HETEROGENEITY IN GENOMIC DNA OF RUST FUNGI

A thesis submitted for the degree of Doctor of Philosophy of The Australian National University.

PETER ALEX ANDERSON

March 1991

Table of Contents

Statement of originality

To the best of my knowledge the work presented in this thesis does not appear elsewhere in the literature. The work is entirely my own and where contributions have been made by others due reference is given.

P. A. Anderson

Peter A. Anderson

Acknowledgements

Abstract

Rust fungi that infect major agricultural crop plants are among the world's most destructive and economically significant disease-causing agents. The available literature on rust fungi indicates that the majority of research has concentrated on the genetic basis of avirulence, the major genes controlling pathogenicity. There has been very little published information about the structure and organization of the rust genome.

The results of this thesis provide some insight into the size, complexity, and heterogeneity within the genome of a rust fungus. The size of the maize rust genome was measured to be 4.5×10^7bp, with approximately 20% of this representing a repeated DNA fraction. The level of repeated DNA was higher than has been reported for other non-pathogenic fungi, and this result was discussed in relation to the suggestion that high levels of repeated DNA may be in some way related to pathogenicity.

Randomly selected cDNA probes from several rust species were used to detect Restriction Fragment Length Polymorphisms (RFLPs) between races of maize, wheat and flax rust which differed in avirulence specificity. The RFLP analysis revealed that a high level of heterogeneity exists in the rust genome. This was particularly evident in maize rust, and an explanation of the basis of this heterogeneity was obtained in part by a genetic study of flax rust. This study indicated that the major contribution to this heterogeneity was heterozygosity at RFLP loci. Over 50% of the RFLP loci in flax rust were heterozygous. This is similar to levels of heterozygosity at avirulence loci, but significantly higher than levels reported using isozyme markers.

One RFLP in maize rust was characterized at the molecular level and found to be caused by the insertion of a 496bp sequence. This sequence was highly repeated in maize rust, but was not homologous to genomic DNA from other rust fungi. The sequence showed several structural similarities to transposable elements that have been characterized in other organisms, however transposition of this sequence has not been demonstrated. The possibility was introduced that such a sequence, if shown to be mobile, may be implicated in generating mutations in the rust genome.

The thesis demonstrates the successful application of molecular techniques to the study of the genetic organization of rust fungi.

Chapter 1 : General Introduction

Plant diseases caused by rusts, smuts and mildews have greatly influenced the domestication of agricultural crop plants and therefore the development of human civilization. While it is clear that these fungal pathogens have coevolved with their plant hosts, it is probable that the occurrence of devastating disease is associated with the appearance of monocultures of agricultural plants. Moses, some 3500 years ago, warned the Israelites of the potential destruction of their field crops " with smuts and rust " (Deut. 28:22). The ancient Romans created the god Robigus specifically to protect their crops from the devastating effects of rust disease. Today methods to combat rust fungi have changed somewhat. Agricultural plants have been genetically manipulated to contain an array of genes conferring resistance to pathogen attack. Despite the impact that rust fungi have had on the selection and breeding of modern agricultural plants, little if anything is known about the molecular basis of disease and resistance.

Rust fungi are a diverse and specialized group of pathogenic organisms. They are members of the basidiomycete class of fungi and divided into approximately 4600 species in over 100 genera. Each rust species has evolved a highly specialized interaction with one or several plant species, and most families within the plant kingdom are infected by at least one species of rust. In the middle of this century, Harold Flor discovered that the genetic basis of the interaction between a rust fungus and its host plant was an interplay between the expression of a single gene in the host and a single gene in the pathogen. He called this the 'gene for gene' model. Immediately it became apparent that an understanding of the molecular determinants of the 'gene for gene' model might lead to a control of these pathogens. However, subsequent progress has been slow. A major reason for this is the difficulty of manipulating rust fungi. Unlike the saprophytic free-living fungi such as *Neurospora* and *Aspergillus*, the growth of rust fungi is obligated to the host plant. However, with the advent of the techniques of molecular biology we are in a position to develop new methods to study these organisms.

The work presented in this thesis describes initial experiments in applying the techniques of molecular biology to the study of rust fungi. This technology has not previously been applied to rust fungi. Most of our present understanding of fungal DNA and the analysis of fungal genomes comes from work on *Saccharomyces*, *Neurospora* and *Aspergillus*, all saprophytic ascomycetes. A small body of data is beginning to accumulate on basidiomycete fungi, represented mainly by *Schizophyllum*, *Ustilago* and *Coprinus*. In order to place this work in the framework of the literature, the current understanding of rust genetics will be discussed in some detail. However, the topics of fungal gene and genome structure will require a broader discussion of the available data from the fungal kingdom. Where possible, emphasis will be placed on basidiomycete and phytopathogenic fungi.

1.1: Sexual genetic systems in rust fungi

A eukaryotic diploid cell contains two copies of each chromosome. During meiosis each chromosome of this pