contrast, the insert size of pBdrC did not correspond to either of the rDNA hybridising bands of genomic DNA suggesting it reflected the occurrence of an *Eco* RI polymorphism, with the fragment present in the plant from which the donor DNA was prepared and perhaps other plants, but absent from the DNA sample analysed in Figure 5.1.

It was evident that the smaller 4 kb *B. dichromosomatica* band that hybridised to GhR1 in genomic DNA (see Figure 5.1) was not cloned using this technique. Replating of λBdrE, a strong initial hybridiser (Figure 5.2), recovered a clone (pBdrE) containing an

Figure 5.2:

Plaque hybridisation with the insert from the cotton rDNA clone pGhR1 to a *Eco* RI partial DNA library constructed in λZap®II. Putative *B. dichromosomatica* ribosomal DNA clones which were picked for further analysis are indicated (A-E).


D
C
B
A

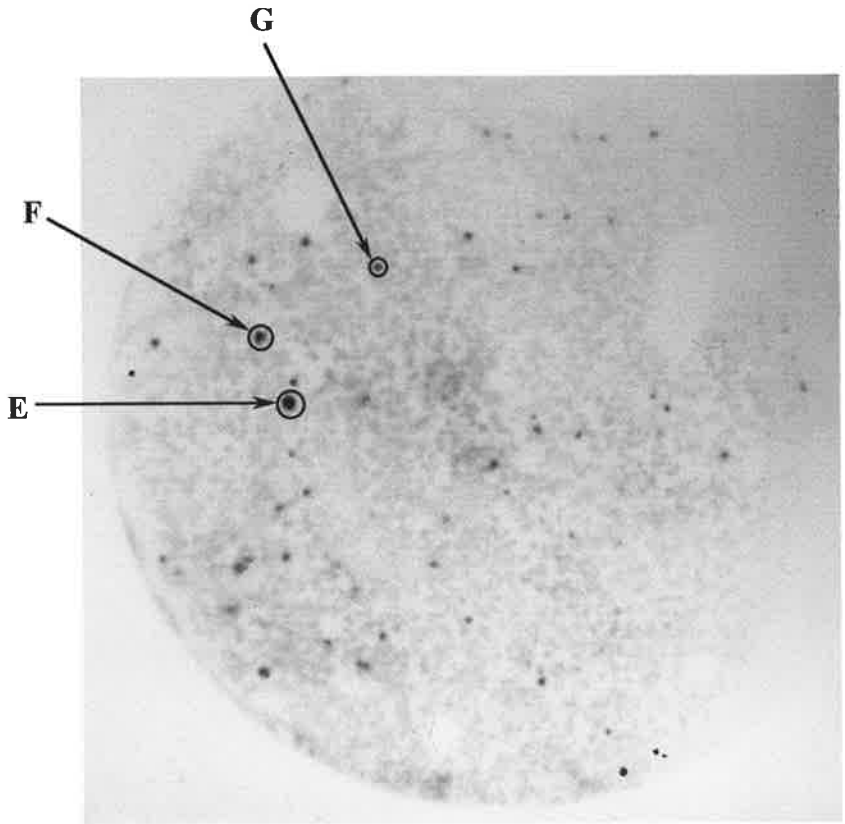

G
F
E

Figure 5.3:

A. Agarose gel showing duplicate *Eco* RI digests of the rescued putative *B. dichromosomatica* rDNA clones in pBluescript®SK(-).

The numbers on the left of the gel represent the molecular sizes (kb) of lambda bacteriophage DNA restricted with *Hin*d III. The identity of the clone in each lane is indicated.

A'. Hybridisation of a Southern blot of the gel in A with the cotton rDNA repeat unit (GhR1).

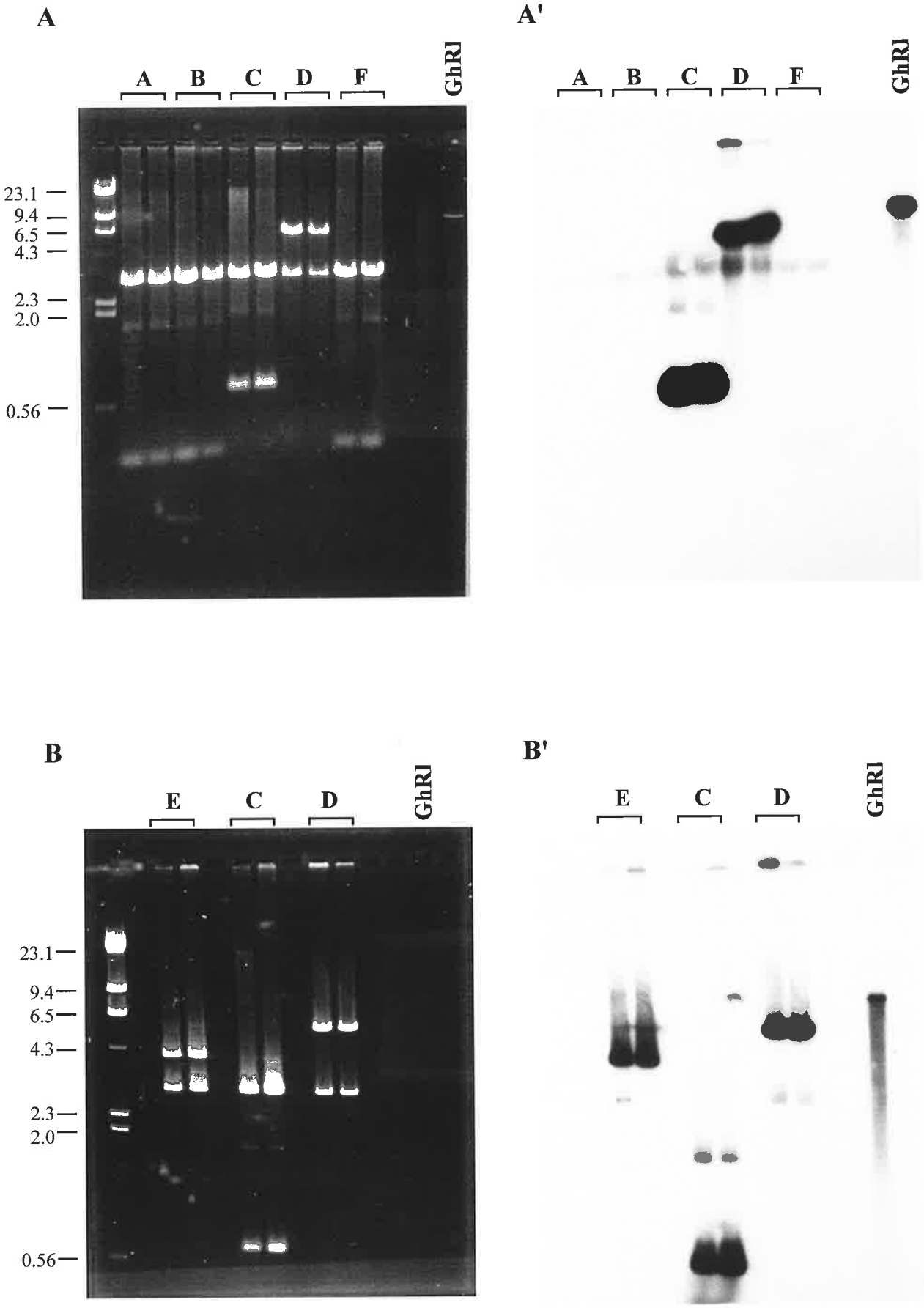
The positive control is the insert from pGhR1.

B. Agarose gel showing duplicate *Eco* RI digests of the rDNA clones pBdrE, pBdrC and pBdrD.

The numbers on the left of the gel represent the molecular sizes (kb) of lambda bacteriophage DNA restricted with *Hin*d III. The identity of the clone in each lane is indicated.

B'. Hybridisation of a Southern blot of the gel in B with the cotton rDNA repeat unit (GhR1).

The positive control is the insert from pGhR1.


A
A B C D F
GhRI
23.1
9.4
6.5
4.3
2.3
2.0
0.56
A'
A B C D F
GhRI
B
E C D
GhRI
23.1
9.4
6.5
4.3
2.3
2.0
0.56
B'
E C D
GhRI

Eco RI insert of approximately 4 kb assumed to be the smaller GhR1-hybridising band (Figure 5.3B, B').

5.3 Analysis of the *B. dichromosomatica* Ribosomal DNA Clones

5.3.1 Hybridisation of BdrD, BdrE and BdrC to 0B Genomic DNA Samples

The inserts of the putative *B. dichromosomatica* ribosomal clones pBdrD, pBdrE and pBdrC (Figure 5.3A, A', B, B') were consecutively hybridised to 0B genomic DNA restricted with *Eco* RI to determine which of the *Eco* RI ribosomal DNA fragments they represented. As predicted BdrD hybridised exclusively to the 6 kb fragment (Figure 5.4A), confirming that it was a clone of the larger of the two *Eco* RI bands. Surprisingly BdrE also hybridised to the same 6 kb *Eco* RI fragment (Figure 5.4B). This was unexpected, especially considering BdrE was cloned from the same genomic DNA and was 4 kb in length. The amount of hybridisation of BdrE to the template (Figure 5.4B) was clearly lower than that of BdrD (Figure 5.4A) or BdrC (Figure 5.4C), suggesting a lower level of identity between the probe and the genomic DNA. Hybridisation of BdrC to *Eco* RI digested genomic DNA again showed strong hybridisation to the 6 kb rDNA band (Figure 5.4C). It was therefore thought that BdrE and BdrC represented rare rDNA polymorphisms and significant sequence differences within the rDNA population of this plant.

Upon hybridisation of BdrD, BdrE and BdrC to 0B genomic DNA cut with *Xba* I, BdrE and BdrC both hybridised to a 9.5 kb fragment and at a lower level to a larger fragment of approximately 12 kb (Figure 5.4B, C). BdrD hybridised to all the bands to which BdrE and BdrC hybridised and to two extra fragments of approximately 2.5 kb in size (Figure 5.4A). The pattern of hybridisation supported the hypothesis that BdrD contained regions with identity to BdrE and BdrC, as well as having additional unique

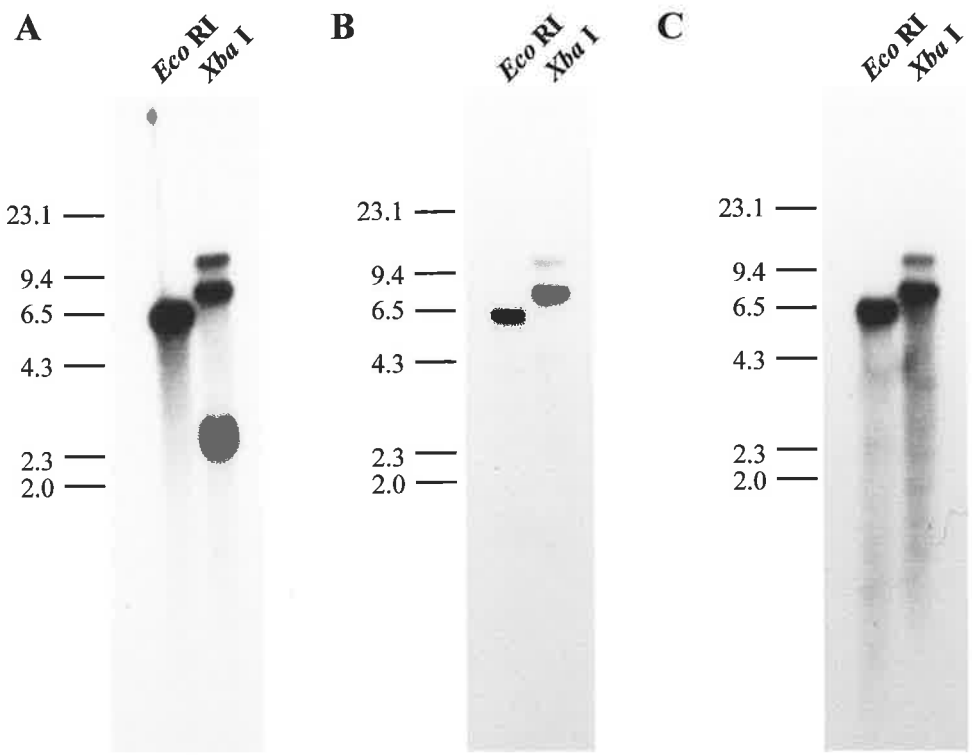
Figure 5.4:

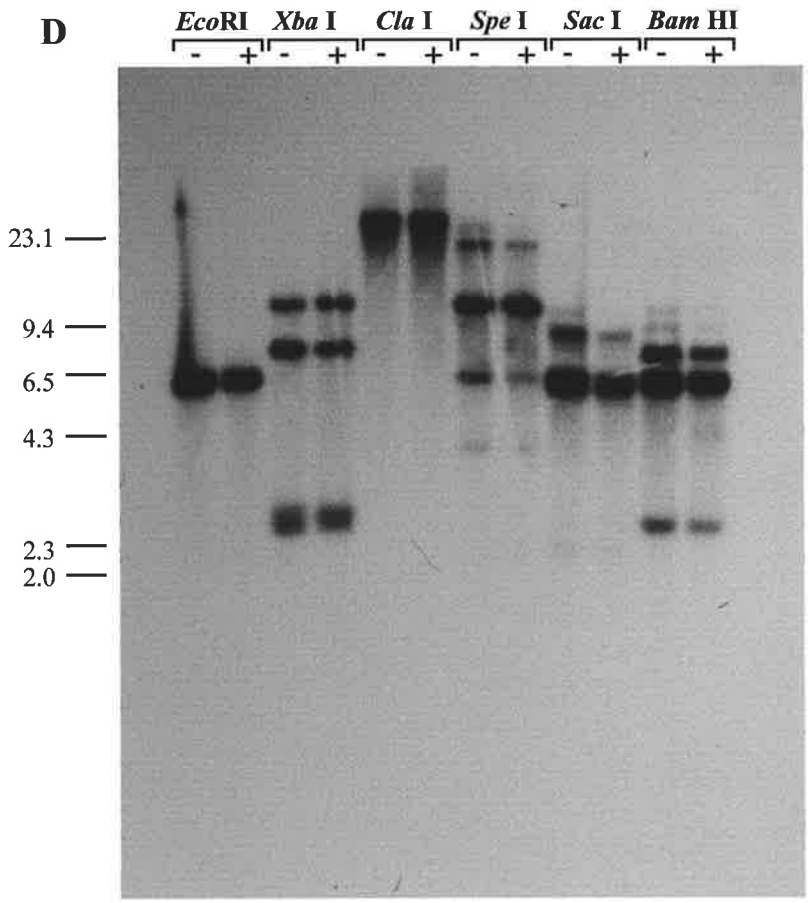
A. Southern hybridisation of *B. dichromosomatica* 0B genomic DNA probed with the insert from pBdrD. Each lane is loaded with 2 μg of total genomic DNA without B chromosomes restricted with the endonucleases indicated. The numbers on the left represent the molecular sizes (kb) of lambda bacteriophage DNA restricted with *Hin*d III.

B. Southern hybridisation of *B. dichromosomatica* 0B genomic DNA probed with the insert from pBdrE. Each lane is loaded with 2 μg of total genomic DNA without B chromosomes restricted with the endonucleases indicated. The numbers on the left represent the molecular sizes (kb) of lambda bacteriophage DNA restricted with *Hin*d III.

C. Southern hybridisation of *B. dichromosomatica* 0B genomic DNA probed with the insert from pBdrC. Each lane is loaded with 2 μg of total genomic 0B DNA restricted with the endonucleases indicated. The numbers on the left represent the molecular sizes (kb) of lambda bacteriophage DNA restricted with *Hin*d III.

D. Southern hybridisation of *B. dichromosomatica* genomic DNA probed with the insert of *B. dichromosomatica* rDNA clone pBdrD.
Each lane was loaded with 3 μg of total genomic DNA derived from a 0B or 3B *B. dichromosomatica* plant and digested with the restriction endonucleases indicated. The numbers on the left represent the molecular sizes (kb) of lambda bacteriophage DNA restricted with *Hin*d III.


A
Eco RI
Xba I
23.1
9.4
6.5
4.3
2.3
2.0
B
Eco RI
Xba I
23.1
9.4
6.5
4.3
2.3
2.0
C
Eco RI
Xba I
23.1
9.4
6.5
4.3
2.3
2.0


D
EcoRI
Xba I
Cla I
Spe I
Sac I
Bam HI
- + - + - + - + - + - +
23.1
9.4
6.5
4.3
2.3
2.0

regions commensurate with its larger size. The level of BdrE hybridisation was again lower than that of BdrD or BdrC (Figure 5.4A, B, and C), supporting the idea that there was sequence diversity between the BdrE probe and the majority of rDNA sequences.

It was therefore apparent that all the available *B. dichromosomatica* rDNA clones obtained contained regions which showed identity to the 6 kb *Eco* RI fragment and no clone represented the 4 kb *Eco* RI fragment (Figure 5.1).

5.3.2 Comparison of BdrD Hybridisation to 0B and +B Genomic DNA Samples

The hybridisation pattern of BdrD to 0B and +B genomic DNA cut with *Eco* RI, *Xba* I, *Cla* I, *Spe* I, *Sac* I and *Bam* HI was indistinguishable between samples with and without B chromosomes (Figure 5.4D) supporting previous evidence that the rDNA regions on A and B chromosomes could not be distinguished by this method (Donald *et al.*, 1995).

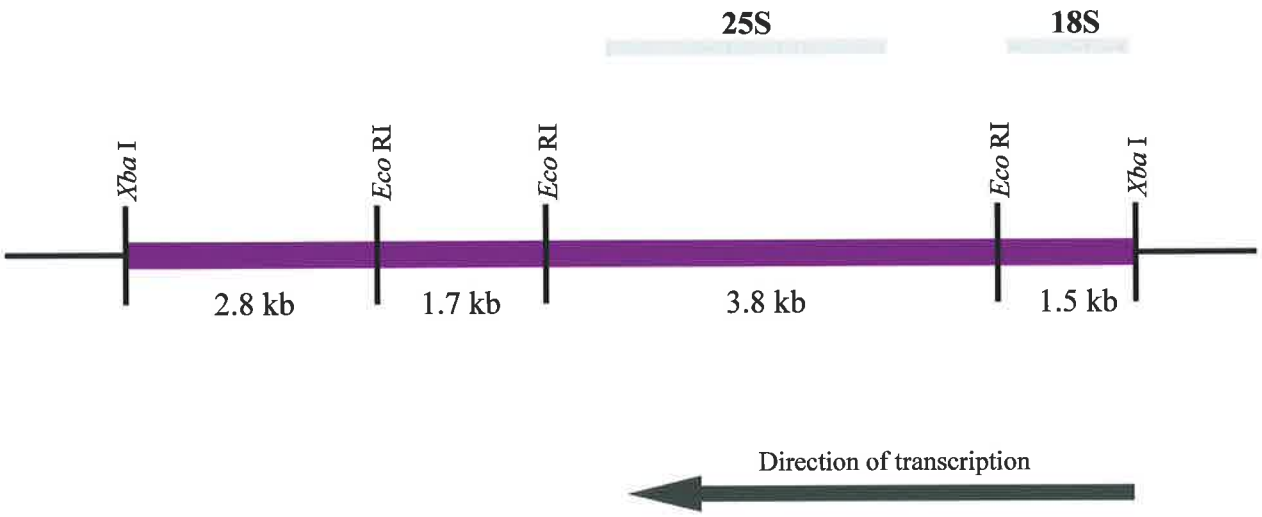
As discussed in Chapter 3, a B chromosome contains approximately 3% of the total rDNA in a plant with one B chromosome. The greater proportion of rDNA on the A chromosomes is likely to overshadow any minor length variation in this region specific to the B chromosomes. Of particular interest was the IGS region which contains RNA polymerase I and other rRNA gene regulatory elements. Mapping of the *B. dichromosomatica* rDNA clones was undertaken to define and then subclone the IGS region.

5.4 Mapping BdrD and BdrE

Restriction enzymes which cut within BdrD and BdrE once or twice, namely *Eco* RI, *Xba* I, *Xho* I, *Cla* I (pBdrD) and *Spe* I (pBdrE), were chosen to map the clones. Physical maps were also constructed using probes specific for each rRNA-coding region derived from the cotton rDNA repeat unit (GhR1, Figure 5.5).

Figure 5.5:

Physical map of the cotton ribosomal repeat unit GhRI showing recognition sites for the endonucleases *Eco* RI and *Xba* I. The regions containing sequence identity to 18S and 25S rRNA coding regions are indicated (adapted from Orford, 1996).


25S
18S
Xba I
Eco RI
Eco RI
Eco RI
Xba I
2.8 kb
1.7 kb
3.8 kb
1.5 kb
Direction of transcription

Initially pGhR1 was digested with *Eco* RI and *Xba* I and the resulting GhR1 products of 3.8 kb, 2.8 kb, 1.7 kb and 1.5 kb (Figure 5.5) were individually eluted from gel sections and used as probes to the *B. dichromosomatica* rDNA clones. The 1.5 kb *Eco* RI/ *Xba* I GhR1 fragment, which contains the 18S rRNA gene, hybridised to the 1.2 kb *Eco* RI/ *Xba* I fragment of BdrD (Figure 5.6B) and the 1.6 kb *Xba* I/ *Eco* RI fragment of BdrE (Figure 5.7B). Hybridisation of the 1.7 kb *Eco* RI GhR1 fragment, containing part of the intergenic spacer region, was confined to the 1.5 kb *Eco* RI/ *Xba* I fragment of BdrD (Figure 5.6C) and the 0.2 kb *Eco* RI/ *Xho* I fragment of BdrE (Figure 5.7C). The 2.8 kb *Xba* I/ *Eco* RI fragment of GhR1, which also contains a portion of the intergenic spacer region, displayed a significant level of hybridisation to the 2.5 kb *Xba* I fragment of BdrD (Figure 5.6D) as well as to the 0.3 kb *Spe* I/ *Xba* I fragment of BdrE (Figure 5.7D).

The 1.7 kb *Eco* RI and 2.8 kb *Xba* I/ *Eco* RI fragments of GhR1 contain mostly intergenic spacer (Figure 5.5) and, due to the low level of sequence conservation in the IGS region across species, it was not expected that this region from cotton would hybridise to the *B. dichromosomatica* rDNA clones. The significant levels of hybridisation seen, despite stringent washing conditions, implied the presence of some 18S, 5.8S and/or 25S rDNA sequences within each of these regions of GhR1.

Of significance is the observation that neither BdrD or BdrE showed hybridisation to the 3.8 kb GhR1 fragment which contains a significant proportion of the 25S rRNA gene of cotton or to either the ITS1 or ITS2 regions of *B. dichromosomatica* (results not shown). It was therefore confirmed that the 25S, 5.8S and ITS regions were not cloned and it was probable that the uncloned 4 kb *B. dichromosomatica Eco* RI fragment which hybridised to GhR1 (Figure 5.1), contained these regions.

Figure 5.6:

A. An agarose gel showing DNA fragments resulting from restriction of pBdrD.

The numbers on the left represent the molecular sizes (kb) of lambda bacteriophage DNA restricted with *Hin*d III. The remaining lanes each contain approximately 3 μg of pBdrD DNA digested with the following restriction enzymes: 1. *Eco* RI; 2. *Xba* I; 3. *Xho* I; 4. *Cla* I; 5. *Eco* RI + *Xba* I; 6. *Eco* RI + *Xho* I; 7. *Eco* RI + *Cla* I; 8. *Xba* I + *Xho* I; 9. *Xba* I + *Cla* I; 10. *Xho* I + *Cla* I; 11. *Eco* RI + *Xba* I + *Xho* I; 12. *Eco* RI + *Xba* I + *Cla* I; 13. *Eco* RI + *Xho* I + *Cla* I; 14. *Xba* I + *Xho* I + *Cla* I.

B. Southern hybridisation of the gel in A with the 1.5 kb GhR1 fragment.

Lanes and molecular size markers are as above.

C. Southern hybridisation of the gel in A with the 1.7 kb GhR1 fragment.

Lanes and molecular size markers are as above.

D. Southern hybridisation of the gel depicted in A with the 2.8 kb GhR1 fragment.

Lanes and molecular size markers are as above.

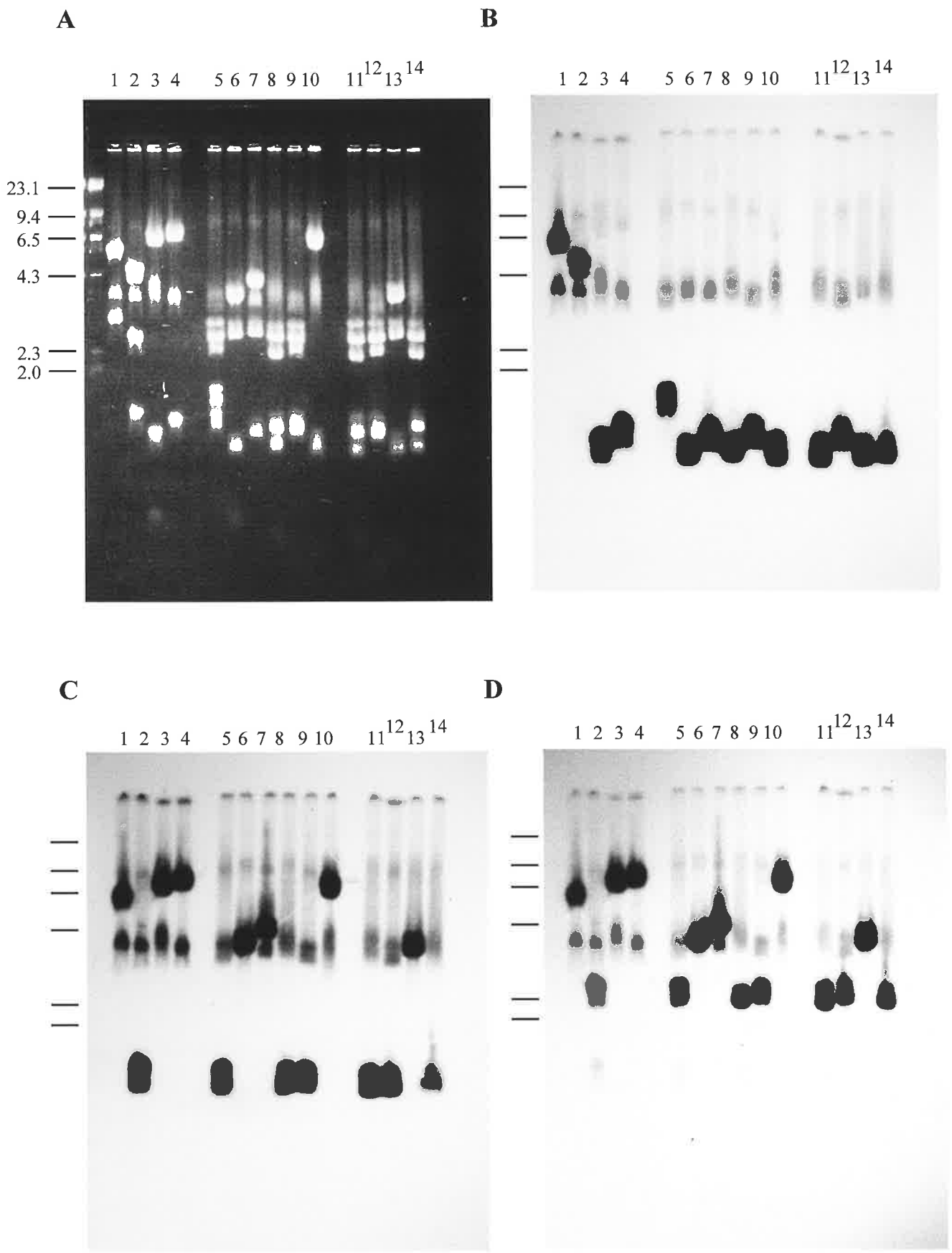

A
B
C
D
1 2 3 4 5 6 7 8 9 10 11 12 13 14
23.1
9.4
6.5
4.3
2.3
2.0

Figure 5.7:

A. An agarose gel showing DNA fragments resulting from restriction of pBdrE.

The numbers on the left represent the molecular sizes (kb) of lambda bacteriophage DNA restricted with *Hin*d III. The remaining lanes each contain approximately 3 μg of pBdrE DNA digested with the following restriction enzymes: 1. *Eco* RI; 2. *Xba* I; 3. *Xho* I; 4. *Spe* I; 5. *Eco* RI + *Xba* I; 6. *Eco* RI + *Xho* I; 7. *Eco* RI + *Spe* I; 8. *Xba* I + *Xho* I; 9. *Xba* I + *Spe* I; 10. *Xho* I + *Spe* I; 11. *Eco* RI + *Xba* I + *Xho* I; 12. *Eco* RI + *Xba* I + *Spe* I; 13. *Eco* RI + *Xho* I + *Spe* I; 14. *Xba* I + *Xho* I + *Spe* I.

B. Southern hybridisation of the gel in A with the 1.5 kb GhR1 fragment.

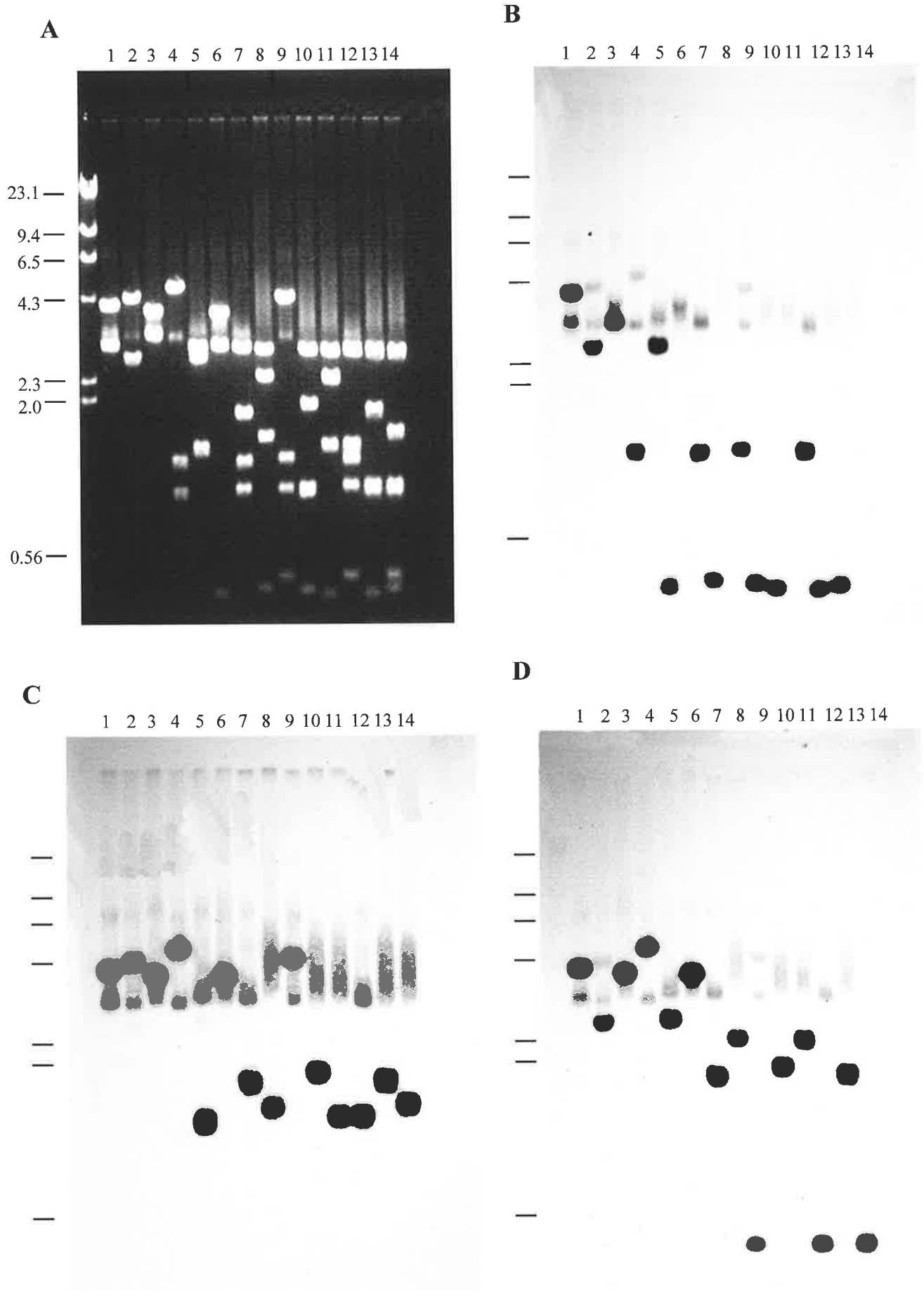
Lanes and molecular size markers are as above.

C. Southern hybridisation of the gel in A with the 1.7 kb GhR1 fragment.

Lanes and molecular size markers are as above.

D. Southern hybridisation of the gel in A with the 2.8 kb GhR1 fragment.

Lanes and molecular size markers are as above.


A
1 2 3 4 5 6 7 8 9 10 11 12 13 14
23.1
9.4
6.5
4.3
2.3
2.0
0.56
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14
C
1 2 3 4 5 6 7 8 9 10 11 12 13 14
D
1 2 3 4 5 6 7 8 9 10 11 12 13 14

5.5 Sequence Analysis of BdrD

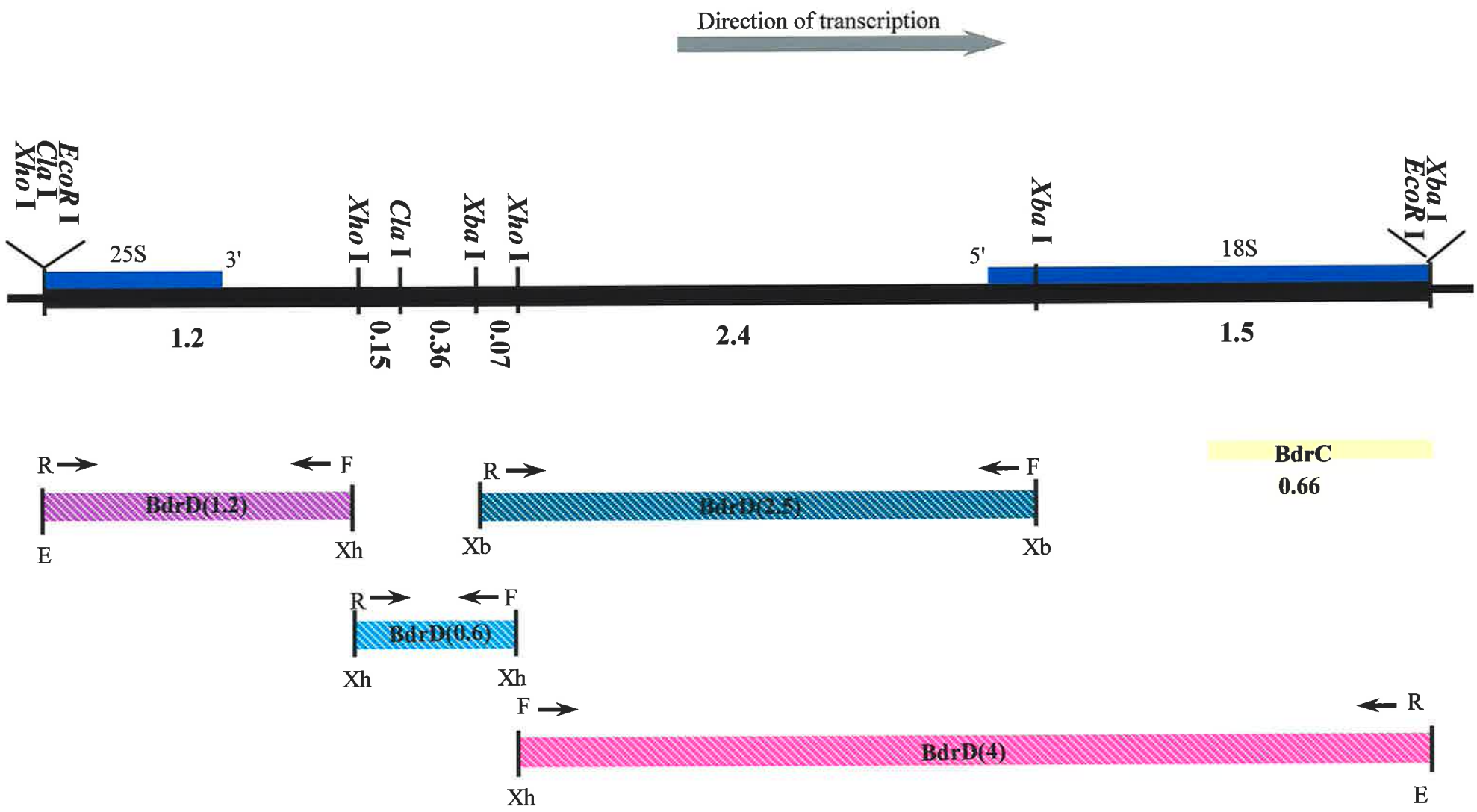
The physical map of BdrD was confirmed by subcloning and sequencing (Figure 5.8). Sequencing was carried out from both ends of the pBdrD(4) subclone (Figure 5.8). The 550 bp of sequence obtained using the reverse sequencing primer (RSP) (Figure 5.9) showed greater than 90% identity to previously characterised 18S rRNA genes from other plants (GenBank and EMBL, 1997) and terminated approximately 200 bp short of the 3' end of the 18S rRNA gene. Sequence obtained from BdrD(4) using the (-20) primer showed no significant identity with any previously characterised sequences (GenBank and EMBL, 1997). The (-20) primer sequence of subclone pBdrD(2.5) resulted in a further 800 bp of sequence, 160 bp of which corresponded to the 5' end of the 18S rRNA gene (Figure 5.13). The remaining BdrD(2.5) sequence showed a low level of similarity to the intergenic spacer region of previously characterised plant rRNA genes (GenBank and EMBL, 1997).

The sequence data obtained disagreed with previous physical positioning of the cotton 18S coding region within the 1.5 kb *Eco* RI/ *Xba* I fragment of GhR1 (Orford, 1996). The sequence of the 1.5 kb *Eco* RI/ *Xba* I fragment of BdrD, which hybridised to the 1.7 kb *Eco* RI GhR1 fragment (Figure 5.6C), indicated that it contained the 18S coding region. Restriction of pGhR1, pBdrD and pBdrE with *Eco* RI and *Xba* I, followed by hybridisation with the cucumber 18S coding region (Kavanagh and Timmis, 1986) revealed strong probe identity to the 1.7 kb fragment of GhR1, the 1.5 kb fragment of BdrD and the 1.6 kb fragment of BdrE (Figure 5.10A, A'). The implication of this result was that the coding regions of the cotton rRNA repeat unit were not arranged as previously thought and located the 18S in the 1.7 kb *Eco* RI fragment rather than the 1.5 kb *Eco* RI/ *Xba* I (Figure 5.5; Orford, 1996).

Figure 5.8:

Physical map of BdrD with restriction sites for the endonucleases *Eco* RI, *Xba* I, *Xho* I and *Cla* I marked.

The fragment sizes given are in kb and the regions containing sequence identity to 18S and 25S rRNA coding regions are indicated. The relative positions of BdrC and the subcloned DNA fragments, in addition to the ends of the subclones which were sequenced with the reverse (R) and (-20) forward (F) sequencing primers, are indicated.


Direction of transcription
EcoR I
Cla I
Xho I
25S
3'
Xho I
Cla I
Xba I
Xho I
Xba I
5'
18S
Xba I
EcoR I
1.2
0.15
0.36
0.07
2.4
1.5
R
F
BdrD(1.2)
E
Xh
R
F
BdrD(2.5)
Xb
Xb
BdrC
0.66
R
F
BdrD(0.6)
Xh
Xh
F
R
BdrD(4)
Xh
E

```
  1  TTCCTCGTTT AAGACCAACA ATTGCAATGA TCTATCCCCA TCACGATGAA
 51  ATTTCAAAGA TTTCCCGGGC CTGTCGGCCA AGGCTATATA CTCGTTGAAT
101  ACATCAGTGT AGCGCGCGTG CGGCCCAGAA CATCTAAGGG CATCACAGAC
151  CTGTTATTGC CTCAAACTTC CGTGGCCTAA AAGGCCATAG TCCCTCTAAG
201  AAGCTGGCCG TGGAGGGATA CCTCCACATA GCTAGTTAGC AGGCTGAGGT
251  CTCGTTCGTT AACGGAATTA ACCAGACAAA TCGCTCCACC AACTAAGAAC
301  GGCCATGCAC CACCACCCAT AGAATCAAGA AAGAGCTCTC AGTCTGTCAA
351  TCCTTACTAT GTCTGGACCT GGTAAGTTTC CCCGTGTTGA GTCAAATTAA
401  GCCGCAGGCT CCACTCCTGG TGGTGCCCTT CCGTCAATTC CTTTAAGTTT
451  CAGCCTTGCG ACCATACTCC CCCCGGAACC AAAAACTTTG ATTTCTCATA
501  AGGTGCCAGC GGAATCCTAA AAGCAACATC CGCTGATCCC TGGTCGGCAT
```

Figure 5.9:

Partial sequence of the pBdrD(4), 4 kb subclone, from the RSP primer of pBluescript®SK(-). The reverse complement of the sequence depicted showed greater than 90% identity to plant 18S rRNA sequences.

Figure 5.10:

A. An agarose gel of pGhR1, pBdrD and pBdrE restricted with *Eco* RI and *Xba* I. The numbers on the left represent the molecular sizes (kb) of lambda bacteriophage DNA restricted with *Hin*d III. The remaining lanes contain approximately 3 μg of the digested recombinant plasmid DNA indicated.

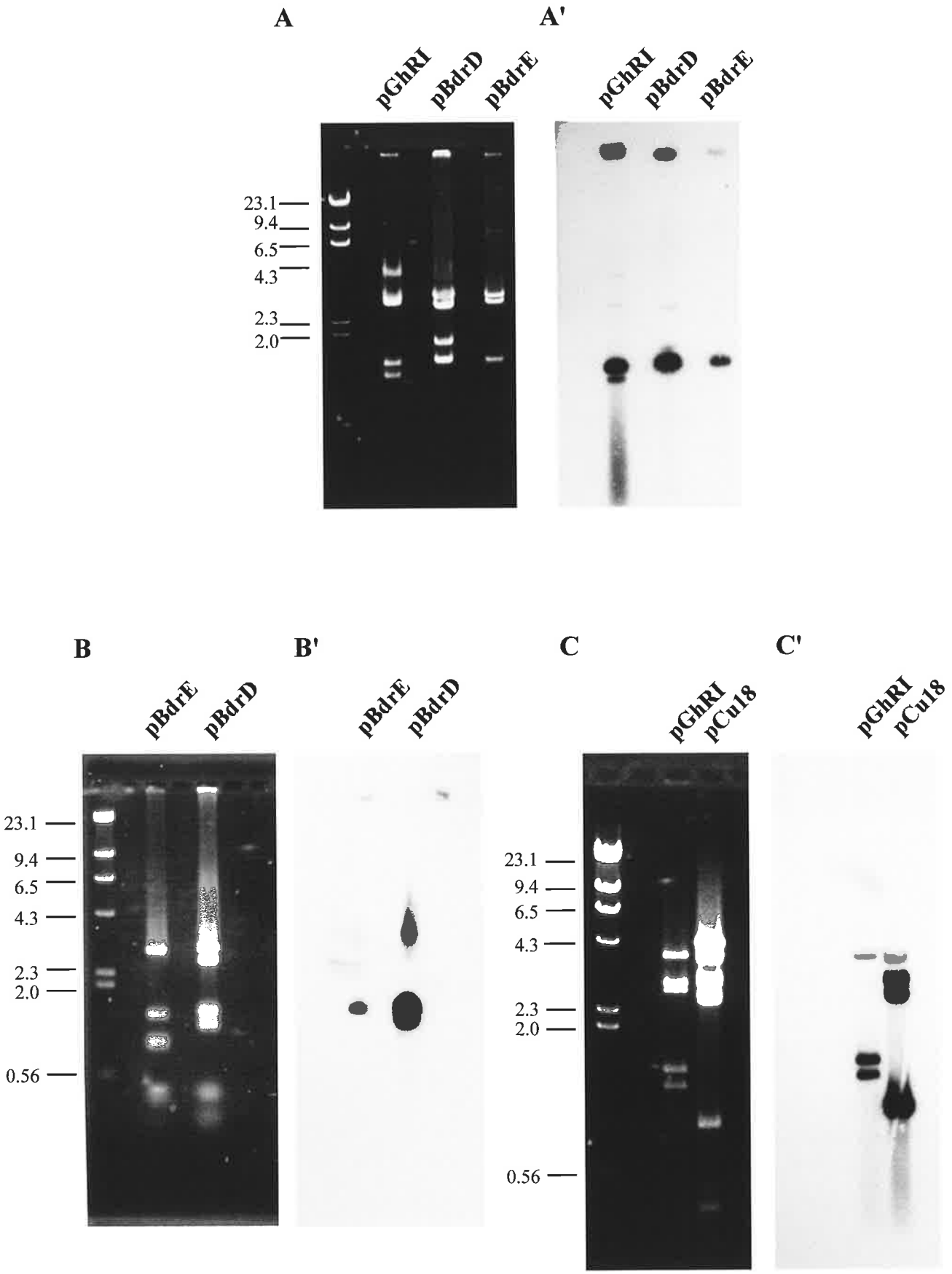
A'. Southern hybridisation of the gel in A with the 18S region of pCU18. Lanes and molecular size markers are as above.

B. An agarose gel of pBdrE and pBdrD restricted with *Eco* RI, *Xba* I, *Xho* I and *Spe* I (pBdrE) or *Cla* I (pBdrD). The numbers on the left represent the molecular sizes (kb) of lambda bacteriophage DNA restricted with *Hin*d III. The remaining lanes contain approximately 3 μg of the digested recombinant plasmid DNA indicated.

B'. Southern hybridisation of the gel in B with end-labelled total RNA from *B. dichromosomatica*. Lanes and molecular size markers are as above.

C. An agarose gel of pGhR1, and pCU18 restricted with *Eco* RI and *Xba* I. The numbers on the left represent the molecular sizes (kb) of lambda bacteriophage DNA restricted with *Hin*d III. The remaining lanes contain 2-3 μg of the digested recombinant plasmid DNA indicated.

C'. Southern hybridisation of the gel in C with end-labelled total RNA from *B. dichromosomatica*. Lanes and molecular size markers are as above.


A
A'
pGhRI
pBdrD
pBdrE
pGhRI
pBdrD
pBdrE
23.1
9.4
6.5
4.3
2.3
2.0
B
B'
C
C'
pBdrE
pBdrD
pBdrE
pBdrD
pGhRI
pCu18
pGhRI
pCu18
23.1
9.4
6.5
4.3
2.3
2.0
0.56
23.1
9.4
6.5
4.3
2.3
2.0
0.56

Sequencing of subclone BdrD(1.2) with the RSP indicated that the clone consisted of a sequence of 483 bp which showed greater than 90% similarity to the extreme 3' end of the 25S rRNA gene of other plants (see Figure 5.13), beyond which the sequence showed no significant identity with any sequences in the database (GenBank and EMBL, 1997). Hybridisation of the 1.5 kb GhR1 *Eco* RI/ *Xba* I fragment to the 1.2 kb *Xho* I/ *Eco* RI fragment of pBdrD suggested that this fragment contained the 3' region of the cotton 25S. This agreed with the original physical position of 25S rDNA in pGhR1 (see Figure 5.5) but placed it in the opposite orientation to that proposed, consistent with the 18S being located in the 1.7 kb *Eco* RI GhR1 fragment and not in the 1.5 kb fragment.

Sequencing of pBdrD(2.5) using the RSP and pBdrD(1.2) using the (-20) forward primer, showed no significant identity with any sequences in the database (GenBank and EMBL, 1997; Figure 5.13). The small size of subclone pBdrD(0.6) enabled the complete sequence of both strands to be obtained. The total 592 bp of this clone also showed no significant identity with the database (GenBank and EMBL, 1997; Figure 5.13).

Hybridisation of end-labelled total *B. dichromosomatica* RNA to pBdrD DNA restricted with *Eco* RI, *Xba* I, *Xho* I and *Cla* I and pBdrE DNA restricted with *Eco* RI, *Xba* I, *Xho* I and *Spe* I enabled confirmation of the regions of the clones which were represented in the 25S, 5.8S and 18S rRNAs. Hybridisation of labelled RNA to the 1.5 kb and 1.2 kb fragments of BdrD (Figure 5.10B, B') supported the sequencing information described above, suggesting that these fragments contained the 18S and part of the 25S coding regions respectively. There was also a lower level of RNA hybridisation to the 2.5 kb *Xba* I fragment (Figure 5.10B, B') accounted for by the presence of 160 bp of the 18S coding region in this fragment (Figure 5.8). Hybridisation of RNA was only seen to the 1.6 kb fragment of BdrE, which contains the 18S region of the clone. Labelled RNA was also hybridised to pGhR1 and pCU18 (Kavanagh and Timmis, 1986) restricted with

Eco RI and *Xba* I (Figure 5.10C). Strong hybridisation was seen to the 1.7 kb and 1.5 kb fragments of pGhR1 as well as to the 3.8 kb fragment (Figure 5.10C') thought to contain a large proportion of the 25S coding region (Figure 5.8). The hybridisation seen to GhR1 supported the evidence presented, suggesting that the 1.7 kb *Eco* RI fragment contained the 18S coding region. RNA hybridisation was observed to all CU18 fragments of the rDNA repeat unit (Figure 5.10C, C'), consistent with the physical map (Kavanagh and Timmis, 1986).

The presence of approximately 4 kb of sequence within pBdrD, between the 5' end of the 18S coding region and the 3' end of the 25S coding region (Figure 5.8), which displayed no apparent identity with the database, supported the physical map and suggested that this region contained the IGS region of *B. dichromosomatica* rDNA.

5.6 Sequence Analysis of BdrE

The physical map of BdrE was also confirmed by subcloning and sequencing (result not shown). Reverse and (-20) primer sequences of pBdrE(1.6) showed extensive similarity to the 18S rRNA gene of a range of fungal, rather than plant, 18S rRNA genes. RSP sequence of pBdrE(3.9) revealed that it contained the 3' end of the 25S rRNA gene, which again showed high levels of identity with fungal rRNA sequences. The remaining sequence obtained showed no apparent identity with any sequences in the database, suggesting that this clone contained the intergenic spacer of a fungal rRNA gene. The predicted size of the IGS of pBdrE was approximately 1.9 kb (result not shown) which is very small in comparison to the 4 kb IGS of pBdrD (Figure 5.8). The smaller size of the IGS of pBdrE, along with the database search results obtained suggested that pBdrE was not from *B. dichromosomatica* DNA but probably from contaminant fungal DNA.

5.7 Sequence Analysis of BdrC

Sequencing of pBdrC confirmed that the fragment resulted from a *Eco* RI polymorphism within BdrD corresponding to the 3' 665 bp of pBdrD(4) (Figure 5.8). The additional *Eco* RI site was identified as the deletion of an A residue within the sequence GAAATTC of BdrD.

5.8 Attempts to Clone the Remainder of the Ribosomal DNA Repeat Unit

Further attempts to clone the remainder of the rDNA repeat unit within the 4 kb *Eco* RI fragment were carried out. The probe chosen was the 2.95 kb *Eco* RI/ *Xho* I fragment of pCU18 which contains the 25S and 5.8S coding regions plus ITS1 and ITS2 (Kavanagh and Timmis, 1986) which therefore would not be expected to hybridise to any of the previously cloned regions in BdrD (Figure 5.8). Total 0B genomic DNA was restricted with *Eco* RI and ligated into *Eco* RI-restricted pBluescript®SK(-). Screening of approximately 1500 recombinants yielded two positives named pBdr85 and pBdr89. Upon digestion with *Eco* RI, pBdr85 was revealed to have an insert of approximately 150 bp and the insert in pBdr89 could not be excised from the vector using *Eco* RI. Subsequent sequencing of pBdr85 revealed that it contained 148 bp of the 18S rRNA gene not present in pBdrD (result not shown). The clone pBdr89 contained a 451 bp insert that was thought to be a cloning artefact. The sequence of Bdr89 corresponded to the extreme 3' end of the 18S gene plus ITS1 and the 5' end of the 5.8S rRNA coding region (result not shown). No other putative ribosomal clones were obtained. The inability to clone the complete region between the 3' end of the 18S and the 5' end of the 25S therefore necessitated a PCR approach to allow comparison of these regions on the A and B chromosomes.

5.9 PCR Analysis of the ITS1 and 5.8S Regions on the B Chromosomes

In the Chapters 3 and 4, microdissected B chromosome DNA enabled the ITS2 sequence of the B chromosome to be analysed. This approach overcame problems associated with the presence of the A chromosomes in whole plant genomic preparations.

A PCR based approach was undertaken to examine the B chromosome rDNA sequence in the region between the 3' end of the 18S and the 5' end of the 25S using genomic DNA containing Bs. Difficulty in identifying sequences unique to the Bs, due to the presence of A chromosome DNA in the template, was overcome by using the *Sfc* I site within the ITS2, which was previously shown to be a specific identifier of B chromosome rDNA (Chapter 3).

Initially the region between the 18S and 25S, which contains the ITS1, 5.8S and ITS2 (see Figure 3.1A) was amplified from 0B, 1B, 2B, 3B and 4B *B. dichromosomatica* genomic DNA samples using the XF18S and ER25S primers (Figure 3.2, 5.11A). The resulting product was approximately 700 bp in length (Figure 5.11A, B). Taking into account the 218 bp ITS2, 300 bp ITS1 (Section 3.2) and primer lengths, this suggested that the 5.8S coding region is approximately 150 bp that is in accordance with the normal range in plants (Kavanagh and Timmis, 1988). The B chromosome sequences were selected by restricting the 700 bp product amplified from the 1B, 2B, 3B and 4B templates with *Sfc* I which resulted in three fragments, the largest one (700 bp) representing the A chromosome product which did not contain the *Sfc* I site and hence was unrestricted. The second band of approximately 600 bp was the B chromosome region from the *Sfc* I site of the ITS2 to the XF18S primer (Figure 5.11A). The remaining 98 bp fragment, which was difficult to

Figure 5.11:

A. A diagram of the PCR products in part B of this figure. The positions of the XF18S and ER25S primers used to amplify the region, the relative positions of the *Sfc* I and *Spe* I restriction enzyme sites and the sizes of the *Sfc* I and *Spe* I restriction fragments are indicated.

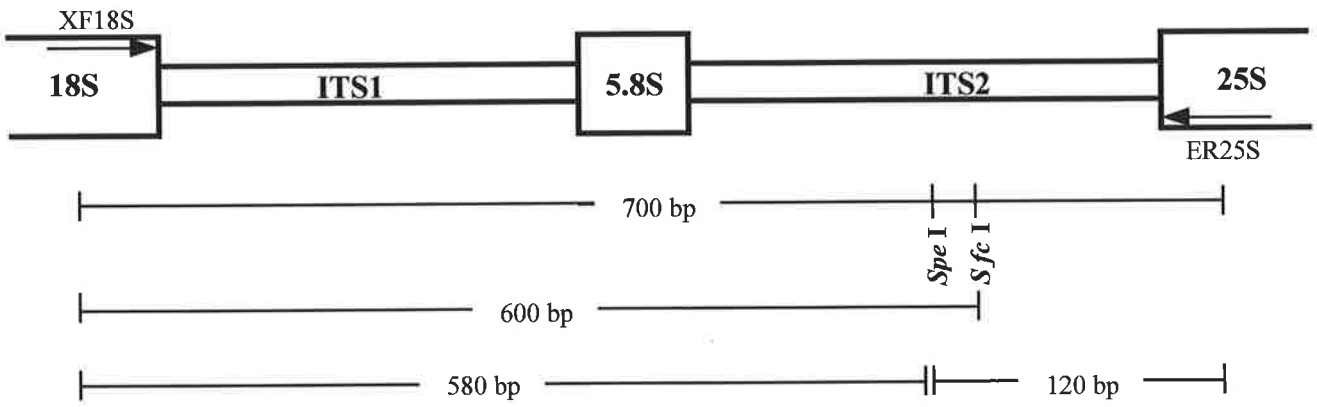
B. An agarose gel of the PCR products resulting from amplification of *B. dichromosomatica* genomic DNA with XF18S and ER25S.

The first lane contains molecular size markers (100 bp DNA ladder), consisting of fragments that range in size from 100 bp to 1000 bp in 100 bp increments, plus an additional fragment of 1500 bp. The remaining lanes each contain 10 μl of a 25 μl PCR reaction mix. The chromosome constitutions of the template DNA and restriction enzymes used are:

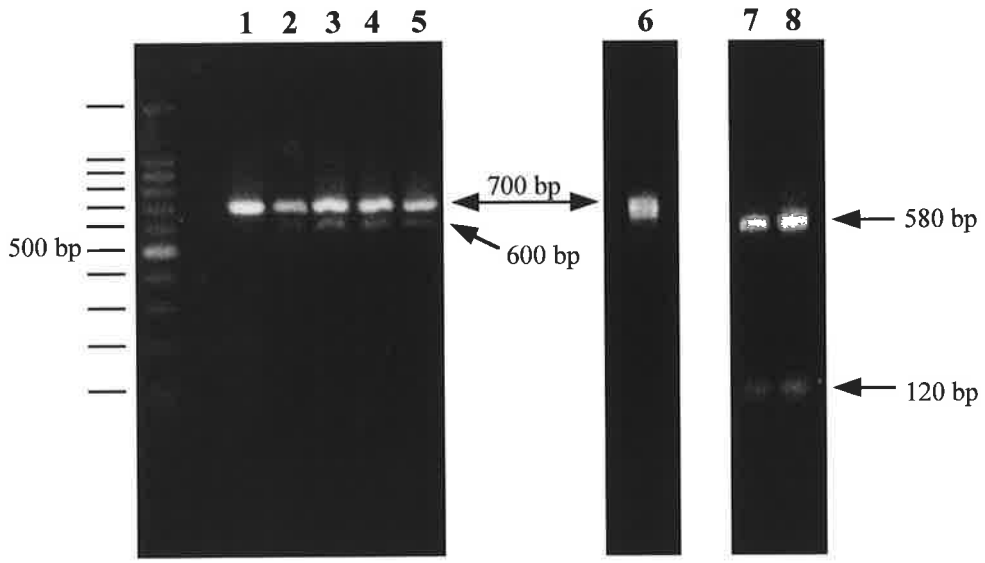
1. 0B(1) unrestricted.
2. 1B restricted with *Sfc* I.
3. 4B restricted with *Sfc* I.
4. 3B restricted with *Sfc* I.
5. 2B restricted with *Sfc* I.
6. 0B(2) unrestricted.
7. 0B(1) restricted with *Spe* I.
8. 0B(2) restricted with *Spe* I.

The double headed arrow (⟷) indicates the 700 bp PCR product amplified with XF18S and ER25S. The 600 bp fragment, which resulted when the 700 bp PCR products amplified from +B templates were digested with *Sfc* I, is arrowed and labelled. The arrows on the right indicate the 580 bp and 120 bp fragments produced when the 700 bp PCR products amplified from the 0B templates were restricted with *Spe* I.

A


XF18S
18S
ITS1
5.8S
ITS2
25S
ER25S
700 bp
Spe I
Sfc I
600 bp
580 bp
120 bp

B


1 2 3 4 5
6
7 8
500 bp
700 bp
600 bp
580 bp
120 bp

visualise on an agarose gel, contained the region from the ER25S primer to the *Sfc* I site of the B chromosomes.

The 600 bp fragment isolated from 1B, 2B, 3B and 4B PCR products restricted with *Sfc* I was gel purified and cloned in pBluescript®SK(-). Parallel experiments using 0B template DNA utilised an *Spe* I site in the ITS2 region, 5' of the B chromosome *Sfc* I site, to clone and sequence the equivalent region containing the 5.8S and ITS1 region derived from the A chromosome (Figure 5.11A). A *Cla* I restriction site within the 5.8S region (Figure 5.12) enabled subcloning and confirmation of sequence data.

Sequencing revealed that this region of the B chromosome showed no apparent rearrangements or sequence polymorphisms that were B-specific. There was, however, a low level of polymorphism seen between individual products derived from A and B chromosomes (Figure 5.12). The nucleotides that were polymorphic in different samples showed no apparent regularity and therefore may represent PCR-based errors, rather than true polymorphism in the region.

The 149 bp of 5.8S sequence obtained was greater than 95% identical to other plant 5.8S sequences in the database (GenBank and EMBL, 1998). The 5.8S sequence is thought to be only 10% divergent across angiosperms (Hershkovitz and Lewis, 1996) and therefore would be expected to show high levels of identity between closely related species. The high levels of identity of the 5.8S sequence from *B. dichromosomatica* A and B chromosomes, despite the inactivity of the rRNA genes, suggests that the B-rRNA genes remain under strong selection.

The design of the PCR primers used in the analysis of the ITS1 and 5.8S regions was based on the standard organisation of the rDNA repeat unit. It is therefore possible that the PCR products obtained were not representative of the entire population of B rRNA genes between 18S and 25S-encoding regions. For example, if large rearrangements in the rDNA

Figure 5.12:

Alignment of the 1TS1-5.8S-ITS2 nucleotide sequence derived from a range of individual *B. dichromosomatica* template DNAs.

The chromosome constitutions of the plants from which the template DNAs were prepared are indicated and the sequences of the ITS2 region from A (A1) and B chromosomes (B1) incorporated (see Figure 3.4). Polymorphisms between individual clones are marked.

Due to the way in which the A and B chromosome regions were selected for cloning the sequences are different lengths. The A chromosome derived clones (0B1 and 0B2) terminate at the *Spe* I site within the ITS2, whereas the B chromosome derived clones (1B, 2B, 3B and 4B) terminate at the *Sfc* I site within the ITS2.

```
   XF18S
0B1   TAACAAGGTT TCCGTAGGTG AACCTGCGGA AGGATCATTG TCGAAGCCTG
0B2   TAACAAGGTT TCCGTAGGTG AACCTGCGGA AGGATCATTG TCGAAGCCTG
1B    TAACAAGGTT TCCGTAGGTG AACCTGCGGA AGGATCATTG TCGAAGCCTG
2B    TAACAAGGTT TCCGTAG-TG AACCTGCGGA AGGATCATTG TCGAAGCCTG
3B    TAACAAGGTT TCCGTAGGTG AACCTGCGGA AGGATCATTG TCGAAGCCTG
4B    TAACAAGGTT TCCGTAGGTG AACCTGCGGA AGGATCATTG TCGAAGCCTG

0B1   CAAAGCAGAA CGACCCGCGA ACATGTTATA ACAACAATGC CAGGATGGGG
0B2   CAAAGCAGAA CGACCCGCGA ACATGTTATA ACAACAATGC CAGGATGGGG
1B    CAAAGCAGAA CGACCCGCGA ACATGTTATA ACAACAATGC CAGGATGGGG
2B    CAAAGCAGAA CGACCCGCGA ACATGTTATA ACAACAATGC CAGGATGGGG
3B    CAAAGCAGAA CGACCCGCGA ACATGTTATA ACAACGATGC CAGGATGGGG
4B    CAAAGTAGAA CGACTCGCGA ACATGTTATA ACAACAATGC CAGGATGGGG

0B1   GCGTCAGTTC GATCCTCGTG GCACACCGTC AGTGTGCGTC CCAGATGACC
0B2   GCGTCAGTTC GATCCTCGTG GCACACCGTC AGTGTGCGTC CCAGATGACC
1B    GCGTCAGTTC GATCCTCGTG GCACACCGTC AGTGTGCGTC CCGGATGACC
2B    GCGTCAGTTC GATCCTCGTG GCACACCGTC AGTGTGCGTC CCAGATGACC
3B    GCGTCAGTTT GATCCTCGTG GCACACCGTC AGTGTGCGTC CCAGATGACC
4B    GCGTCAGTTC GATCCTCGTG GCACACCGTC AGTGTGCGTC CCAGATGACC

0B1   CATTGGGTGT CTTGGTCGTT TCTTTGACGT AACAAAACCC CGGCACGATG
0B2   CATTGGGTGT CTTGGTCGTT TCTTTGACGT AACAAAACCC CGGCACGATG
1B    CATTGGGTGT CTTGGTCGTT TCTTTGACGT AACAAAACCC CGGCACGATG
2B    CATTGGGTGT CTTGGTCGTT TCTTTGACGT AACAAAACCC CGGCACGATG
3B    CATTGGGTGT CTTGGTCGTT TCTTTGACGT AACAAAACCC GGGCACGATG
4B    CATTGGGTGT CTTGGTCGTT TCTTTGACGT AACAAAACCC GGGCACGATG

0B1   TCAAGGAAAT TTAAATTGAA GAATTGCTTG TTCCATGATG TCCCGTTCGC
0B2   TCAAGGAAAT TTAAATTGAA GAATGGCTTG TTCCATGATG TCCCGTTCGC
1B    TCAAGGAAAT TTAAATTGAA GAATGGCTTG TTCCATGATG TCCCGTTCGC
2B    TCAAGGAAAT TTAAATTGAA GAATGGCTTG TTCCATGATG TCCCGTTCGC
3B    TCAAGGAAAT TTAAATTGAA GAATGGCTTG TTCCATGATG TCCCGTTCGC
4B    TCAAGGAAAT TTAAATTGAA GAATGGCTTG TTCCATGATG TCCCGTTCGC

0B1   GGTGTGCTCA TGGGGCGTGG CTTCTTTGTA ATCTAAAACG ACTCTCGGCA
0B2   GGTGTGCTCA TGGGGCGTGG CTTCTTTGTA ATCTAAAACG ACTCTCGGCA
1B    GGTGTGCTCA TGGGGCGTGG CTTCTTTGTA ATCTAAAACG ACTCTCGGCA
2B    GGTGTGCTCA TGGGGCGTGG CTTCTTTGTA ATCTAAAACG ACTCTCGGCA
3B    GGTGTGCTCA TGGGGCGTGG CTTCTTTGTA ATCTAAAACG ACTCTCGGCA
4B    GGTGTGCTCA TGGGGCGTGG CTTCTTTGTA ATCTAAAACG ACTCTCGGCA
                                                   5.8S

          ER58S
0B1   ACGGGATATC TCGCGTCACG CATCGATGAA GAACGTAGCA AAATGCGATA
0B2   ACGGGATATC TCGCGTCACG CATCGATGAA GAACGTAGCA AAATGCGATA
1B    ACGGGACATC TCGCGTCACG CATCGATGAA GAACGTAGCA AAATGCGATA
2B    ACGGGATATC TCGCGTCACG CATCGATGAA GAACGTAGCA AAATGCGATA
3B    ACGGGATATC TCGCGTCACG CATCGATGAA GAACGTAGCA AAATGCGATA
4B    ACGGGATATC TCGCGTCACG CATCGATGAA GAACGTAGCA AAATGCGATA
                            Cla I
```

```
0B1    CTTGGTGTGA ATTGCAGAAT CCCGTGAACC ATCGAGTTTT TGAACGCAAC
0B2    CTTGGTGTGA ATTGCAGAAT CCCGTGAACC ATCGAGTTTT TGAACGCAAG
1B     CTTGGTGTGA ATTGCAGAAT CCCGTGAACC ATCGAGTTTT TGAACGCAAG
2B     CTTGGTGTGA ATTGCAGAAT CTCGTGAACC ATCGAGTTTT TGAACGCAAG
3B     CTTGGTGTGA ATTGCAGAAT CCCGTGAACC ATCGAGTTTT TGAACGCAAG
4B     CTTGGTGTGA ATTGCAGAAT CCCGTGAACC ATCGAGTTTT TGAACGCAAG

                                                XF58S
0B1    GTGCGCCCGA AGCCACTCGG CCGAGGGCAC GTCTGCCTGG GCGTCACGCA
0B2    TTGCGCCCGA AGCCACTCGG CCGAGGGCAC GTCTGCCTGG GCGTCACGCA
1B     TTGCGCCCGA AGCCACTCGG CCGAGGGCAC GTCTGCCTGG GCGTCACGCA
2B     TTGCGCCCGA AGCCACTCGG CCGAGGGCAC GTCTGCCTGG GCGTCACGCA
3B     TTGCGCCCGA AGCCACTCGG CCGAGGGCAC GTCTGCCTGG GCGTCACGCA
4B     TTGCGCCCGA AGCCACTCGG CCGAGGGCAC GTCTGCCTGG GCGTCACGCA
A1     .......... .......... .......... .......... ........CA
B1     .......... .......... .......... .......... .......CA
                                                     5.8S

0B1    TCGCGTCGCC CCCCACCATT CTTTCCTTCG GGATGATTGG CTGGGGGCGG
0B2    TCGCGTCGCC CCCCACCATT CTTTCCTTCG GGATGATTGG CTGGGGGCGG
1B     TCGCGTCGCC CCCCAGCATT CTTTCCTTCG GGATGATTGG CTGGGGGCGG
2B     TCGCGTCGCC CCCCAGCATT CTTTCCTTCG GGATGATTGG CTGGGGGCGG
3B     TCGCGTCGCC CCCCACCATT CTTTCCTTCG GGATGATTGG CTGGGGGCGG
4B     TCGCGTCGCC CCCCACCATT CTTTCCTTCG GGATGATTGG CTGGGGGCGG
A1     TCGCGTCGCC CCCCACCGTT CTTTCCTTCG GGATGATTGG CTGGGGGCGG
B1     TCGCGTCGCC CCCCACCATT CTTTCCTTCG GGATGATTGG CTGGGGGCGG

0B1    ATACTGGTCT CCCGTTCCTC ACCGAGCGGT TGGCCAAAAC AAGAGTCCCT
0B2    ATACTGGTCT CCCGTTCCTC ACCGAGCGGT TGGCCAAAAC AAGAGTCCCT
1B     ATACTGGTCT CCCGTTCCTC ACCGAGCGGT TGGCCAAAAC AAGAGTCCCT
2B     ATACTGGTCT CCCGTTCCTC ACAGAGCGGT TGGCCAAAAC AAGAGTCCCT
3B     ATACTGGTCT CCCGTTCCTC ACCGAGCGGT TGGCCAAAAC AAGAGTCCCT
4B     ATACTGGTCT CCCGTTCCTC ACCGAGCGGT TGGCCAAAAC AAGAGTCCCT
A1     ATACTGGTCT CCCGTTCCTC ACCGAGCGGT TGGCCAAAAC AAGAGTCCCC
B1     ATACTGGTCT CCCGTTCCTC ACCGAGCGGT TGGCCAAAAC AAGAGTCCCT

0B1    TTTGAAGGAT GCACGA.... .......... .......... ..........
0B2    TTTGAAGGAT GCACGA.... .......... .......... ..........
1B     TTTGAAGGAT GCACGACTAG TGGTGGTTGA CTAAAC.... ..........
2B     TTTGAAGGAT GCACGACTAG TGGTGGTTGA CAAAAC.... ..........
3B     TTTGAAGGAT GCACGACTAG TGGTGGTTGA CAAAAC.... ..........
4B     TTTGAAGGAT GCACGACTAG TGGTGGTTGA CAAAAC.... ..........
A1     TTTGAAGGAT GCACGACTAG TGGTGGTTGA CAAAACTACG GAATCGTGTC
B1     TTTGAAGGAT GCACGACTAG TGGTGGTTGA CAAGACTACA GAATCGTGTC
                        Spe I                   Sfc I

0B1    .......... .......... .......... .......... ..........
0B2    .......... .......... .......... .......... ..........
1B     .......... .......... .......... .......... ..........
2B     .......... .......... .......... .......... ..........
3B     .......... .......... .......... .......... ..........
4B     .......... .......... .......... .......... ..........
A1     GTGTGTCTTT TCAAAAAGGT GCTTCTTAAT AGACCCAACG TGTTGTCATT
B1     GTGTGTCTTT TCAAAAAGGT GCATCTTAAT AGACCCAACG TGTTGTCATT
```

```
0B1     ............ .....
0B2     ............ .....
1B      ............ .....
2B      ............ .....
3B      ............ .....
4B      ............ .....
A1      TGACAACGCTT CGACC
B1      TGACAACGCTT CGACC
                         ←————
                         ER25S
```

were present on the Bs they would not necessarily have been amplified. Therefore heterogeneity in this region on the B chromosomes, though unlikely, cannot be completely ruled out. However, it was concluded that a proportion of the ITS2 regions which contains the B-specific *Sfc* I site are present in apparently intact rDNA repeat units in the region between the 18S and 25S coding regions and hence sequence diversity and rearrangements in this region do not account for the transcriptional inactivity.

5.10 Analysis of the Intergenic Spacer on the A Chromosomes

The organisation of eukaryotic IGS regions, including the relative positions of the rRNA promoter and associated elements, is broadly conserved (Wu and Fallon, 1998). Relative to the rDNA coding regions however, the nucleotide sequences of the IGS evolve rapidly and show both inter- and intra-specific variability. Variation in IGS length has also been seen within individual plants (Kavanagh and Timmis, 1986; Rogers and Bendich, 1987a; Rogers and Bendich, 1987b; Wu and Fallon, 1998).

5.10.1 Sequence Analysis of the Complete A Chromosome IGS within BdrD

The complete sequence of the A chromosome IGS was obtained from pBdrD, through subcloning and the design of a series of internal primers within the 2.5 kb clone (Figure 5.13).

A search of the GenBank database, with the complete A chromosome derived IGS sequence, showed low levels of identity with other plant IGS regions but the organisation of the region overall was highly conserved in comparison with other plant IGS regions. Ribosomal RNA transcription initiation sites (TIS) have been determined within the IGS of a number of different plant species (Sollner-Webb and Tower, 1986; Delcasso-Tremousaygue *et al.*, 1988; Doelling et al., 1993; Perry *et al.*, 1990; Borisjuk and Hemleben, 1993; Cordesse *et al.*, 1993; Polanco and de la Vega, 1994; Capesius, 1997)

Figure 5.13:

Complete sequence of the *B. dichromosomatica* A chromosome IGS region.

Initially sequence was obtained from pBdrD using the (-20) forward primer and RSP from pBluescript®SK(-). Additional primers designed internally to sequence the region are underlined in blue and the primers PIGS1, PIGS2, PIGS3 and P18S are underlined in red. The three repeat regions (corresponding to the regions identified in Figure 5.14) are underlined in pink, green and black respectively. Putative TIS regions are highlighted in green and restriction sites used for subcloning are boxed (*Xba* I = TCTAGA, *Xho* I = CTCGAG, *Cla* I = ATCGAT). The 3' end of the 25S and 5' end of the 18S are marked.

1 TTCACCAAGT GTTGGATTGT TCACCCACCA ATAGGGAACG TGAGCTGGGT

51 TTAGACCGTC GTGAGACAGG TTAGTTTTAC CCTACTGATG ACAGTGTTGC

101 AATAGTAATT CAACCTAGTA CGAGAGGAAC CGTTGATTCG CACAATTGGT

151 CATCGCGCTT GGTTGAAAAG CCAGTGGCGC GAAGCTACCG TGCGCTGGAT

201 TATGACTGAA CGCCTCTAAG TCAGAATCCG GGCTAGAAGC GACGCGTGTG

251 CTCGCCGCCT GTTTGCCGAC CAGCAGTAGG GGCCTCGGCC CCCAAAGGCA

301 CGTGTCGTTG GCAAAGCCTG TTCGACGGAC GAGTCGTTCA GGCCGCCTTG

351 AAGTACAATT CTCATCAAGC GGCGAGTAGA ATCCTTTGCA GACGACTTAA

401 ATACGCGACG GGGTATTGTA AGTGGCAAAA TGGCCTTGCT GCCACGATCC

PIGS1 25S

451 ACTGAGATTC AGCCCTTTGT CGCTCCGATC CGTCCCTCCC CCCCATCTAC

501 ACATCTAATT ATTTCCCCAA CCATTTATTG CGAAGCTGTA CCCCCATACT

551 TAGAGAAATT TACAAGTGTT GGGATAAAAA AAACCTACAC GGTAACAACT

601 ACGTTCCAAC CAACGCCTAA AAGAAACAAA GGATGGGTTA CATTTATTGT

651 GAGGCTGTTC CCCATACTTA GAAAAATTTA CAAGTGTTTG GATAAAAAAA

701 AACCTACACG GTAACAACTA TGTACGTTCC AACCAACCCC TAGACGAAAT

751 AAAGGGCCGA TTGCGTTGGG TTACCTTTAT GAACTTGGAA AGTCCAAGGT

801 AGACCAAGAT CAATCCATAC GGGGTCAAGT GAAACGACCT ACGGTAGTTT

851 GCCCCCCCCC CCCCCCCAAA ACTGCGAGTC GAAACGGGCC CAACACATAC

901 TAGACATAAA CGAGTCCGAA CGGGCCCGAC ACATCCGAAT AACGAGCCAT

951 CACAGATAAG ACCCACGTAA GGTTGGAAGA CAACCCGACA TCGTTGTATC

1001 CTTTTCGTTC GTCATACAGA AGGCCGACAG GGGCCTGACC CGTCCGAACT

1051 CGATATTCGG GCCGACTCGA CCCGAAAACC AACTTCTCGA TTTGGAAGCC

1101 CCTCTATGTG TGTTAGATAA GTGTTTCGCA AGTTGCATCA CATTGTTGAC

1151 TAAAATGAGT CGAAACGGTC CCGTCACGTC CTAACCCGGG GTGACGGGCC

1201 CATTCGACAC GAAAAACATG TTCTCGAGTT GAAAGTGGCG ATGTGTGTTG

1251 GATAAGTGCC AATTAAAGAT TCGGGTATAT TCTTTATTAA CATACGTTGT

1301 CGTAACGGCC GGACCCGTCC CAACCCGACA AACGGGCTGA ATGGACCAAG

1351 ATCCAACTTA CCGAGATGAA GTACATCGAT GTATGCGTTG GATGAGTGTT

1401 TCGTATCTTG TATCACCCTC TTGACATAAA CGAGCCGAAA ACGGTCCCGA

1451 GCCATCCTAA CGGGCCGATT CGACCCGGAG ACCAACTTCC CGATTTGGAA

1501 ACCCACCTAT GTGTGATAGA TATGTGTTTC GTAAGTTGCA TCACGCTCTT

1551 GACGTAAGCG AGTCGAAACG GGCCCAACTT CCCGATAAGT CCCAAGTAAA

1601 TTTTCATGTA TCACGTTCTT TACATAAAAT GGCCGAAACG GGCCCGACCC

1651 ATCCTAACCC GACAACGGGC CGAAATGACC CGAAAACCAA CGCTCGATGT

1701 ATGTCTTGGG ATTGCTTTTT CGTAAAGCCG TATCACCGTC TAGACATAAA

1751 CGACCTTAAA CGGGCCCGAC CCGGTCCAAC CCGACAACGG GCCGAATCGA

PIGS2

1801 CCCGAAACCC AACGCTCGAG TTGGAAGACG CTCGCTCTAC CATTTGTGAC

PIGS3

1851 AATGTTGGTG TTTCGTAAAG TTACAGCTCG ACCCGACAAC CTTGAACGAA

1901 CCCTCTTCTT GAACTACACC GCCCGGAACG GCCCCACATG GGGTAACCCG

1951 ACAACGGGCC GAATCGACCC GAAAAAATAA AACGTCTTTT CGAAAGTTAA

2001 TATATTTTTG TTTGCGAAGA CACACAAAAA ATTTTGTGCA AATTGGACAT

2051 AACTTCAAAA ATTATGATTA TTCAAGAAAT CAATGTCTGT TCCTGTCACT

2101 CTGCTTTTTC GCCAATATGT ATATATAGGG GAGTGGTGTT CCCCAAGCAA

2151 GGTTGCCGAG CAAAAGGTGT CATGACACCG CACACAGCCC CATTTCCTTG

2201 GTCGTTTCCG AGCAAAAGGT GTCATGACAC CGCACACAGC CCCATTTCCT

2251 TGGTCGTTTC CGAGCAAAAG GTGTCACGAC ACCGCAAAAG GTGTCACGAA

2301 CTCGTTCACA GCCCCATTTC CTTGGTCGTT GCCGAGCAAA AGGTGTCATG

2351 ACCACCTTTT GCTTGGTCGT TGCCGAGCAA AAGGTGTCAT GACACCCATT

2401 TCCTTGGTCG TTGCCGAGCA AAAGGTGTCA TGACACCCTT TTCCTTGGTC

2451 GTTGCCGAGC AAAAGGTGTC ATGACACCCT TTTCCTTGGT CGTTGCCGAG

2501 CAAAAGGTGT CATGACACCC TTTTCCTTGG TCGTTGCCGA GCAAAAGGTG

2551 TCATGACACC CATTTCCTTG GTCGTTGCCG AGCAAAAGGT GTCATGACAC

2601 CCATTTCCTT GGTCGTTGCC GAGCAAAAGG TGTCATGACA CCCTTTTGCT

2651 TGGTCGTTGC CGAGCAAAAG GTGTCATGAC ACCCATTTCC TTGGTCGTTG

2701 CCGAGCAAAA GGTGTCATGA CACCCTTTTC CTTGGTCGTT GCCGAACAAA

2751 AGGTGTCATG ACACCCTTTT CCTTGGTCGT TGCCGAACAA AAGGTGTCAT

2801 GACACCCTTT TCCTTGCCCA GACGACGTCC TGATCAAAAA AACTTTGATT

2851 ATTCAAGAAA TCAATGTCTG TTCCTGTCAC TTTGCTTTTT CTCCAATATG

2901 TATATATAGG GGGCAGTGG GTGTTGGCTG GTGGGTGATC TTGAAGTCAC

2951 GGGGATCTTC TAGAAGGGGG AGGGGTGTTT GGCTTAACGT ATAAGGCTTG

3001 GTAGTCCCTC CCGAGGGGAC TTCGGTGTTG GGAAAGGAAA TAGGGGTGGG

3051 TGCGACAAAG AAAGATCTTG ATCAAGTCGG TAGGATGGGC AGGTACCTGG

3101 ATGGGGACTC GGCTAGGCAT GTCGTATCTT TTTCACAAGA TTCATGCGTG

3151 TCTCTTGCCC GATCGTCAAA AAGCTATCGA ACGCACCATC CGAGTGGTGC

3201 CCGGTTTTAT TTTGTGGTGT TGGCCACACC GGGAATACTG CGAAAATGAT

3251 CTGTTCGTTT AATGATGCAT TCTTGTCGAA AGGCTAGTAG ATTGCTGAGA

3301 ACGTCCCTAG ATCAAACCCG GATTGCGTTA AATGATGTAT GTGGATGTGG

3351 ATGAGGGTAA GAGTCTCAAC GTGTGACCTT TTGTAGGATT GACGTGTTTC

3401 TTCTTGCACC GACCAGTCAT ACAACGATCG TAAGACGCAT TTCGGGTGGA

3451 TGCCTTAGTG TTGGTGGCTT TTGTTCAACT TGGTTCTCTT CGTATCGTGC

3501 GGTACATTTT GGGGTCGCAT ATGGGTTTCG GTTGTTCGCT TGGCTCTTGA

3551 GCCTTGCGGG TGGTTGTTTC CCAAGGCGGC TTCGGGGATG TATTTCGTAA

3601 AAGATGCATG AGTGGTGTTT GGTTTGTTAC GGGTGGTTGG CTCCCCTGCT

3651 TGTGCAGGCA CGGCCACGCG TTCATTCCTT TTCAGTTTTG CGGCAAGACT

3701 TGTATCGTTT TGATCGTATA TGAGGGTTTA CTGTGTTGCA TACCTGATCA

3751 ATGGCATCGC ATTGATAAAA AAATGTACCT TTTTCGTCAG GCGTTGCACC

3801 AGCAACGTCG GATGAACCAC GAAGCATTCT CTTTGATCCT TACGATTGAT

3851 GGTTAGTTCT GTTGATTGTT TGGCCCGTTG AGGGTGGCTT GGTCTCTTGG

3901 ATATGGAAGC CAAAGTGGGC ATGGGTCTTA TGATCCTTAT ACTGCCCTCG

3951 TTGAGCGTTC TCGCTTCTCT TACACGACCG CCTTTCACGT ATGGCTCGTC

4001 TCAGCGTGTC TGGCTACGTC GAAGAGGAAT GCTACCTGGT TGATCCTGCC

P18S

4051 AGTAGTCATA TGCTTGTCTC AAAGATTAAG CCATGCATGT GTAAGTATGA

4101 ACAAATTCAG ACTGTGAAAC TGCGAATGGC TCATTAAATC AGTTATAGTT

4151 TGTTTGATGG TACCTGCTAC TCGGATAACC GTAGTAATTC T

18S

and they generally contain a conserved region with the sequence TATATAGGGG or TATATAAGGG. Two putative transcription initiation sites of the consensus sequence TATATAGGGG were identified at nucleotide numbers 2122 and 2903 of the *B. dichromosomatica* A chromosome IGS (Figure 5.13) and it was assumed that the one closest to the 5' end of the 18S was functional based on the position of the TIS determined in other species (McMullen et al., 1986; Delcasso-Tremousaygue *et al.*, 1988; Gerstner *et al.*, 1988; Perry and Palukaitis, 1990; Zentgraf *et al.*, 1990; Borisjuk and Hemleben, 1993). Furthermore, the region downstream of the second putative TIS exhibited conservation with other members of the Asteraceae (GenBank and EMBL, 1998).

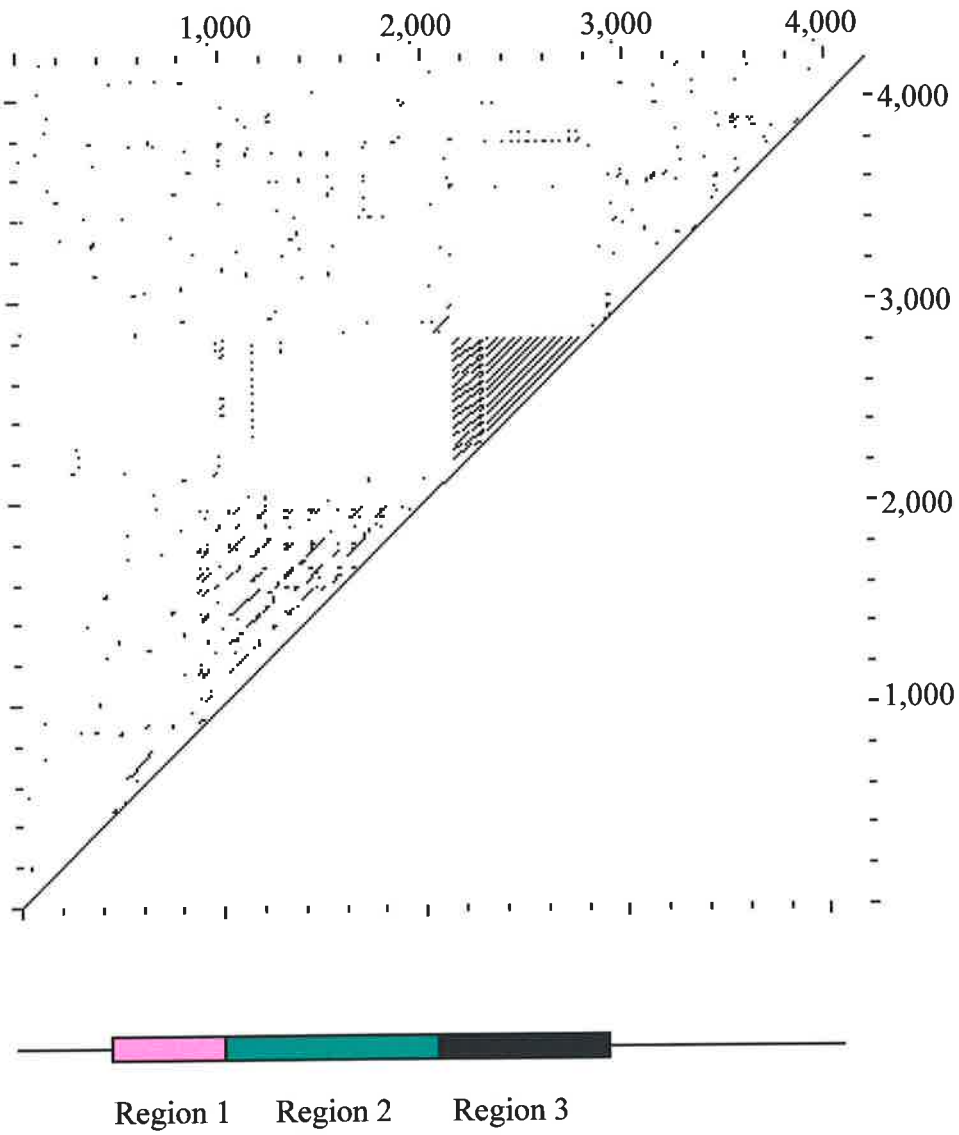
Additional sequences, in common with other plant rDNAs, were located 3' to the 25S and 5' to the 18S rRNA domains. The pyrimidine-rich region, consisting of ten cytosine residues out of eleven nucleotides, directly following the 3' terminus of the 25S, as well as the ten nucleotides (AGAGGAATGC) immediately upstream of the 18S domain (Figure 5.13), were common to a range of other plant species (GenBank and EMBL, 1998; McMullen *et al.*, 1986; Appels *et al.*, 1986; Delcasso-Tremousaygue *et al.*, 1988; Perry and Palukaitis, 1990). These sequences appear to be under selection in comparison with other sequences within the IGS, suggesting a role in the processing of the 40S precursor rRNA.

Dot matrix analysis enabled the complete intergenic spacer sequence to be compared within itself to determine regions which contain high levels of repetitive elements and to aid in defining the boundaries of the repeats (Figure 5.14; Maizel and Lenk, 1981). The matrix helped identify three major regions of repeats. The first, commencing directly after the 3' end of the 18S, consisted of two imperfect repeats of approximately 100 bp in length (Figure 5.13, 5.14). The second region, from bases 1000 to approximately base 2000, included of a number of 6-8 bp sequences present twice within approximately 50

Figure 5.14:

Dot matrix analysis of the DNA sequence of the intergenic spacer of a *B. dichromosomatica* A chromosome rRNA gene.

A self comparison of the IGS sequence (nucleotides 483 - 4032 in Figure 5.13) was made and the three blocks of repetitive regions are indicated by sets of diagonal lines. A schematic representation of the spacer is shown under the plot.


1,000
2,000
3,000
4,000
4,000
3,000
2,000
1,000
Region 1
Region 2
Region 3

nucleotides (Figure 5.13, 5.14). The third region, from base 2321 to approximately 2811, consisted entirely of 12 repeats of a 41 bp sequence, which was often imperfect differing by one or a few bases (Figure 5.13, 5.14). Overall the structure of the A chromosome *B. dichromosomatica* IGS was consistent with all other plant species studied where at least one array of tandem repeats is found upstream of the TIS. Repeats within the IGS region, such as those identified in *B. dichromosomatica* by dot matrix analysis (Figure 5.14), are ubiquitous in plants and their function remains unknown. However, they have been hypothesised to function as RNA polymerase I enhancers therefore playing an important role in the regulation of rRNA transcription (Flavell *et al.*, 1986; Hemleben *et al.*, 1988; Sardana *et al.*, 1993).

5.11 Analysis of the IGS region in 0B and +B Genomic DNA Samples

Localisation of the IGS within pBdrD (Figure 5.8) enabled the inserts of subclones containing IGS sequences to be used as probes to genomic DNA from 0B and +B plants, to look for variations in hybridisation patterns associated with the presence of Bs. The genomic DNA of twenty nine plants with and without B chromosomes, selected from a range of *B. dichromosomatica* populations, was screened for polymorphisms in the IGS region by Southern hybridisation. The DNA samples were separately restricted with *Spe* I and *Xba* I and probed with BdrD(2.5) or BdrD(0.6), which contain different segments of the IGS of *B. dichromosomatica* (see Figure 5.8).

5.11.1 Analysis of the IGS in 0B and +B Genomic DNA Restricted with *Spe* I

Genomic DNA restricted with *Spe* I was run on an agarose gel (Figure 5.15A), Southern blotted and initially hybridised with BdrD(2.5) which contained 160 bp of the 18S coding region and approximately 2.4 kb of IGS sequence (Figure 5.8). Hybridisation was observed to two main bands one greater than 23.1 kb and the other approximately

Figure 5.15:

A. An agarose gel of *B. dichromosomatica* genomic DNA restricted with *Spe* I.

The numbers on the left represent the molecular sizes (kb) of lambda bacteriophage DNA restricted with *Hin*d III. The relative quantities and degree of enzyme digestion can be seen between different samples. The presence (+) or absence (-) of B chromosomes, which was confirmed by Bd49 hybridisation (John *et al.*, 1990), is indicated at the top of individual lanes.

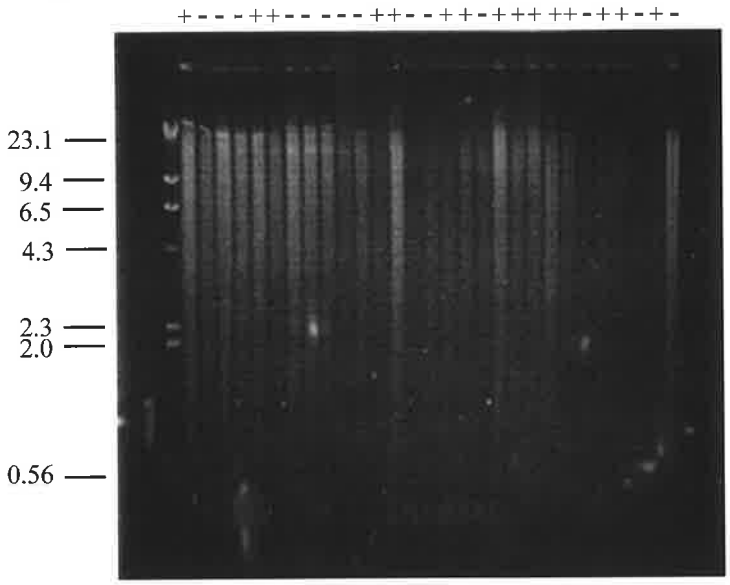
B. Southern hybridisation of the gel in A with BdrD(2.5).

Lanes, chromosome constitutions and molecular size markers are as above.

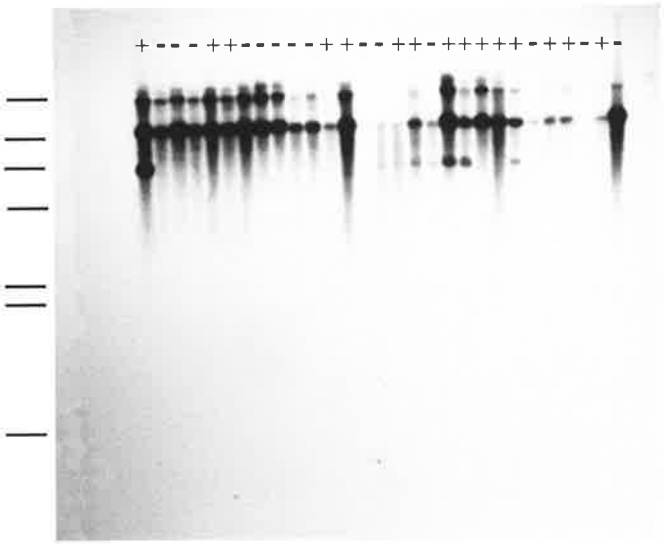
C. Southern hybridisation of the gel in A with BdrD(0.6).

Lanes, chromosome constitutions and molecular size markers are as above.

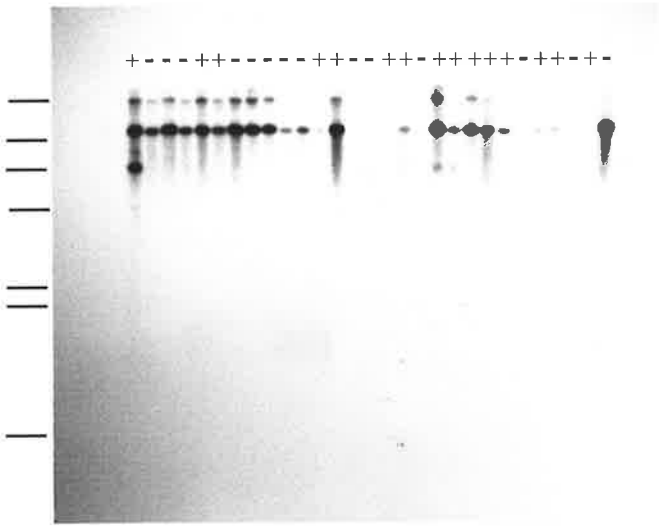
A



B



C



9.5 kb (Figure 5.15B), the larger of which was thought to be due to partial DNA digestion. In several tracks there was also an additional hybridising band of approximately 6 kb. However, there were no polymorphisms detected that were consistently present when the DNA was from plants with Bs and absent from plants without Bs (Figure 5.15B).

The same Southern blot was hybridised with BdrD(0.6) which contained 600 bp of IGS sequence upstream of the putative start of transcription (Figure 5.8). One major BdrD(0.6)-hybridising fragment of 9.5 kb was observed, as well as a larger one in several tracks resulting from partial digestion of some samples (Figure 5.15C). Although there were BdrD(0.6) hybridising fragments that were polymorphic between individual samples, none were specific to the presence of B chromosomes.

5.11.2 Analysis of the IGS in 0B and +B Genomic DNA Restricted with *Xba* I

Hybridisation of BdrD(2.5) to genomic DNA restricted with *Xba* I (Figure 5.16A) was consistently to two bands of approximately 2.5 kb (Figure 5.16B). In addition the probe hybridised to a fragment of approximately 9.5 kb (Figure 5.16B). The larger hybridising band present in several tracks was likely to represent a large number of repeats lacking the *Xba* I site at one end of BdrD(2.5) (Figure 5.8). In several samples there were also one or two larger hybridising bands thought to be due to additional polymorphisms or to partial *Xba* I digestion. However, there were no BdrD(2.5) hybridising fragments associated specifically with the presence of B chromosomes.

Hybridisation of the *Xba* I-digested genomic DNA samples with BdrD(0.6) detected hybridisation to the same bands as BdrD(2.5) (Figure 5.16B) due to sequences present in common to the two probes (Figure 5.16C). In addition, BdrD(0.6) also hybridised to a fragment of approximately 6.7 kb in all samples (Figure 5.16C). Importantly there was also a polymorphic band of approximately 4.4 kb present in all +B samples, that was absent from every 0B sample examined (Figure 5.16C), suggesting the presence of an *Xba* I

Figure 5.16:

A. An agarose gel of *B. dichromosomatica* genomic DNA restricted with *Xba* I.

The numbers on the left represent the molecular sizes (kb) of lambda bacteriophage DNA restricted with *Hin*d III. The relative quantities and degree of enzyme digestion can be seen between different samples. The presence (+) or absence (-) of B chromosome DNA, which was confirmed by Bd49 hybridisation (John *et al.*, 1990), is indicated at the top of individual lanes.

B. Southern hybridisation of the gel in A with BdrD(2.5).

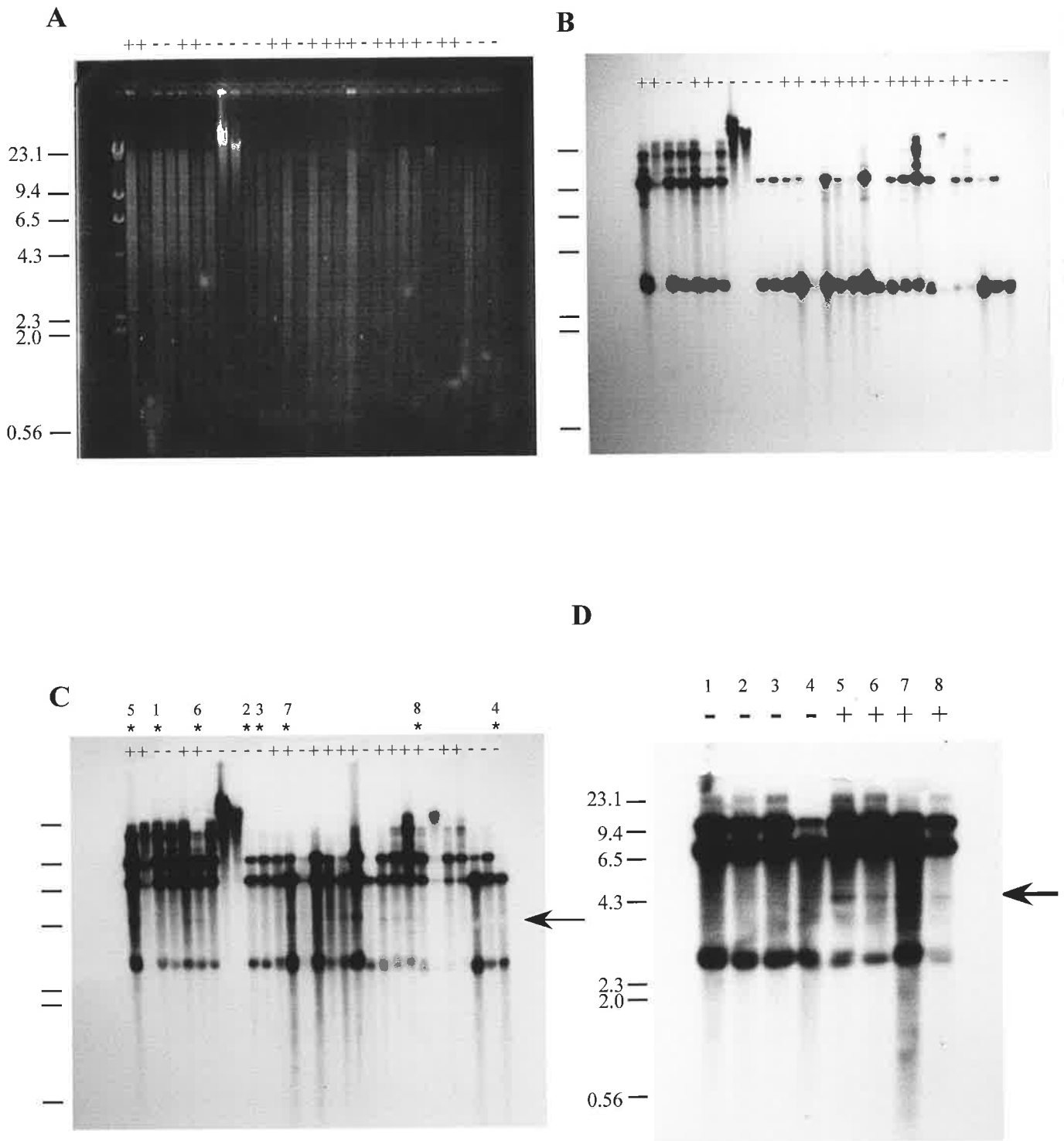
Lanes, chromosome constitutions and molecular size markers are as above.

C. Southern hybridisation of the gel in A with BdrD(0.6).

Lanes, chromosome constitutions and molecular size markers are as above. The putative B chromosome specific band is arrowed.

D. Southern blot of eight samples (marked with * in C) restricted with *Xba* I and hybridised with the 500 bp *Xba* I/ *Xho* I fragment in BdrD(0.6).

The numbers on the left represent the molecular sizes (kb) of lambda bacteriophage DNA restricted with *Hin*d III. The putative B chromosome specific band is arrowed.


A
++ - - ++ - - - - - ++ - ++++ - ++++ - ++ - - -
23.1
9.4
6.5
4.3
2.3
2.0
0.56
B
++ - - ++ - - - - - ++ - ++++ - ++++ - ++ - - -
C
5 1 6 2 3 7 8 4
* * * * * * * *
++ - - ++ - - - - - ++ - ++++ - ++++ - ++ - - -
D
1 2 3 4 5 6 7 8
- - - - + + + +
23.1
9.4
6.5
4.3
2.3
2.0
0.56

polymorphism in this region that was specific to the B chromosomes. Variations in DNA loadings made it difficult to visualise the putative +B specific hybridising band in some tracks (Figure 5.16C). Subsequent selection of four 0B and four +B samples enabled confirmation of the +B-specific, *Xba* I polymorphism (Figure 5.16D).

The chromosome constitution of each genomic DNA sample was checked by subsequent hybridisation of the same filters with Bd49, a *B. dichromosomatica* B-specific repeat sequence (John *et al.*, 1991), expected to hybridise only to +B samples. All the +B samples hybridised strongly to this probe and the 0B samples were negative (result not shown).

5.12 PCR Analysis of IGS on A and B Chromosomes

5.12.1 Attempted Identification of the B Chromosome IGS Polymorphism

To identify the origin of the putative +B-specific *Xba* I polymorphism (Figure 5.16C, D), which was possibly located within a B-specific IGS, a PCR approach was undertaken. Primers were designed to the 5' end of the 25S coding region (PIGS1, Table 2.4b) and to the 5' end of the pBdrD(0.6) sequence (PIGS2, Table 2.4b) of the A chromosome rDNA. In combination, PIGS1 and PIGS2 were designed to amplify a 1.3 kb product from the IGS region represented in BdrD (Figure 5.13).

PCR with PIGS1 and PIGS2 resulted in two major amplification products, of approximately 1.3 kb and 1.2 kb in length, from all 0B and +B templates analysed (Figure 5.17A, lanes 1-4). In contrast, only the 1.2 kb fragment and several smaller fragments were amplified from microdissected B chromosome DNA, with no trace of the 1.3 kb amplicon (Figure 5.17A, lane 5). To determine which of the products were specific to the IGS region, the gel was Southern blotted and probed with the 500 bp *Xba* I/ *Xho* I fragment of the BdrD(0.6) subclone (Figure 5.17A). Hybridisation was seen to both the

Figure 5.17:

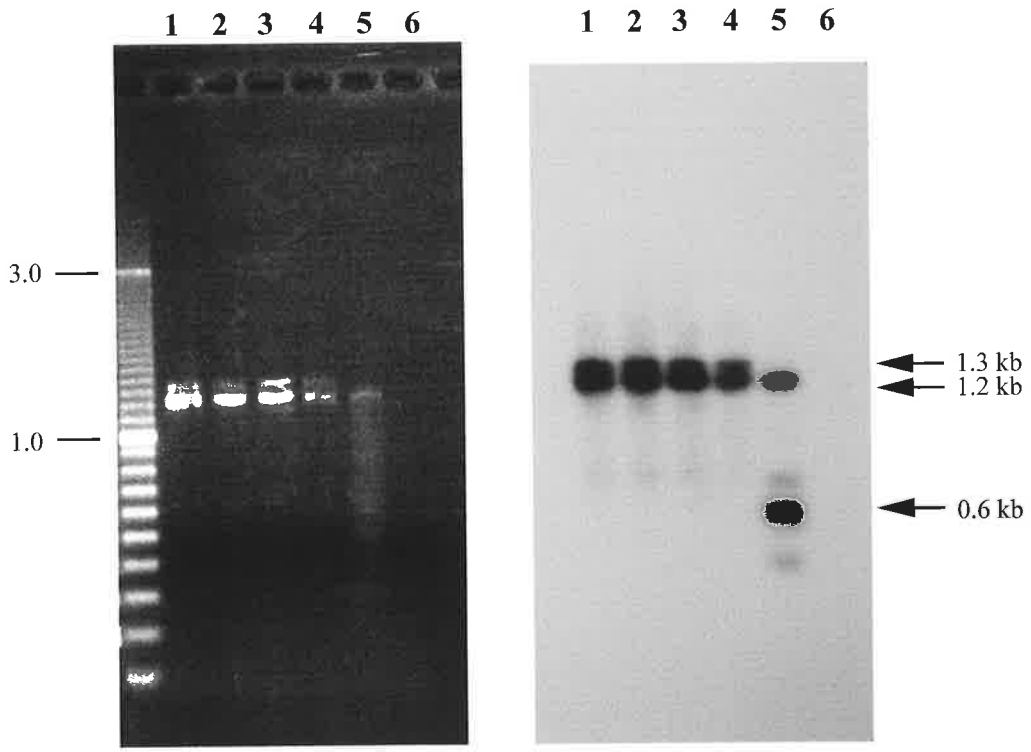
A. An agarose gel and Southern hybridisation of PCR products resulting from amplification with PIGS1 and PIGS2, hybridised with the 500 bp *Xba* I/ *Xho* I fragment of BdrD(0.6).

The first lane contains molecular size markers (100 bp ladder) which consists of fragments from 100 bp to 3000 bp in 100 bp increments. The remaining lanes each contain 10 μl of a 25 μl reaction mix. The numbers on the right indicate the sizes of hybridising fragments. The *B. dichromosomatica* template DNAs used were: **1**. 0B genomic DNA; **2**. 0B genomic DNA; **3**. +B genomic DNA; **4**. +B genomic DNA; **5**. microdissected B chromosome DNA; **6**. no template DNA (-ve control).

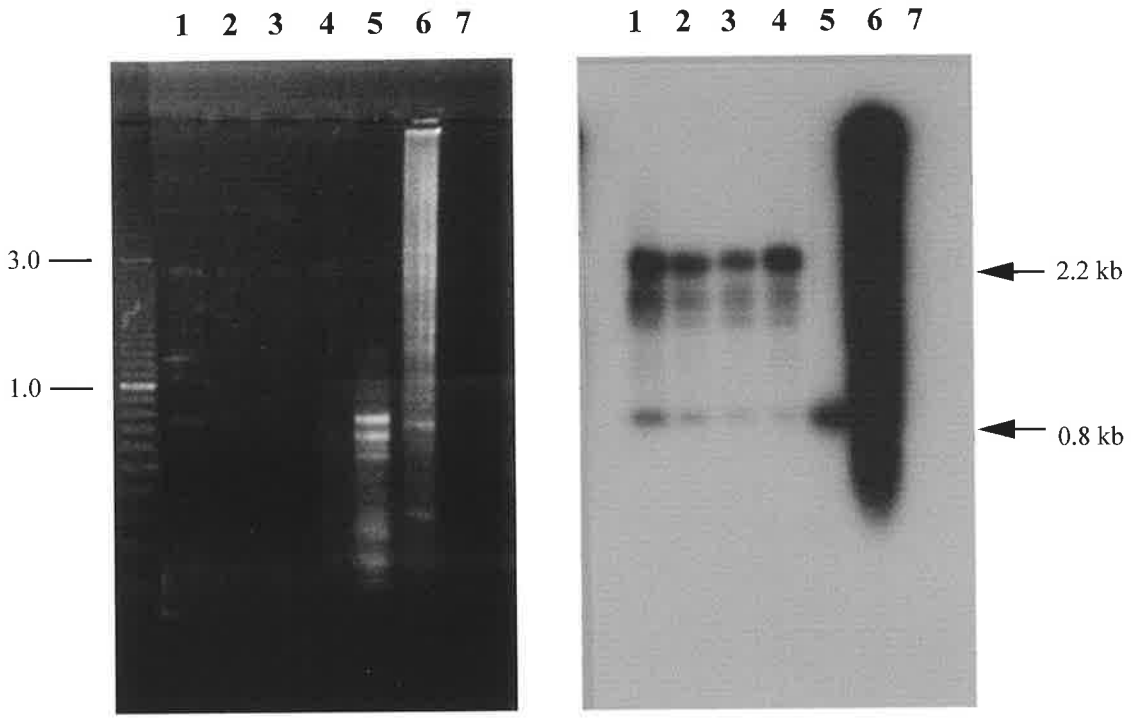
B. An agarose gel and Southern hybridisation of PCR products resulting from amplification with PIGS3 and P18S, hybridised with BdrD(2.5).

The first lane contains molecular size markers (100 bp ladder) which consists of fragments from 100 bp to 3000 bp in 100 bp increments. The remaining lanes each contain 10 μl of a 25 μl reaction mix. The numbers on the right indicate the sizes of hybridising fragments. The *B. dichromosomatica* template DNAs used were: **1**. 0B genomic DNA; **2**. 0B genomic DNA; **3**. +B genomic DNA; **4**. +B genomic DNA; **5**. microdissected B chromosome DNA; **6**. pBdrD (+ve control); **7**. no template DNA (-ve control)

A


1 2 3 4 5 6
1 2 3 4 5 6
3.0
1.0
1.3 kb
1.2 kb
0.6 kb

B


1 2 3 4 5 6 7
1 2 3 4 5 6 7
3.0
1.0
2.2 kb
0.8 kb

1.3 kb and 1.2 kb products amplified from all samples tested and there was no apparent difference in hybridisation between 0B and +B samples (Figure 5.17A). The 1.2 kb product, amplified from the microdissected B-DNA, also hybridised the probe in addition to a smaller band of 600 bp (Figure 5.17A). The 1.3 kb and 1.2 kb products amplified from a 0B template, and the 1.2 kb and 0.6 kb products from microdissected B-DNA, were cloned and sequenced.

The nucleotide sequence of the 1.3 kb clone (pBdK3) was closely similar to the sequence of the IGS from BdrD (Figure 5.18). The sequence data showed that the 1.2 kb product (pBdK2) was the result of a second PIGS2 binding site, internal to the site to which the primer was designed. The 1.2 kb product amplified from microdissected B-DNA (BdBIGS1) was identical in sequence to that from the 0B plant (Figure 5.18). One possible reason that the 1.3 kb fragment was not able to be amplified from the microdissected B-DNA is that the IGS region is truncated on the Bs. It is also possible, although unlikely, that the region was too large to be represented within the DOP-PCR-amplified DNA sample.

The sequence of the microdissected B-DNA-derived 0.6 kb PCR product (BdBIGS2) was similar to the BdBIGS1 but terminated 600 bp into the sequence. The last 11 nucleotides of BdBIGS2, leading into the PIGS1 primer region, differed from the sequence of BdBIGS1, BdK3 and BdK2 suggesting a DNA sequence morph specific to the B chromosomes (Figure 5.18).

The region amplified using PIGS1 and PIGS2 included the sequence of BdrD(0.6). Therefore it is possible that the variations between the A and B-derived PCR products, detected using these primers (Figure 5.17A), were responsible for the *Xba* I, +B-specific polymorphism identified by Southern hybridisation with BdrD(0.6) (Section 5.11.2) but this could not be experimentally established.

Figure 5.18:

Alignment of sequences of BdBIGS2, BdBIGS1, BdK3 and BdK2 with the IGS sequence (Figure 5.13). Primer binding sites of PIGS2 are underlined in red and PIGS3 in green. Primer binding site of PIGS1, within BdBIGS2, is underlined in blue.

```
                  .         .         .         .         .         .
BIGS2      TTCAGCCCTTTGTCGCTCCGATCCGAGGTTGACATGTGGCGATGTGTGCTTGATAAGTG
BIGS1     CCCATTCGACACGAAAAACATGTTCTCGAGTTGAAAGTGGCGATGTGTGTTGGATAAGTG
K3        CCCATTCGACACGAAAAACATGTTCTCGAGTTGAAAGTGGCGATGTGTGTTGGATAAGTG
K2        CCCATTCGACACGAAAAACATGTTTTCGAGTTGAAAGTGGCGATGTGTGTTGGATAAGTG
IGS       CCCATTCGACACGAAAAACATGTTCTCGAGTTGAAAGTGGCGATGTGTGTTGGATAAGTG
                                                 ************************

                  .         .         .         .         .         .
BIGS2     CAATTAAAGATTCGGGTATATTCTTTATTAACATACGTTGTCGTAACGGCCGGACCCGTC
BIGS1     CAATTAAAGATTCGGGTATATTCTTTATTAACATACGTTGTCGTAACGGCCGGACCCGTC
K3        CAATTAAAGATTCGGGTATATTCTTTATTAACATACGTTGTCGTAACGGCCGGACCCGTC
K2        CAATTAAAGATTCGGGTATATTCTTTATTAACATACGTTGTCGTAACGGCCGGACCCGTC
IGS       CAATTAAAGATTCGGGTATATTCTTTATTAACATACGTTGTCGTAACGGCCGGACCCGTC
          ************************************************************

                  .         .         .         .         .         .
BIGS2     CCCAACCCGACAAACGGGCTGAATGGACCAAGATCCAACTTACCGAGATGAAGTACATCG
BIGS1     CCCAACCCGACAAACGGGCTGAATGGACCAAGATCCAACTTACCGAGATGAAGTACATCG
K3        CCCAACCCGACAAACGGGCTGAATGGACCAAGATCCAACTTACCGAGATGAAGTACATCG
K2        CCCAACCCGACAAACGGGCTGAATGGACCAAGATCCAACTTACCGAGATGAAGTACATCG
IGS       CCCAACCCGACAAACGGGCTGAATGGACCAAGATCCAACTTACCGAGATGAAGTACATCG
          ************************************************************

                  .         .         .         .         .         .
BIGS2     ATGTATGCGTTGGATGAGTGTTTCGTATCTTGTATCACCCTCTTGACATAAACGAGCCGA
BIGS1     ATGTATGCGTTGGATGAGTGTTTCGTATCTTGTATCACCCTCTTGACATAAACGAGCCGA
K3        ATGTATGCGTTGGATGAGTGTTTCGTATCTTGTATCACCCTCTTGACATAAACGAGCCGA
K2        ATGTATGCGTTGGATGAGTGTTTCGTATCTTGTATCACCCTCTTGACATAAACGAGCCGA
IGS       ATGTATGCGTTGGATGAGTGTTTCGTATCTTGTATCACCCTCTTGACATAAACGAGCCGA
          ************************************************************

                  .         .         .         .         .         .
BIGS2     AAACGGTCCCGAGCCATCCTAACGGGCCGATTCGACCCGGAGACCAACTTCCCGATTTGG
BIGS1     AAACGGTCCCGAGCCATCCTAACGGGCCGATTCGACCCGGAGACCAACTTCCCGATTTGG
K3        AAACGGTCCCGAGCCATCCTAACGGGCCGATTCGACCCGGAGACCAACTTCCCGATTTGG
K2        AAACGGTCCCGAGCCATCCTAACGGGCCGATTCGACCCGGAGACCAACTTCCCGATTTGG
IGS       AAACGGTCCCGAGCCATCCTAACGGGCCGATTCGACCCGGAGACCAACTTCCCGATTTGG
          ************************************************************

                  .         .         .         .         .         .
BIGS2     AAACCCACCTATGTGTGATAGATATGTGTTTCGTAAGTTGCATCACGCTCTTGACGTAAG
BIGS1     AAACCCACCTATGTGTGATAGATATGTGTTTCGTAAGTTGCATCACGCTCTTGACGTAAG
K3        AAACCCACCTATGTGTGATAGATATGTGTTTCGTAAGTTGCATCACGCTCTTGACGTAAG
K2        AAACCCACCTATGTGTGATAGATATGTGTTTCGTAAGTTGCATCACGCTCTTGACGTAAG
IGS       AAACCCACCTATGTGTGATAGATATGTGTTTCGTAAGTTGCATCACGCTCTTGACGTAAG
          ************************************************************

                  .         .         .         .         .         .
BIGS2     CGAGTCGAAACGGGCCCAACTTCCCGATAAGTCCCAAGTAAATTTTCATGTATCACGTTC
BIGS1     CGAGTCGAAACGGGCCCAACTTCCCGATAAGTCCCAAGTAAATTTTCATGTATCACGTTC
K3        CGAGTCGAAACGGGCCCAACTTCCCGATAAGTCCCAAGTAAATTTTCATGTATCACGTTC
K2        CGAGTCGAAACGGGCCCAACTTCCCGATAAGTCCCAAGTAAATTTTCATGTATCACGTTC
IGS       CGAGTCGAAACGGGCCCAACTTCCCGATAAGTCCCAAGTAAATTTTCATGTATCACGTTC
          ************************************************************

                  .         .         .         .         .         .
BIGS2     TTTACATAAAATGGCCGAAACGGGCCCGACCCATCCTAACCCGACAACGGGCCGAAATGA
BIGS1     TTTACATAAAATGGCCGAAACGGGCCCGACCCATCCTAACCCGACAACGGGCCGAAATGA
K3        TTTACATAAAATGGCCGAAACGGGCCCGACCCATCCTAACCCGACAACGGGCCGAAATGA
K2        TTTACATAAAATGGCCGAAACGGGCCCGACCCATCCTAACCCGACAACGGGCCGAAATGA
IGS       TTTACATAAAATGGCCGAAACGGGCCCGACCCATCCTAACCCGACAACGGGCCGAAATGA
          ************************************************************

                  .         .         .         .         .         .
BIGS2     CCCGAAAACCAACGCTCGATGTGGAA----------------------------------
BIGS1     CCCGAAAACCAACGCTCGATGTGGAA----------------------------------
K3        CCCGAAAACCAACGCTCGATGTATGTCTTGGGATTGCTTTTTCGTAAAGCCGTATCACCG
K2        CCCGAAAACCAACGCTCGATGTGGAA----------------------------------
IGS       CCCGAAAACCAACGCTCGATGTATGTCTTGGGATTGCTTTTTCGTAAAGCCGTATCACCG
          **********************
```

```
                    .         .         .         .         .         .
BIGS2               ------------------------------------------------------------
BIGS1               ------------------------------------------------------------
K3                  TCTAGACATAAACGACCTTAAACGGGCCCGACCCGGTGCAACCCGACAACGGGCCGAATC
K2                  ------------------------------------------------------------
IGS                 TCTAGACATAAACGACCTTAAACGGGCCCGACCCGGTCCAACCCGACAACGGGCCGAATC

BIGS2               ------------------------------------------------------------
BIGS1               ------------------------------------------------------------
K3                  GACCCGAAACCCAACGCTCGAGATGGAA--------------------------------
K2                  ------------------------------------------------------------
IGS                 GACCCGAAACCCAACGCTCGAGTTGGAAGACGCTCGCTCTACCATTTGTGACAATGTTGG
```

5.12.2 Further PCR Analysis of the B Chromosome IGS

The primers PIGS3 and P18S (Figure 5.13, 5.18; Table 2.4b) were designed to further elucidate the structure of the IGS on the B chromosomes and, in particular, to compare the RNA polymerase I promoter regions of the A and B chromosomes. PCR with primers PIGS3 and P18S from 0B and +B genomic DNA templates amplified a product of approximately 2.2 kb: equivalent in length to the region in an A chromosome rDNA repeat unit (Figure 5.13). A smaller product of approximately 800 bp was present in all 0B and +B tracks (Figure 5.17B). When the microdissected B-DNA was used as a template for these primers only the 800 bp product was amplified. Cloning and sequencing of the microdissected B-DNA-derived 800 bp PCR product revealed that it was due to a second binding site for the primer PIGS3. The results obtained using the PIGS3 and P18S primers were therefore inconclusive. The inability to amplify the 2.2 kb product from the microdissected B-DNA may represent a B chromosome polymorphism or alternatively the small size of DNA in the template. In support of the latter, Southern hybridisation was unable to identify any B-specific polymorphisms when BdrD(2.5) was used as a probe to *Xba* I and *Spe* I digested 0B and +B genomic DNA (Figure 5.15, 5.16), suggesting that no sequence variation exists in this region of the IGS between the A and B chromosomes.

5.13 *IN SITU* HYBRIDISATION ANALYSIS OF THE IGS REGION ON THE A AND B CHROMOSOMES

Previously, fluorescence *in situ* hybridisation (FISH) using the cotton ribosomal repeat unit as a probe revealed the presence of rDNA at a major cluster near the satellited end of the short arm of the *B. dichromosomatica* B chromosome and, to a lesser extent, proximally on the same arm (Donald *et al.*, 1995; Houben *et al.*, 1998). The heterologous cotton probe would only be expected to hybridise to the conserved coding regions and not

to the IGS region, due to the high degree of divergence in this region even between closely related organisms (Section 3.1, 5.1). Therefore FISH, using BdrD(0.6) and BdrD(2.5) as probes, was used to confirm the presence of IGS sequences on the Bs of *B. dichromosomatica*. In addition, Southern hybridisation and PCR analysis suggested that the organisation of the B chromosome IGS may differ from that on the As (Section 5.11, 5.12). In the light of these results it was also of interest to determine if there were any detectable differences between the hybridisation of BdrD(0.6) and BdrD(2.5) to the Bs. If similar results were obtained, this would strengthen the evidence that the rDNA on the B is in complete, essentially normal, genes rather than fragments of disorganised rDNA-related sequences.

As expected hybridisation of both BdrD(2.5) and BdrD(0.6) was visualised at the NOR on the larger of the two A chromosomes (Figure 5.19). In the case of the B chromosome BdrD(2.5) and BdrD(0.6) both hybridised to the secondary constriction at the end of one arm and, to a lesser degree, a region closer to the centromere (Figure 5.19), identical to the hybridisation position of the heterologous cotton rDNA probe (Donald *et al.*, 1995). When the hybridisation signals of the BdrD(2.5) and BdrD(0.6) were overlayed there were no regions where either one of the probes hybridised differentially (Figure 5.19). The results indicated that the rDNA on the B chromosomes contains intact IGS regions at a level proportionate to the signal seen when they were hybridised with GhR1 (Donald *et al.*, 1995).

The hybridisation of BdrD(2.5) and BdrD(0.6) to the B chromosome at equivalent levels and patterns showed that *in situ* hybridisation was not able to detect differences in this region. What was clear was that the proximal region of the Bs, which hybridised the cotton rDNA probe, also contained IGS sequences and it was therefore confirmed that there was a second locus which most likely contained intact rRNA genes on the B chromosomes.

Figure 5.19:

In situ hybridisation with BdrD(0.6) and BdrD(2.5) as probes to metaphase spreads of *B. dichromosomatica*.

BdrD(0.6) was labelled with biotin and hybridisation is seen as a green signal. BdrD(2.5) was labelled with DIG and hybridisation is seen as a red signal. Using Adobe Photoshop the images were overlayed and positions where both probes hybridise appear yellow.

A. Complete 2B metaphase spread (2n=4+2B) with B chromosomes arrowed. Signals from hybridisation of BdrD(2.5) and BdrD(0.6) are overlayed.

B. Complete 2B metaphase spread with B chromosomes arrowed. The DAPI stained spread and the probes visualised in each frame are indicated.

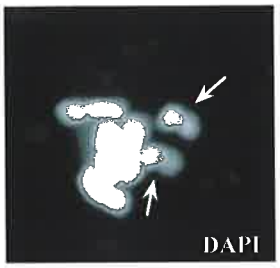
C. Complete 2B metaphase spread plus an interphase cell and an incomplete cell, with B chromosomes arrowed. The DAPI stained spread and the probes visualised in each frame are indicated.

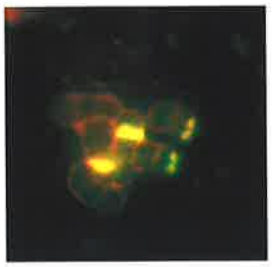
D. Incomplete metaphase spread with two A chromosomes and one B chromosome which is arrowed. The DAPI stained spread and the probes visualised for each frame are indicated.

A

BdrD(2.5)/BdrD(0.6)

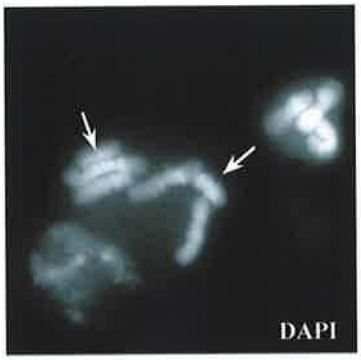
B

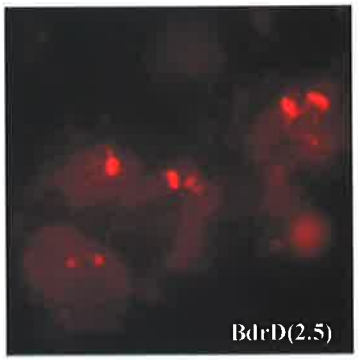


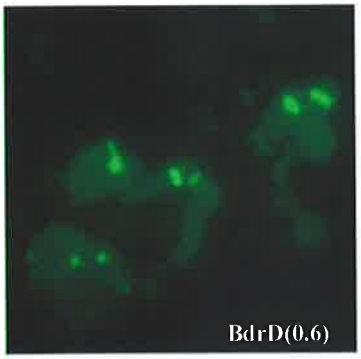


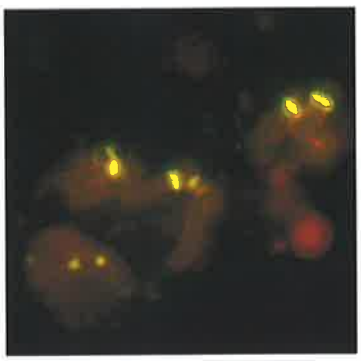
BdrD(2.5)/BdrD(0.6)

C









BdrD(2.5)/BdrD(0.6)

5.13 CONCLUSION

Cloning of part of the A chromosome rDNA repeat unit enabled the detection of an IGS-related *Xba* I polymorphism that was exclusively associated with the presence of B chromosomes. PCR and *in situ* analysis was not successful in determining the origin of the polymorphism but PCR experiments demonstrated that there was a degree of sequence variation between the regions amplified from the A and microdissected B-DNA templates.

Although the level of hybridisation of BdrD(0.6) to the +B-specific, *Xba* I fragment was low, it is possible that the polymorphism is representative of every IGS sequence on the B chromosomes, especially as previously it was estimated that the Bs contain only approximately 3% of the rRNA genes in a plant with one B chromosome (Chapter 3). Ultimately the identification of the source of the *Xba* I polymorphism, that is present in the IGS region of the B chromosomes, is crucial in determining whether it has any role in rDNA inactivity. However, the low level of probe hybridisation to the polymorphic band in genomic DNA, which suggests low copy number in the genome or high sequence divergence from the majority of the rDNA sequences, have so far precluded its characterisation.

CHAPTER 6:

Isolation of Non-Repetitive Sequences from the B Chromosomes

6.1 INTRODUCTION

Previously, in *B. dichromosomatica*, differential hybridisation and sequence enrichment methods isolated a B chromosome-specific sequence named Bd49 (Section 1.6.1b; John *et al.*, 1991). The nature of differential hybridisation causes a bias towards the isolation of high copy number repeat sequences and in accordance with this Bd49 was found to be a 149 bp, tandemly repeated sequence. *In situ* hybridisation enabled the localisation of Bd49 to the proximal region of the B chromosomes but no transcriptional activity has been detected and no functions have been deduced from the sequence (Section 1.6.1b; Leach *et al.*, 1995; Franks *et al.*, 1996). It was thought that the evolutionary origin of the B chromosomes may have been revealed by characterisation of Bd49 and its native sequence context within the B chromosome DNA. However, the flanking sequences were either highly repeated on the A chromosomes, retroviral or chloroplast derived (Franks *et al.*, 1996) and thus were not useful in determining B chromosome origin.

Despite efforts using subtractive cloning, differential screening techniques and analysis of sequences associated with Bd49 on the B, no further *B. dichromosomatica*, B-specific sequences have since been isolated from *B. dichromosomatica* (Franks *et al.*, 1996). There is therefore a requirement for non-repetitive sequences, specific to the Bs or present on the A and B chromosomes to be isolated to help in determining the origin of the *B. dichromosomatica* B chromosome and to shed light on its function.

This chapter discusses the use of two PCR-based approaches that were undertaken to isolate non-repetitive sequences specific to the B chromosomes and/or those that are located on both the A and B chromosomes. The techniques chosen involve the comparison of PCR-amplification products from genomic DNA samples with and without B chromosomes, in order to identify amplification products correlated with the presence of Bs.

Ideally, the PCR template DNA samples should be homogeneous with respect to A chromosome sequences, so that the only difference between the 0B and +B plants is the presence of B chromosomes, eliminating the possibility that differences found between the amplification products from 0B and +B genomic DNA are due to polymorphisms between the As of different individuals. However, due to the outbreeding nature of *B. dichromosomatica* it is difficult to obtain a population of 0B and +B plants with homogeneous A chromosomes. The advantage of using individuals heterogeneous for As is that polymorphisms between these chromosomes are unlikely to be incorrectly associated with the presence of Bs because they will only be amplified from one or a few individual plant DNA samples. The +B genomic DNA samples used in the following experiments were derived from plants which were cultivated from an individual plant with a single B chromosome suggesting that, although the A chromosomes were heterogeneous, the Bs were homogeneous. It was therefore not expected that there would be any polymorphisms between the B chromosomes from the individual +B samples. In addition, the use of 3B genomic DNA meant that B chromosome sequences were well represented in the template DNA and any B chromosome-specific sequences would be more readily detected.

6.2 Amplified Fragment Length Polymorphisms

Amplified fragment length polymorphism (AFLP) analysis is a method by which a selection of restriction fragments of total genomic DNA are detected by amplification using PCR (Vos *et al.*, 1995). The genomic DNA is initially cut with two different restriction enzymes (in this case *Pst* I, a rare cutter, and *Mse* I, a frequent cutter) and two double stranded adaptors (one specific for each enzyme) are ligated to the digested DNA. Following adaptor ligation, the mixture contains a selection of fragments, most of which have *Mse* I- *Mse* I ends. In addition, small proportions of fragments have *Pst* I-*Mse* I ends

and even less have *Pst* I-*Pst* I ends. Fragments with *Pst* I ends would conceivably represent low copy sequences, in comparison to *Mse* I-*Mse* I fragments which would be more likely to represent repetitive regions due to the frequent cutting nature of *Mse* I.

AFLP analysis subsequently employs PCR amplification using two oligonucleotide primers, one corresponding to each adaptor. To allow the visualisation of AFLP products after electrophoresis and autoradiography, the primer corresponding to the rare-cutting enzyme's adaptor (*Pst* I) is radioactively labelled in the final amplification step (Section 2.3.5), thereby reducing the complexity of the mixture so that only the fragments with a rare cutting enzyme site on one or two ends (representing mainly low copy sequences) are visualised. The AFLP 'profiles' of different genomic samples are then compared to look for polymorphisms.

It was considered possible that the AFLP technique would enable detection of sequences unique to Bs as well as low copy polymorphic sequences present on both the A and B chromosomes. The latter were expected to be present at greater copy number in a +B plant as well as showing polymorphism associated with location on the two chromosome types.

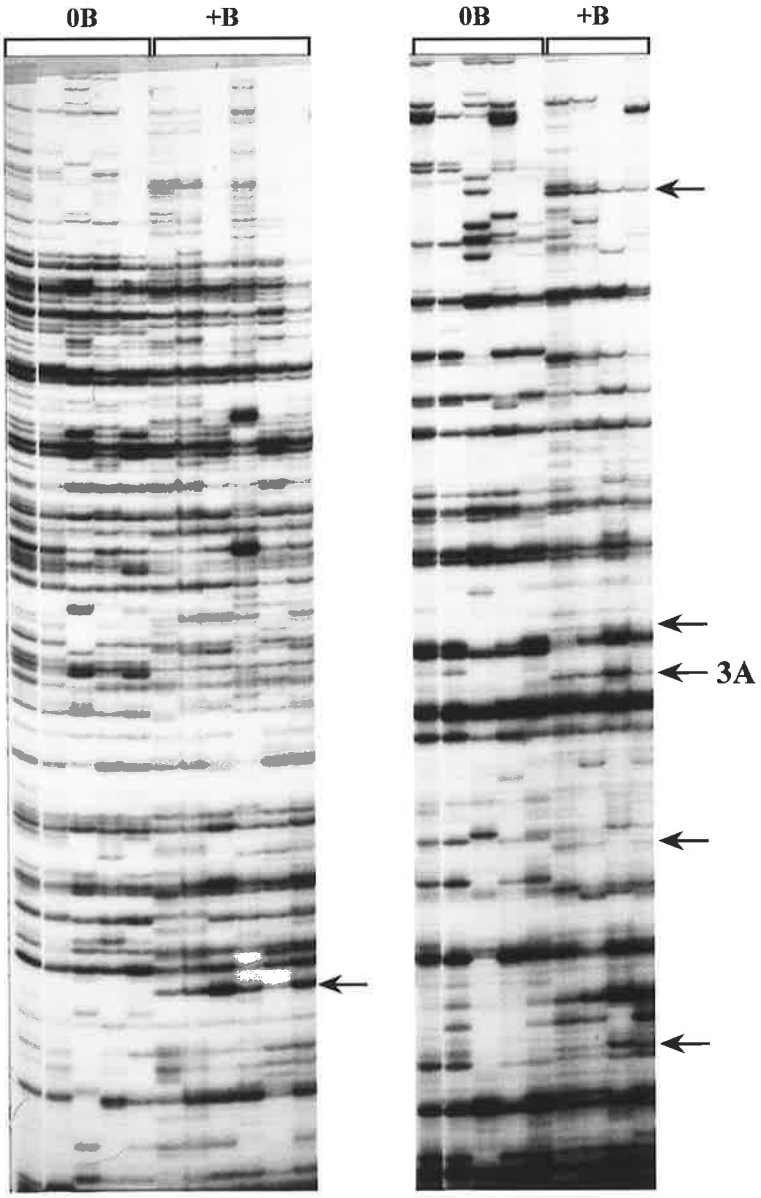
The AFLP products amplified from individual 0B and 3B templates, using all possible primer combinations (Table 2.4c), were run on acrylamide gels. The polymorphic nature of the population was highlighted by a number of bands being amplified from one or a few genomic DNA samples and not from others (Figure 6.1). However, the AFLP patterns were overall very similar for all primer combinations suggesting that the A chromosomes within the 0B and +B samples were, to a certain extent, homogeneous and that the B chromosome consists predominantly of sequences also on the A chromosomes.

The AFLP bands, 15 in all, which were present in 3B and absent in 0B amplification products (examples indicated in Figure 6.1) were gel excised, eluted from the gel slice and

Figure 6.1:

Examples of polyacrylamide gels of AFLP products using the primer *Pst* I+AA in combination with *Mse* I+CAT and *Mse* I+CGA.

The template DNA samples were derived from individual *B. dichromosomatica* plants without (0B) or with (+B) B chromosomes as indicated at the top of the gels. Examples of putative B chromosome specific AFLP products are arrowed. The band 3A, which was analysed further, is arrowed and labelled.



Mse I+CAT *Mse* I+CGA

then reamplified using the same primers that were used for the original amplification. The reamplified products were subsequently labelled during PCR with [α-^{32}P]dATP and used individually as probes to 0B and +B genomic DNA restricted with a range of endonucleases to determine B chromosome specificity (results not shown).

Hybridisation of most of the bands excised showed no differential hybridisation to 0B and +B genomic DNA. However one AFLP fragment, named 3A (Figure 6.1), which was amplified using the primers *Pst* I+AA and *Mse* I+CGA (Table 2.4c), hybridised to a band in the *Hin*d III +B track which was absent from the corresponding 0B track (Figure 6.2A, band a). A range of 0B and +B samples was subsequently restricted with *Hin*d III to determine if the 3A polymorphism seen was consistently correlated with the presence of B chromosomes. Unlike the example shown in Figure 6.2A, the hybridisation pattern of 3A was indistinguishable between 0B and +B genomic DNA restricted with *Hin*d III (Figure 6.2B) suggesting that the 0B DNA sample used in Figure 6.2A was not representative of all 0B plants. However, hybridisation levels of 3A to the putative polymorphic band (Figure 6.2A, band a) appeared to be higher to +B than 0B genomic DNA, when compared to the hybridisation levels of 3A to a larger band which hybridised the probe at an equal level in all samples (Figure 6.2B, band b). The higher level of hybridisation to +B than 0B genomic DNA in Southern blots (Figure 6.2B) suggested that sequences contained in band 3A (Figure 6.1) are present on both the B and A chromosomes of *B. dichromosomatica*.

The reamplified 3A PCR product was then cloned into pGEM®-T for further analysis. Putative positive clones were size-selected by PCR amplification across the insert with T7 and SP6 primers (Table 2.4a) and Southern hybridisation with the original 3A PCR product (Figure 6.3). The levels of hybridisation to the probe varied, indicating a mixed population

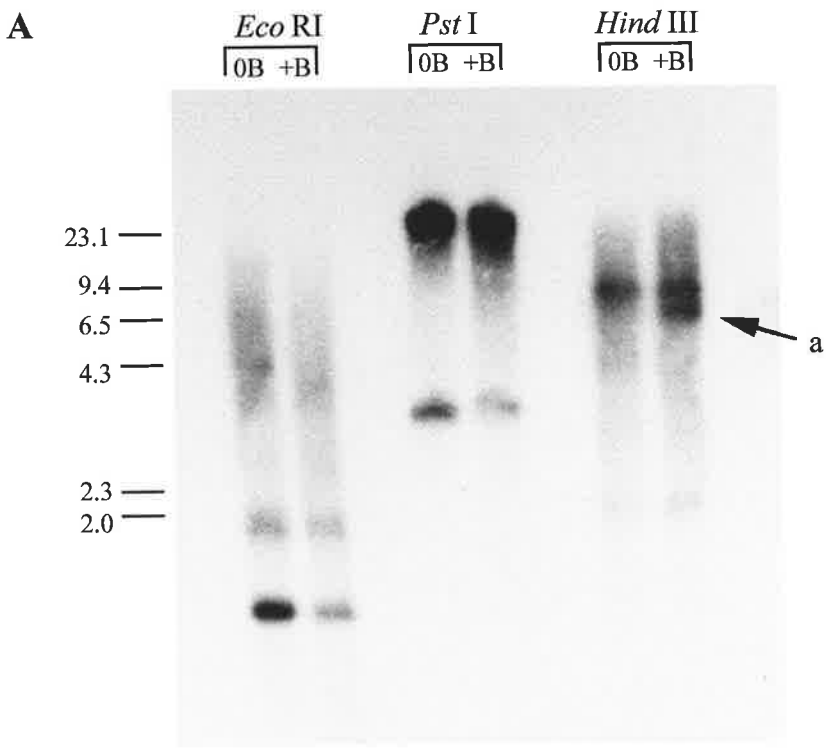
Figure 6.2:

A. Southern hybridisation of *B. dichromosomatica* genomic DNA without (0B) and with (+B) B chromosomes probed with ^{32}P-labelled AFLP product 3A.

Each lane was loaded with 2 μg of total genomic 0B or +B DNA from single plants restricted with the endonucleases indicated. The putative B chromosome specific *Hin*d III restriction fragment is arrowed and corresponds to the similarly-labelled band in part B of this figure. The numbers on the left represent the molecular sizes (kb) of lambda bacteriophage DNA restricted with *Hin*d III.

B. Southern hybridisation of a range of *B. dichromosomatica* genomic DNAs without (0B) and with (+B) B chromosomes restricted with *Hin*d III and probed with ^{32}P-labelled AFLP product 3A.

The relative quantities of DNA and degree of enzyme digestion can be seen between different samples. The 3A hybridising band which corresponds to the putative B chromosome specific fragment is arrowed and labelled (a), and the 3A hybridising band identified in all samples is arrowed and labelled (b).


A
Eco RI
0B +B
Pst I
0B +B
Hind III
0B +B
23.1
9.4
6.5
4.3
2.3
2.0
a

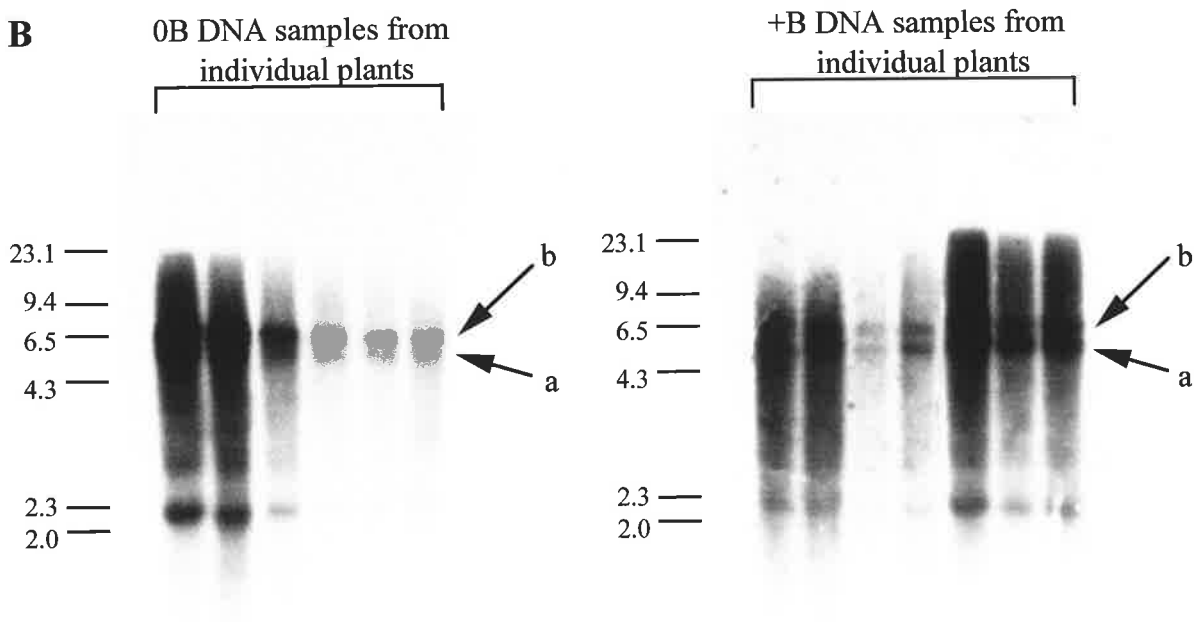
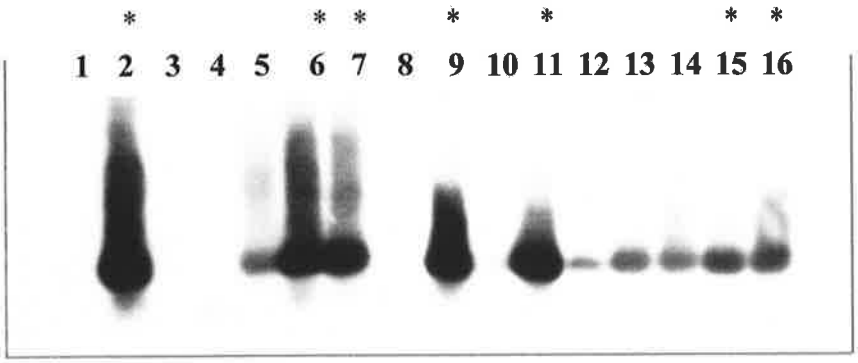

B
0B DNA samples from individual plants
+B DNA samples from individual plants
23.1
9.4
6.5
4.3
2.3
2.0
b
a

Figure 6.3:

Southern hybridisation of PCR products produced by amplification across the inserts of putative paflp3A clones (1-16) with the universal T7 and SP6 primers (Table 2.4a) and probed with AFLP product 3A.

The clones selected for sequencing are marked with an asterisk and their numbering corresponds with the names of the clones. Lanes were equally loaded with DNA, as determined by EtBr staining of the agarose gel.


* * * * * * *
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

of clones, seven of which were selected for further analysis (p2aflp3, p6aflp3, p7aflp3, p9aflp3, p11aflp3, p15aflp3 and p16aflp3; Figure 6.3).

Sequence analysis revealed that clones p2aflp3 and p11aflp3 had inserts similar to each other, with 94% nucleotide identity across the 105 bp (Figure 6.4). Clones p16aflp3 and p15aflp3 also had similar inserts which showed 99% nucleotide identity (Figure 6.4), however their low level of hybridisation to the original 3A-PCR product (Figure 6.3) suggested that they were not responsible for the +B polymorphism and they were not further analysed. The inserts of p6aflp3, p7aflp3 and p9aflp3 were different from each other and from the other cloned 3A inserts (Figure 6.4). As expected, all the clones sequenced had one *Pst* I adaptor sequence and one *Mse* I adaptor sequence, including the selective nucleotides (CGA on the *Mse* I end and TT on the *Pst* I end). The heterogeneity between sequences obtained from cloning the reamplified fragment 3A implied that within the region originally excised from the gel there was a range of fragments of similar size. It was unclear which of the cloned inserts were responsible for the 3A polymorphism between PCR products amplified from 0B and +B templates (Figure 6.1).

Inserts of the four clones p2aflp3 (11aflp3), p6aflp3, p7aflp3 and p9aflp3 (Figure 6.3) were subsequently PCR-labelled and hybridised to Southern blots of AFLP-PCR products from 0B and +B samples resolved on agarose gels. Labelled 2aflp3 (11aflp3) hybridised to AFLP products amplified from all the +B samples and at a lower level to one 0B sample (Figure 6.5A). Hybridisation of 6aflp3 was limited to AFLP products derived from three of the 0B samples (Figure 6.5B). 7aflp3 hybridised to all +B- and no 0B-derived AFLP products (Figure 6.5C) and 9aflp3 hybridised at varying levels to all 0B and all +B amplification products (Figure 6.5D).

The hybridisation of 2aflp3 and 7aflp3 predominantly to AFLP products amplified from +B samples (Figure 6.5) indicated that the ability to amplify 2aflp3 (93% identical to

Figure 6.4:

Sequence of putative paflp3 clones selected from the Southern hybridisation in Figure 6.3. The sequence of 2aflp3 is aligned with 11aflp3, and 15aflp3 is aligned with 16aflp3, and nucleotide differences are marked. Primers designed to amplify 2aflp3 (P2aflp3-1, P2aflp3-2) and 7aflp3 (P7aflp3-1 and P7aflp3-2) are underlined in red in their respective sequences.

```
2aflp     CGATAGTGTGGAAAGGGAAGAAGGCCCTC--TGAACCACTTTTCGCTCGT
11aflp    CGATAGTGTGGAAAGGGAAGAAGGTTACCAATGAACCACTTTTCGCTCGT
              P2aflp3-1

2aflp     GCAGATATTTCAAACCGTTCAAAATCTGCCTAGTGTACTCCGCGACTTGG
11aflp    GCAGATATTTCAAACCGTTCAAAATCTGCCTAGTGTACTCCGCGACTTGG
                                                P2aflp3-2

2aflp     GAATT
11aflp    GAATT 105
```

```
15aflp    CGAAACTTACCTTGATTATTCCTAGCAGTGAATAGTATAGTCCCAGTCTC
16aflp    CGAAACTTACCTTGATTATTCCTAGCAGTGAATAGTATAGTCCCACTCTC

15aflp    GTCTCCAACCAAACATTCTGCAATTCGCATTTGACGATCGTCAGGACGGC
16aflp    GTCTCCAACCAAACATTCTGCAATTCGCATTTGACGATCGTCAGGACGGC

15aflp    CCTT
16aflp    CCTT 104
```

```
6aflp     CGAGTGGTACCGGCTACATTGCTGGCAGAAGTGTAGCTGTTGGCCAGTAA

6aflp     TCGAAACTTGTGGCTCTTGGAATGCAGTTCACAAACCTTATGGCGTTGGT

6aflp     AATACTTT 108
```

```
7aflp     CGATGAAGTAAATGATCGGGCCGCATGAGCTTCTTTGCCTTGCACGATCG
              P7aflp3-1

7aflp     AAAGAGGTAGGCTAAGGAAATGGAATGCTTTGGGGTTCCCTTCTATAAAC
                                                P7aflp3-2

7aflp     GACACTT 107
```

```
9aflp     CGACTAATATTGAGGTCACGGTTACGGTGAACAATCGTGGTCCCCAACCA

9aflp     TTGGCAATCCGCAGTTGATTTTACTTGCACGAGCGGACGCAGGGATGTTA

9aflp     CGTGGAATT 109
```

Figure 6.5:

Southern hybridisation analysis of cloned AFLP products.

Each lane contains equally loaded AFLP products derived from individual genomic DNA samples from plants with the chromosome constitutions indicated. The PCR-labelled probes were:

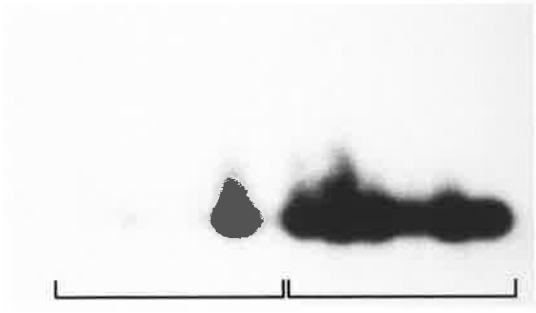
A. 2aflp3

B. 6aflp3

C. 7aflp3

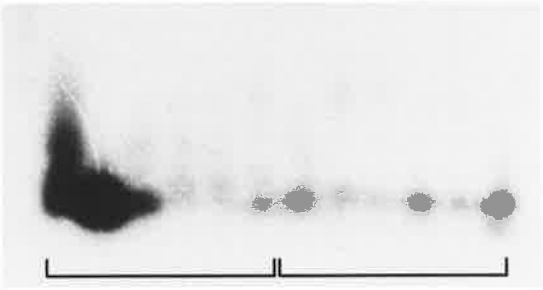
D. 9aflp3

A



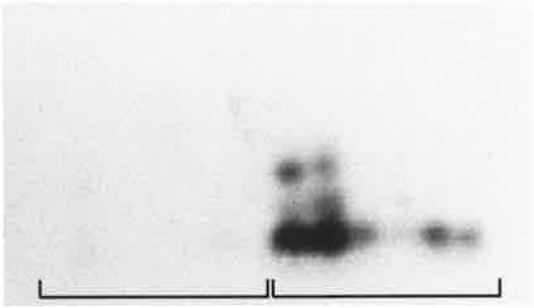
0B +B

B



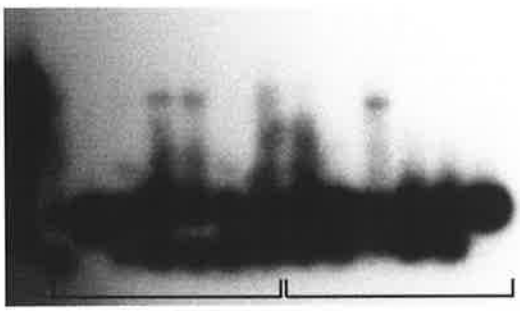
0B +B

C



0B +B

D



0B +B

11aflp3) and 7aflp3 from genomic DNA was closely related to the presence of B chromosomes in the template and may have been responsible for the original AFLP polymorphism (Figure 6.1). It was therefore thought that the 2aflp3 and 7aflp3 sequences may be present on the B chromosomes. The additional hybridisation of 2aflp3 to the AFLP product amplified from one 0B sample (Figure 6.5) suggested that this sequence may also be present on some A chromosomes.

Results from genomic DNA Southern hybridisations using 2aflp3 and 7aflp3 as probes were difficult to interpret as the levels of hybridisation were very low, suggesting that the sequences were at low copy number within the *B. dichromosomatica* genome (result not shown).

6.2.1 PCR Analysis of Putative B Chromosome AFLP Products

The inability to obtain clear results from genomic Southern hybridisations lead to a PCR approach in combination with microdissection (Section 3.2.2) to determine whether 2aflp3 and 7aflp3 sequences were in fact present on the B chromosomes. Primers were designed to 2aflp3 and 7aflp3 (Figure 6.4, Table 2.4c), so that the corresponding sequences could be amplified from genomic DNA. As expected, primers P2aflp-1 and P2aflp-2 amplified a 100 bp product from genomic DNA with B chromosomes and from the clone p2aflp3 (Figure 6.6). Initially a smear was amplified from microdissected B chromosome DNA template, but reamplification resulted in a product of equal size to 2aflp3 (Figure 6.6). The negative control containing no DNA template confirmed that there was no contamination of the PCR reaction constituents (Figure 6.6). Cloning and sequencing of the 2aflp3 product amplified from microdissected B-DNA revealed that the insert of the B-derived 2aflp3 clone showed 100% nucleotide identity to the original 2aflp3 sequence, indicating that the sequence was present on the B chromosomes (result not shown).

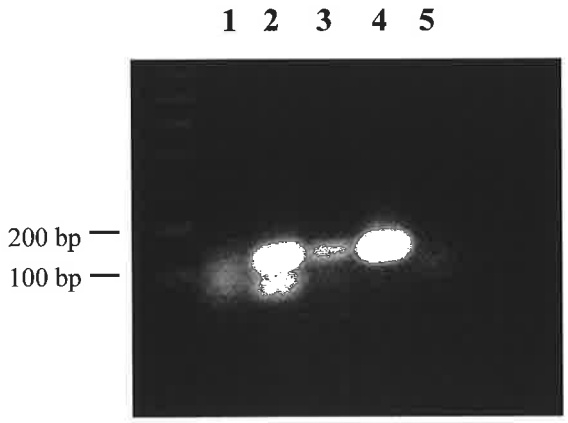
Figure 6.6:

Agarose gel of PCR products amplified with 2aflp3 specific primers (P2aflp3-1 and P2aflp3-2).

The first lane contains molecular size markers (100 bp DNA ladder), consisting of fragments that range from 100 bp to 3000 bp in 100 bp increments.

The template DNA for the remaining lanes were:

1. Microdissected B chromosome DNA.
2. Preamplified microdissected B chromosome DNA from lane 1.
3. *B. dichromosomatica* +B genomic DNA.
4. Clone p2aflp3 (+ve control).
5. No DNA template (-ve control).


1 2 3 4 5
200 bp
100 bp

Primers, P7aflp3-1 and P7aflp3-2, designed to amplify 7aflp3-like sequences from genomic DNA were not able to amplify a product from the microdissected B-DNA, despite several attempts (result not shown), suggesting that the sequence was not present on the B chromosomes. The hybridisation of 7aflp3 exclusively to the AFLP products amplified from +B samples in Figure 6.4C may be the result of a polymorphism which is specific to the A chromosomes present in the +B samples in the experiment. Alternatively, it may be that the sequence could not be amplified from the microdissected B chromosome DNA due to unknown technical difficulties.

6.2.2 Screening of 3B Genomic Library with 2aflp3

The small size of 2aflp3 lead to attempts to isolate larger clones, which would be more revealing in database searches and in studies of differences between the sequence on the A and B chromosomes, which in turn may be useful in determining the origin and evolutionary path of the B chromosomes. However, screening of 50,000 plaques of a 3B *B. dichromosomatica* genomic library was unsuccessful in isolating genomic clones of 2aflp3, supporting the idea that the sequence was of low copy in the *B. dichromosomatica* genome of plants in which they were present.

6.2.3 Summary of AFLP Analysis

In summary, the AFLP technique enabled isolation of a sequence (2aflp3) which was present in all +B samples examined as well as in DNA derived from microdissected B chromosomes. The small size and difficulty in performing Southern analysis with 2aflp3 as a probe made it difficult to determine the extent of the sequence in the population and further analysis requires a larger genomic clone to be isolated.

6.3 Random Amplified Polymorphic DNA Sequences

Random amplified polymorphic DNA sequences (RAPDs) are DNA fragments amplified from genomic DNA by PCR using 10-base oligonucleotides as primers (Williams *et al.*, 1990). The object of the work presented in this section was to isolate B chromosome-specific RAPDs from *B. dichromosomatica*. The majority of RAPDs amplified with individual primers will be shared between 0B and +B samples, representing sequences on the A chromosomes which may or may not also be present on the B chromosomes. Repeat and low copy sequences specific to the B chromosomes which contain, or are flanked by, primer recognition sites, will also be amplified and distinguished as additional RAPDs, amplified only from genomic DNA templates containing B chromosomes.

Initially, collaborators in Germany used 300 10-base primers, to amplify DNA from 0B and 3B pools of genomic DNA templates, in order to determine which primers were candidates for amplifying polymorphisms consistent with the presence of B chromosomes. Fifteen of the primers tested were selected because they each amplified PCR fragments from +B and not from 0B templates (result not shown) and these primers formed the focus of the following experiments. During attempts at repeating the results obtained in Germany only four of the original fifteen primers showed consistent amplification of *B. dichromosomatica* genomic DNA (Figure 6.7A). These primers (V12, Y2, Z6 and AC5) were used to amplify from 'pools' of 0B and 3B genomic DNA samples and the PCR products were resolved on agarose gels (Figure 6.7A, B). RAPDs which were consistently amplified from +B pooled DNA templates and not from 0B pooled DNA (namely 2R, 4R, 6R , 7R, CR, ER and FR; Figure 6.7A, B) were excised from the gel, freeze-squeezed and cloned into the vector pGEM®-T. Inserts of putative clones were confirmed as

Figure 6.7:

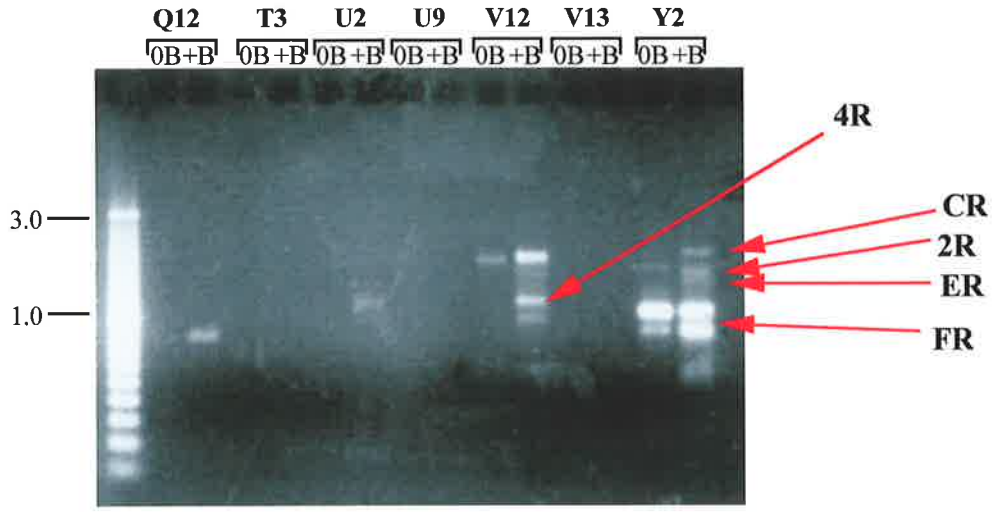
A. Agarose gels of PCR products amplified with the 10-mer primers indicated, from 0B and +B pooled *B. dichromosomatica* genomic DNA samples.

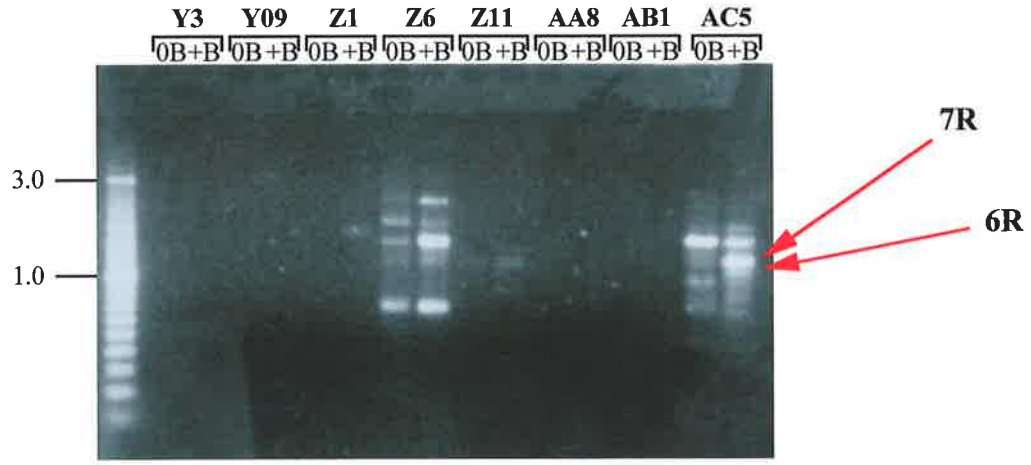
The RAPD bands that were gel excised for further analysis are arrowed and labelled. The first lanes contain molecular size markers (100 bp DNA ladder), consisting of fragments that range from 100 bp to 3000 bp in 100 bp increments.

B. Agarose gel of PCR products amplified with the 10-mer primers indicated, from 0B and +B pooled *B. dichromosomatica* genomic DNA samples.

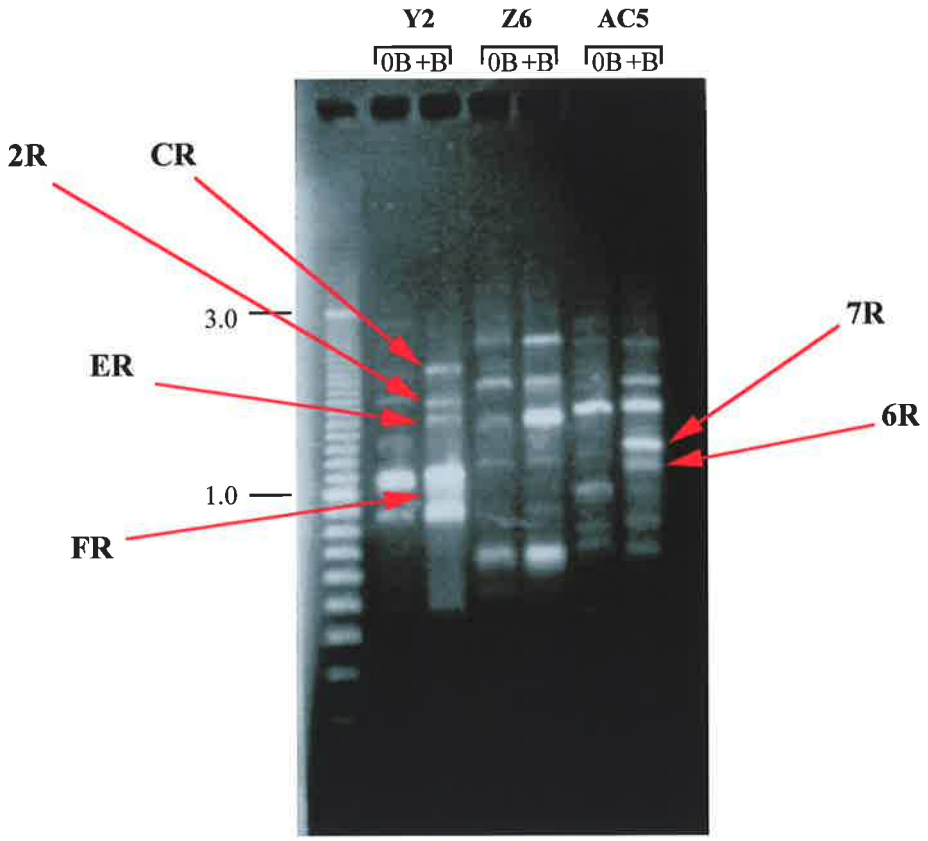
The RAPD bands that were gel excised for further analysis are arrowed and labelled. The first lanes contain molecular size markers (100 bp DNA ladder), consisting of fragments that range from 100 bp to 3000 bp in 100 bp increments.

A


Q12
T3
U2
U9
V12
V13
Y2
0B+B
3.0
1.0
4R
CR
2R
ER
FR


Y3
Y09
Z1
Z6
Z11
AA8
AB1
AC5
0B+B
3.0
1.0
7R
6R

B


Y2
Z6
AC5
0B+B
2R
CR
3.0
ER
1.0
FR
7R
6R

representative of the RAPD fragment by hybridisation with the initial freeze-squeezed products (result not shown).

6.3.1 Analysis of Putative B Chromosome Specific RAPDs

All the putative B-specific RAPDs were analysed to determine their B chromosome status by Southern hybridisation. The inserts from clones pBd2R, pBd4R, pBd6R and pBd7R, originally obtained using RAPD primers V12, Y2 and AC5 (Figure 6.7), were used as probes to *Eco* RI digested genomic DNA with and without B chromosomes to look for differences in hybridisation patterns associated with the presence of B chromosomes (Figure 6.8).

The insert from clone pBd2R hybridised to *Eco* RI fragments of both 0B and +B genomic DNA samples (Figure 6.8B). High levels of hybridisation of Bd2R occurred to a ladder of bands, suggesting that the sequence was a high copy number repeat. The lack of differentiation between 0B and +B samples suggested that the sequence was present on the A chromosomes, or on the A and B chromosomes in a similar organisation (Figure 6.8B).

The insert from clone pBd4R displayed lower levels of hybridisation to *Eco* RI digested genomic DNA (Figure 6.8C) when compared with Bd2R (Figure 6.8B) and showed a smear with several superimposed hybridising fragments. Again, no difference in overall hybridisation pattern was detected between 0B and +B samples, indicating that RAPD fragment 4R was not B-specific. Despite equal amounts of DNA being present in each track (Figure 6.8A) the levels of hybridisation of Bd4R varied considerably between individual genomic DNA samples, suggesting that the sequence varied in copy number between different plants (Figure 6.8C). The apparent heterogeneous nature of the sequence may account for the initial differential 4R RAPD amplification product (Figure 6.6A). Similarly, no differential hybridisation of Bd7R was apparent between 0B and +B genomic DNA samples (Figure 6.8D). The additional band in one of the 3B tracks

Figure 6.8:

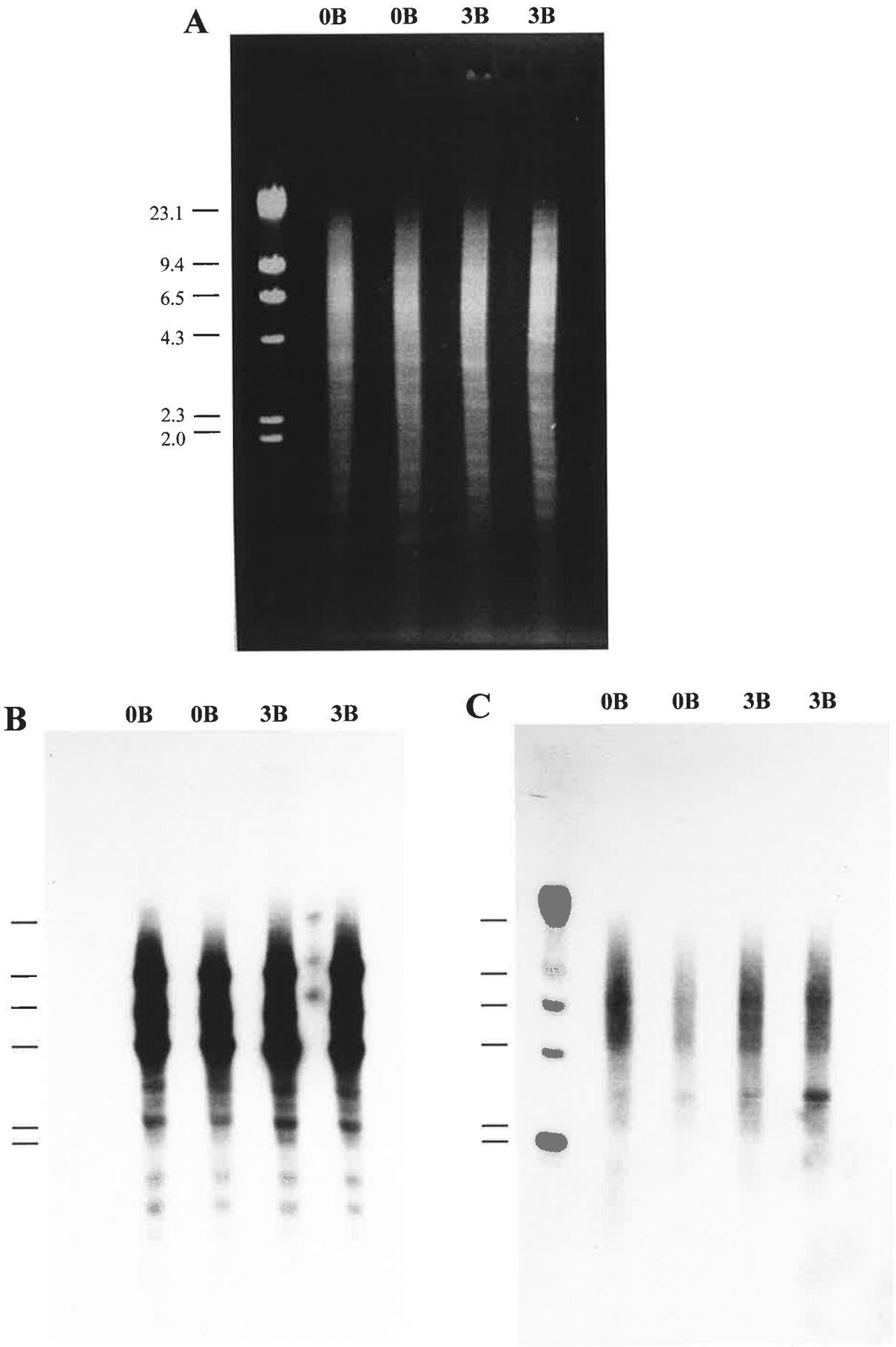
A. Agarose gel showing *B. dichromosomatica* genomic DNA restricted with *Eco* RI. Each lane was loaded with 3 μg of genomic DNA. The numbers on the left represent the molecular sizes (kb) of lambda bacteriophage DNA restricted with *Hin*d III. The chromosome constitutions of the plants from which the genomic DNA was prepared are indicated.

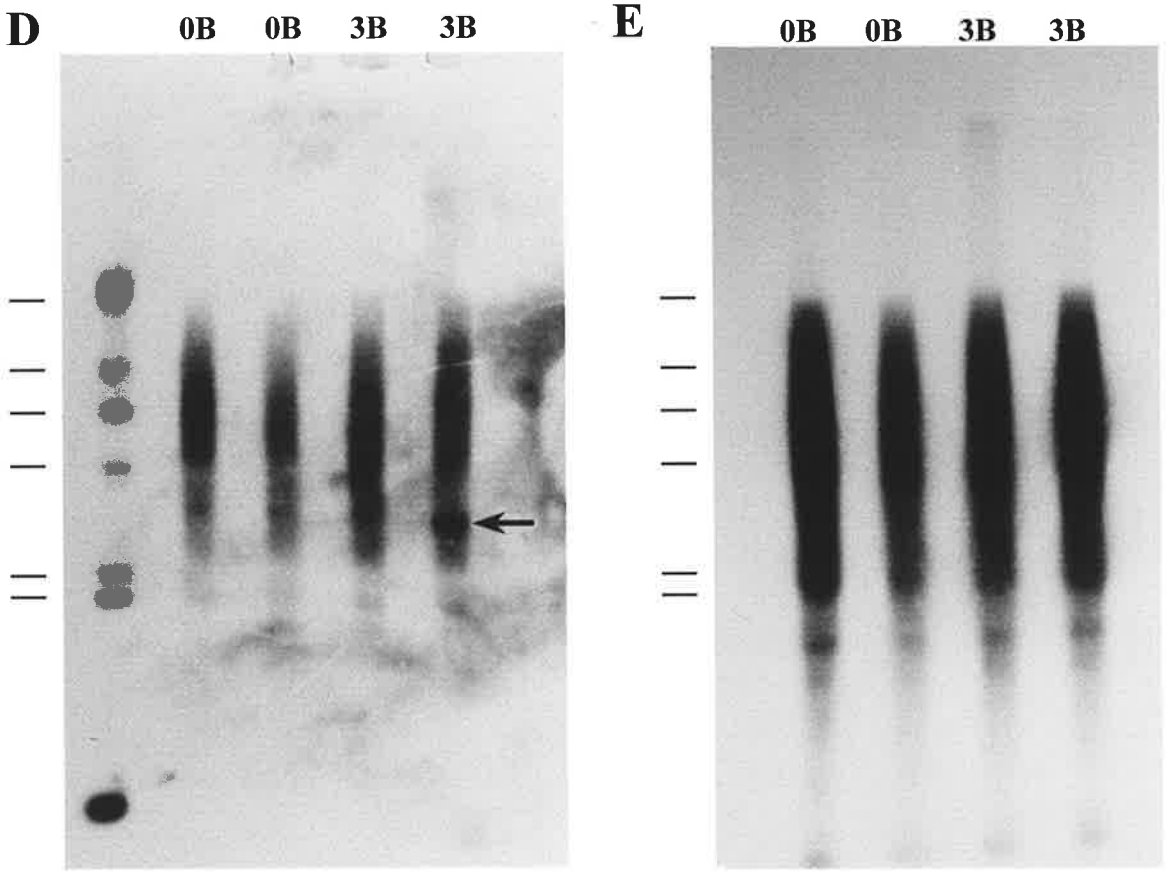
B. Southern hybridisation of the agarose gel in A with the insert from pBd2R. Lanes and molecular size markers are as above.

C. Southern hybridisation of the agarose gel in A with the insert from pBd4R. Lanes and molecular size markers are as above.

D. Southern hybridisation of the agarose gel in A with the insert from pBd7R. Lanes and molecular size markers are as above. The polymorphic band present in one 3B sample is arrowed.

E. Southern hybridisation of the agarose gel in A with the insert from pBd6R. Lanes and molecular size markers are as above.


A
0B
0B
3B
3B
23.1
9.4
6.5
4.3
2.3
2.0
B
0B
0B
3B
3B
C
0B
0B
3B
3B


D
0B
0B
3B
3B
E
0B
0B
3B
3B

(Figure 6.8D) was initially thought to represent a polymorphism between different plants but a longer exposure (not shown) indicated that the band was present in all tracks and was increased in copy number in the DNA of one plant examined.

Bd6R showed increased levels of hybridisation to the genomic DNAs (Figure 6.8E) in comparison to Bd4R (Figure 6.8C) and Bd7R (Figure 6.8D). The smear of hybridisation (Figure 6.8E) suggested that pBd6R contained a repetitious sequence, dispersed throughout the genome. No differences were seen in patterns or levels of hybridisation between 0B and +B genomic DNA.

The inserts from pBdCR, pBdFR and pBdER, all initially amplified with the RAPD-primer Y2 (Figure 6.7), were used as probes to Southern blots of the RAPD-PCR products from 0B and +B pooled samples. BdCR and BdFR, as expected, hybridised strongly to the parent RAPD band, but also showed hybridisation to both 0B and +B products with no apparent differences in the hybridisation patterns between the samples (Figure 6.9A, B). In contrast, hybridisation of BdER to the same Southern blot of the RAPD-PCR gel was confined to the B-containing Y2 RAPD-PCR product, with no sign of any hybridisation to PCR products amplified from 0B samples with the same primer (Figure 6.9C). The hybridisation observed was not specific to the BdER band, but was also seen to a number of other bands within the PCR product, implying that Y2 was amplifying a number of regions from within the +B genome with BdER identity. The implication of the hybridisation of BdER to RAPD products amplified, with primer Y2, from +B and not 0B genomic DNA was that the clone pBdER contained a sequence specific to the B chromosomes.

To determine the extent of BdER within B-containing samples, and to confirm the absence of the sequence in 0B plants, primer Y2 was used in PCRs with DNA templates prepared from the six individual 0B and +B plants present in the original pools. The

Figure 6.9:

Southern hybridisation analysis of cloned RAPD products amplified with the Y2 RAPD primer from 0B and +B pooled *B. dichromosomatica* genomic DNA templates.

Each lane contains equally loaded RAPD products amplified with the Y2 primer from 0B and +B pooled *B. dichromosomatica* genomic DNA templates.

The probes were:

A. BdCR

B. BdFR

C. BdER. The RAPD representing BdER, amplified from the +B pool of samples, is arrowed and labelled.

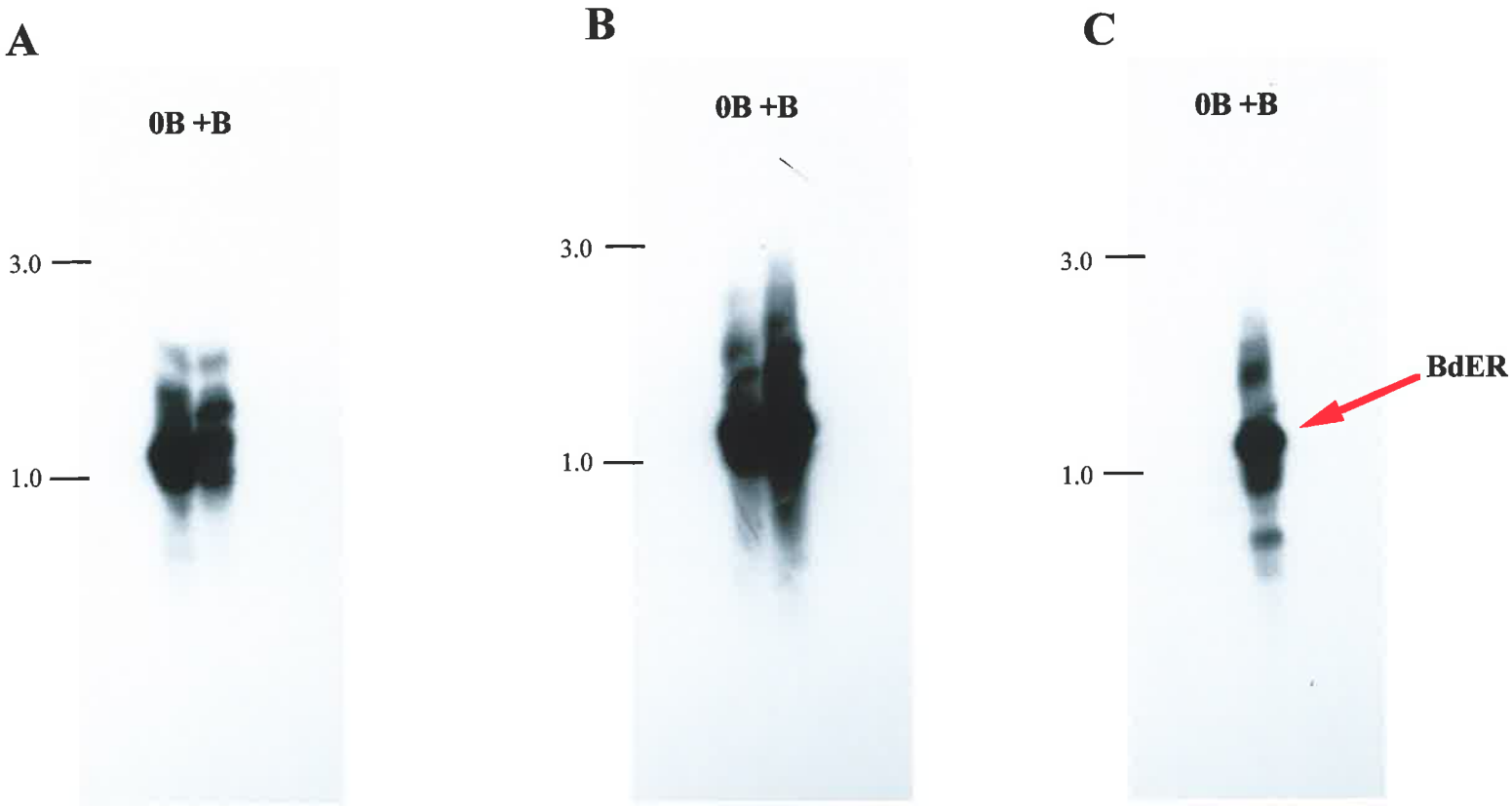

A
0B +B
3.0
1.0
B
0B +B
3.0
1.0
C
0B +B
3.0
1.0
BdER

Figure 6.10:

A. Agarose gel and corresponding Southern hybridisation of PCR products amplified with the Y2 RAPD primer from 0B *B. dichromosomatica* genomic DNA samples from individual plants and probed with the insert from pBdER.

Amplification products from 0B and +B pooled samples are labelled accordingly and a control reaction with no template DNA is labelled N. The RAPD representing BdER, amplified from the +B pool of samples, is arrowed and labelled.

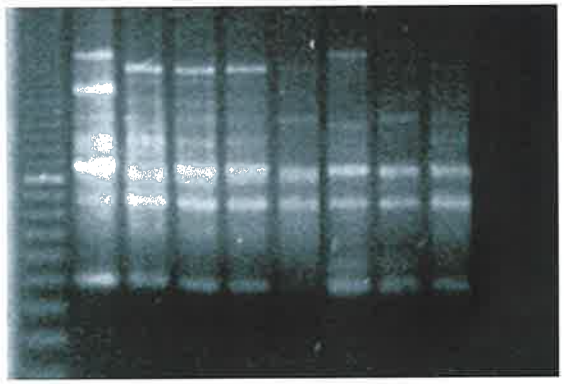
B. Agarose gel and corresponding Southern hybridisation of PCR products amplified with the Y2 RAPD primer from +B *B. dichromosomatica* genomic DNA samples and probed with the insert from pBdER.

Amplification products from 0B and +B pooled samples are labelled accordingly and a control reaction with no template DNA is labelled N. The RAPD representing BdER, amplified from the +B pool of samples, is arrowed and labelled.

A

0B DNA samples from individual plants

1 2 3 4 5 6 0B +B N



0B DNA samples from individual plants

1 2 3 4 5 6 0B +B N

B

+B DNA samples from individual plants

1 2 3 4 5 6 0B +B N

+B DNA samples from individual plants

1 2 3 4 5 6 0B +B N

number and size of the PCR products amplified with primer Y2 varied slightly between individual samples (Figure 6.10A, B) suggesting that the plants from which the DNA was derived were heterogeneous, as would be expected for an outbreeding population. Southern analysis of the PCR products with BdER as a probe showed hybridisation to the products amplified from all six +B templates (Figure 6.10B). Hybridisation at a lower level was also seen to the products amplified from one sample from within the 0B pool (Figure 6.10A). The positive and negative controls were the parallel amplification and hybridisation from the 0B and +B pooled samples and the incorporation of a reaction lacking DNA template to check for contamination of the PCR constituents (Figure 6.10A, B).

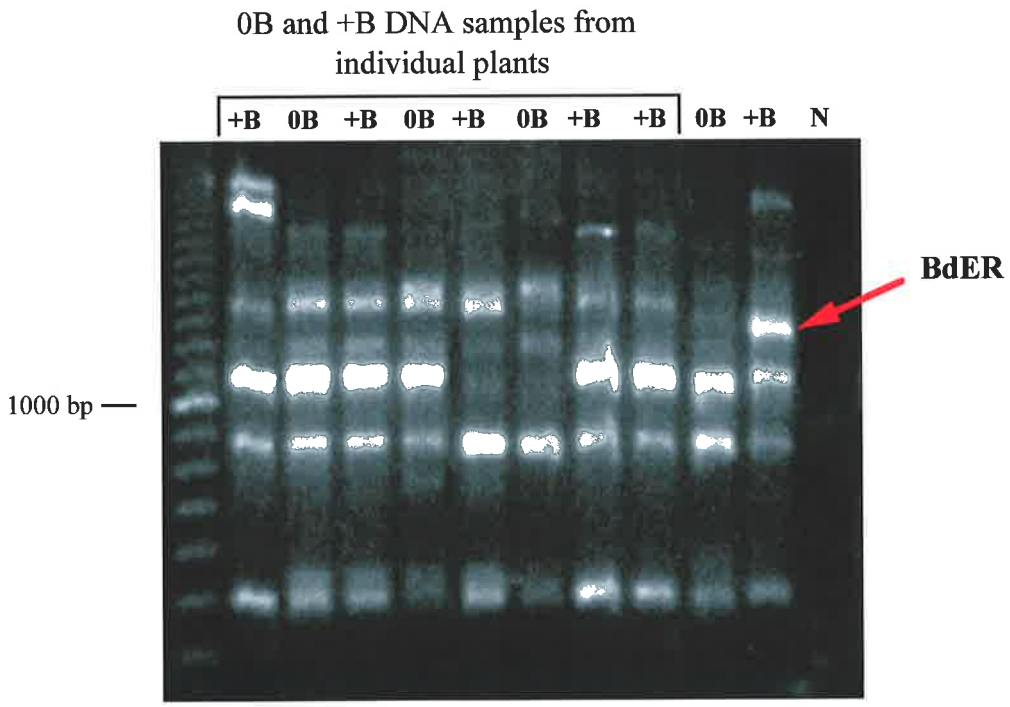
The lack of BdER hybridisation to RAPDs amplified from the 0B pool, despite the hybridisation to products amplified from one of the individual samples present within the pool, suggested that there was a low copy number of the BdER sequence in the genome of one of the 0B plants. In amplification reactions from the 0B pool it is likely that the high proportion of non-BdER sequences with primer Y2 identity outcompeted the BdER sequences for the primer. However, within the individual 0B sample, the percentage of BdER in the total DNA would be greater and, therefore, more readily amplifiable. The total number of BdER-hybridising bands was decreased in the 0B sample, compared with the +B samples (Figure 6.10), indicating lower copy number and less variation amongst the copies of the sequence in DNA from the plant without Bs than the plants with Bs.

In order to ascertain the extent of BdER within the *B. dichromosomatica* population, amplification with primer Y2 was repeated on a number of additional samples with and without B chromosomes. The genomic DNA samples chosen were derived from different populations of *B. dichromosomatica* than the samples used in the initial 0B and +B pools and were therefore expected to be divergent in A, and possibly B, chromosome sequences

Figure 6.11:

Agarose gel of RAPD products amplified with primer Y2 from a range of *B. dichromosomatica* genomic DNA samples without (0B) and with (+B) B chromosomes, in addition to the 0B and +B pooled samples and a no DNA template (N) control.

The first lanes contain molecular size markers (100 bp DNA ladder), consisting of fragments that range from 100 bp to 3000 bp in 100 bp increments. The RAPD representing BdER, amplified from the +B pool of samples, is arrowed and labelled.


0B and +B DNA samples from individual plants
+B 0B +B 0B +B 0B +B +B 0B +B N
BdER
1000 bp

due to the outbreeding nature of the species. Amplification products resulted from all the samples tested and were resolved on agarose gels. Controls included were parallel amplifications from the original 0B and +B pools and a reaction with no DNA template (Figure 6.11).

Overall the number and sizes of the RAPD products amplified were fairly uniform across all samples, including those from the 0B and +B pools (Figure 6.11). The low level of visible polymorphism between the RAPDs amplified from individual templates was indicative of the degree of heterogeneity within the population. Hybridisation of the RAPD products with BdER was strong to the +B pool-PCR products as was seen previously (Figure 6.9, 6.10). However, minimal levels of BdER hybridisation were obtained to all other RAPD products (result not shown). The BdER sequence could be present on the B and/or the A chromosomes, but it was clear that it was not a sequence ubiquitous to either chromosome type in *B. dichromosomatica*. Sequencing of pBdER with (-20) and reverse sequencing primers enabled the full sequence of the insert to be determined (Figure 6.12). The 1275 bp of nucleotide sequence obtained showed significant levels (55-70%) of nucleotide identity with retroviral and mitochondrial sequences in the database (GenBank and EMBL, 1997), suggesting invasion, followed by sequence divergence, of a subpopulation of A and, possibly, B chromosomes with mobile elements or mitochondrial DNA.

In maize, RAPD analysis isolated several putative B-specific RAPDs (Gourmet and Rayburn, 1996; Stark *et al.*, 1996; Lin and Chou, 1997), including one with significant nucleic acid similarity to a maize retroviral element called PREM-1 (Stark *et al.*, 1996). However, the overall composition of the maize B was similar to that of the standard chromosome complement (Stark *et al.*, 1996). Initial RAPD and AFLP analysis in rye suggested that low copy sequences are on average under represented on the B chromosome

```
CATCGCCGCACAGTGGATCGAAAAATGGAATTTTCCGAACATAATTTTTT 50
TAATAAAAATGAAGATTTTGAACAATAAAAATCAGGATGACTTAAAAATG 100
TGTTAAATCATTCATTTTATAACTTTTAAAATTAGTGGAATAACATGGTT 150
TTTACAAGAAAGTATCAACACTTAAATGTAATCAGAGGGGAGGGGGGAAA 200
GTGCTTGTCACGGCCAGAAAAATACATGTTTCTGCCCAGTGCGCGCCTAT 250
TCTTCTCAGAGCCCCAATGAATTAGCGTAGAGGAATCAATCAAAGCGGCC 300
TGTAACGGAGTAAAACACGAGGGCTGCTCTTCCGAAGCAATTCTAACGGA 350
GCCACCATGCTGATTTCTTTGGAAGAGATTAATCCATGTTTTATCGATTT 400
TTCCTCTGGAGATGGCCTTACTTTGTCTTTTCCGCTGAGACAGAGATTTA 450
ATATCATCTACCGACAGCGTGGCCAGTTTTCAGAAAATTATCAGGCTTTT 500
CCTTATCAACTCAATGTTTTTGGCAGAAAATAAATTTTATAAATGTAATA 550
TAATTTATTAATAAAAATTTATAATTTAAATTATAATGTTATGATAATCT 600
AATTATTTAAATGATTTTGGAAAAGAATTTTAAAGAAAAATCTGGCAACA 650
TTGCCTACCGTTGGCTTTGTTGTAAGAACTTATAAAAGCCTTCAGAAATC 700
CTTTTTGATCAATTCAGATGTTTTTAGTCTGCGATAACTTGATTTGGCTA 750
TCGAGTGTCTGATGTGAAGAATGCACACTGTTTCTCAAAAGTTTGGCAAG 800
TGGCCAAAGCGTTCATTGCAATCAGTGGTTTCCTCTAAACGTACGTAAAA 850
AATCGATGGTAGAAGGAGCAATAATCACCTTCAATATTCGAATATCTTAC 900
GCACGTTCAAATGGAGAAAACTTGTTGTTGCCGTAATATACGAGCCGCCA 950
CCGAATGCTATAAATAACAGAAAACATTTGAGAGAATCTAGCATTAATTT 1000
TAGTTGAAAAGACCGGGTGCATCTTAGTTATCATTATGGTATGCAGATTT 1050
TGTTCTTTTCAGGGCTGTCGTTTTGTGTATAATCATATCCCATCAAGAAC 1100
CTTTTCAGGTGCAAGCAGACGGCATGTCAGGTAATTAGGTCTGTCACAAA 1150
AAGCCGCCCCAATGCATCAACTCGAAGATATGATATGATGGGAACGGCAA 1200
GTCCCTCCAAGTGGGCATCCAGCAAGGCAACTGTTAATCATTTTTAAAAA 1250
TCAACACTATCTATCTGCGGCGATG 1275
```

Figure 6.12:

Complete 1275 bp sequence of the insert of pBdER, with the Y2 primer at either end boxed.

and that the euchromatic component of the B shows similar repetitive sequence composition as that of the As (Langdon *et al.*, 1997). Similar to the RAPD results obtained in *B. dichromosomatica*, the pericentromeric region of the rye B chromosome was found to contain organelle related DNA (Langdon *et al.*, 1997).

Sequences which were retroviral or organelle-derived have previously been found on the B chromosomes of *B. dichromosomatica*, flanking Bd49 (Franks *et al.*, 1996) and the wasp, *Nasonia vitripennis*, which contains a B-specific transposable element (*NATE*) (Section 1.7.2). Therefore, it is evident that, in addition to *B. dichromosomatica*, the B chromosomes from a range of species contain retroviral and/or other transposable elements but it is uncertain whether the Bs are more or less prone to accumulation of such sequences. The significance, if any, of these sequences on the Bs is unknown, however, in light of their essentially inert genetic nature it is not surprising that B chromosomes are particularly tolerant of DNA transposition and rearrangement.

6.4 Conclusion

The results obtained using RAPD and AFLP techniques suggest that a large proportion of the B chromosome in *B. dichromosomatica* is composed of sequences which are common to the A chromosomes. This hypothesis is supported by the fact that, apart from the repeat Bd49, no other sequences specific to the *B. dichromosomatica* Bs have been isolated (Franks *et al.*, 1996). Further, the only other sequences located on the Bs of *B. dichromosomatica* are rDNA (Section 1.6.2b; Donald *et al.*, 1995) and Bdm29 (Section 1.6.1b, 1.8; Houben *et al.*, 1997b) both of which are also present on the A chromosomes. The results presented in this chapter are complemented by comparisons of the sequence compositions of the A and B chromosomes in a wide range of species, which support the idea that the B chromosomes consist predominantly of sequences present on both

chromosome types (John *et al.*, 1991; Tsujimoto and Niwa, 1992; Blunden *et al.*, 1993; Cuardro and Jouve, 1994; Jamilena *et al.*, 1994; López-León *et al.*, 1994; McQuade *et al.*, 1994; Bougourd *et al.*, 1995; Stark *et al.*, 1996).

CHAPTER 7:

Concluding Discussion

7.1 Organisation and Expression of Plant B Chromosomes

7.1. 1 Ribosomal DNA Sequences on the B Chromosomes of *B. dichromosomatica*

Previously, fluorescence *in situ* hybridisation revealed the presence of sequences with rDNA identity on the B chromosomes of *B. dichromosomatica*. The preliminary aim of the rDNA work presented in this thesis was to determine if these B chromosome sequences were transcriptionally active.

Initially, analysis of ITS2 identified two nucleotide differences between the sequences derived from the A and B chromosomes. One of these differences involved a *Sfc* I restriction site present in the ITS2 of B-derived rDNA that enabled the ITS2 on the A and B chromosomes to be distinguished. The inability to detect the B-specific ITS2 regions within the 40S precursor rRNA suggested that the B-rDNA was not transcribed in leaf or floral tissue. This was the first molecular evidence of the transcriptional inactivity of rDNA on B chromosomes which had previously only been examined by silver staining techniques. The mechanism for the B-rDNA inactivation is not known but it was of interest to determine if there were any differences in the sequence and/or organisation of the rDNA on the A and B chromosomes which could; (1) explain the inactivity of the B chromosome sequences, (2) shed light on the evolution of these sequences on the Bs, and their relationship with those on the As and (3) suggest a possible origin for the B chromosomes themselves.

Comparison of the region separating the 18S and 25S coding regions of the rDNA on the A and B chromosomes, which is comprised of the ITS1, 5.8S and ITS2, suggested that a proportion, and possibly all, the regions on the Bs were intact and, with the exception of the identified B-specific polymorphisms at bases 142 and 175 within the ITS2, identical in sequence to the corresponding region on the A chromosomes. In addition, comparisons of Southern hybridisation after probing with cotton (Donald *et al.*, 1995) and

B. dichromosomatica rDNA was unable to detect differences in hybridisation between 0B and +B genomic DNA, indicating overall homogeneity within and between the rRNA coding regions on the A and B chromosomes.

To examine the B chromosome IGS region, which is of particular interest as it contains the RNA polymerase I transcription-initiation site as well as other elements important in rRNA transcriptional regulation, Southern hybridisation with IGS-derived probes revealed essentially very similar patterns of hybridisation to 0B and +B genomic DNA. A single restriction fragment polymorphism associated with the presence of Bs was identified but the source of the polymorphism is yet to be determined. Experiments which looked specifically at the B chromosome IGS region, using PCR, supported the hypothesis that there were low levels of variation in the IGS region on the Bs, but these may have reflected technical difficulties associated with the presence of incomplete DNA fragments in the DOP-PCR-amplified microdissected B-DNA. *In situ* hybridisation confirmed the presence of IGS sequences on the Bs and no variation in the hybridisation of the different IGS-subclones was detected.

In summary, evidence from ITS2 characterisation, Southern hybridisation, PCR analysis, nucleotide sequence comparison and *in situ* hybridisation supported the preposition that overall the rDNA on the B chromosomes of *B. dichromosomatica* is organised in intact repeat units. The identification of several putative B-specific polymorphisms in the IGS region implied that variation between A- and B-rDNA may exist and could possibly be associated with the B-rDNA inactivity.

The apparent similarity between A and B chromosome rDNA suggests that despite the inactivity of the rDNA on the Bs, they are under the control of a homogenising mechanism which controls the non-independent evolution of these repetitive DNA sequences. The implication is therefore that the B chromosome rDNA is maintained by

mechanisms similar to those on the As. However, differences between the A and Bs in the region suggest that the two chromosome types may not be evolving in concert with each other.

7.1.2 Isolation of Non-Repetitive Sequences from the B chromosomes

Attempts to isolate non-repetitive sequences from the B chromosomes using RAPD and AFLP analysis were not successful in isolating B-specific sequences but did identify sequences polymorphic within the species, present on the A and possibly the B chromosomes of some individuals. It was apparent from RAPD and AFLP analyses that the number and size of the PCR products amplified from 0B and +B genomic DNA samples, using these techniques, were overall similar. Any polymorphisms that were detected generally represented genetic heterogeneity expected of an outbreeding species and did not involve sequences unique to the B chromosomes, indicating that the A and B chromosomes were overall very similar.

7.1.3 Concluding Discussion

Due to the lack of transcription and in the absence of selection, many of the sequences that comprise Bs would be expected to show high rates of evolutionary change. However, the results from this work indicate that despite the inactivity of the *B. dichromosomatica* B chromosomes, insinuated by the sequence analysis of rRNA and histone acetylation patterns (Houben *et al.*, 1997a), these chromosomes contain a high proportion of DNA, including rDNA, that is both common to the A chromosomes and conserved in sequence. Previously, it was suggested that degeneration of supernumerary chromosomes may proceed via the mechanism of Muller's Ratchet (Green, 1990). This hypothesis predicts that supernumerary chromosomes should lose functional loci, lose sequence homology with the regular genome, and gain heterochromatin over time, resulting in multiple heteromorphic forms of degenerate supernumeraries within and between populations.

Although there are two different B chromosome types present in *B. dichromosomatica* (Section 1.8), evidence suggests the micro B chromosomes are more likely to be forerunners of the larger Bs than degenerate morphs (Houben *et al.*, 1997b). The Bs in *B. dichromosomatica* have no apparent functional loci but still seem to maintain significant sequence identity with the A chromosomes and this scenario is similar in a wide range of other species where the Bs are apparently transcriptionally inactive, but the sequences on the Bs are predominantly common with the As (Chapter 6).

One of the major questions raised by the results in this project is: How are sequences on the Bs maintained when they appear to be inactive and is there any evolutionary or other requirement for this sequence conservation? One possible answer is that the entire B chromosome is under evolutionary control similar to that proposed for rDNA and other repetitive sequences, whereby the intra-genomic diversity is generally low due to concerted evolution within and between loci (Dover, 1982). Alternatively the Bs may be 'young' and have had insufficient time to diverge. However, there is currently no evidence for Bs at different stages of degeneration, which would be predicted if this was the case. Other possibilities are that the structure of the B is important in ensuring its own transmission and this is somehow selected for or that Bs that vary considerably from the As are more detrimental to the organism and therefore selected against.

7.2 Final Comment

Many of the aims outlined prior to the commencement of this research have been achieved, but a number of important questions remain unanswered;

(1) What is the significance, if any, of the very frequent presence of rDNA sequences on B chromosomes in a large number of species?

(2) How is meiotic pairing between the A and B chromosomes prevented at the molecular level, despite the apparent presence of massive sharing of sequences between the two chromosome types?

(3) Is there a stage of the life cycle when the sequences on the B chromosomes are expressed?

Further molecular analysis of B chromosomes is likely to help answer these questions, in addition to increasing general understanding of the universally important processes involved in the control of chromosome pairing, division and evolution. Perhaps the next important step towards this end is to extend the analyses started here to give detailed sequence comparisons of DNA that is present on both chromosome types.

APPENDICES

APPENDIX 1

Phosphorimage analysis of the proportion of ITS2-PCR products, amplified from genomic DNA derived from *B. dichromosomatica* plants with different chromosome constitutions, which was unrestricted and restricted with *Sfc* I.

Chromosome constitutions	Unrestricted (PSL)	Restricted (PSL)	% of ITS2 Restricted with *Sfc* I
0B	20760.0	0	0
	30680.0	0	0
	21180.0	0	0
1B	17980.0	709.20	3.8
	37790.0	1234.60	3.2
	18890.0	657.9	3.4
2B	13010.0	931.90	6.7
	17500.0	1050.4	5.6
	18150.0	1160.0	6.0
3B	23540.0	2099.0	8.2
	28630.0	3298.6	10.3
	41060.0	4509.0	9.9
4B	28750.0	4637.0	13.8
	17070.0	2584	13.3
	16390	2569.5	13.5

Using the MacBas Version 2.X computer program (Fuji Photo Film, Co., Ltd) the radioactivity within specified regions was measured in units of photostimulated luminescence (PSL) and the percentage of the total ITS2 PCR product which was restricted with *Sfc* I was calculated.

APPENDIX 2

Donald TM, Houben A, Leach CR and Timmis JN. 1997. Ribosomal RNA genes specific to the B chromosomes in *Brachycome dichromosomatica* are not transcribed in leaf tissue. *Genome* **40**: 674-681.

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Genome, v. 40(5), pp. 674-681

NOTE:
This publication is included after page 216 in the print copy
of the thesis held in the University of Adelaide Library.

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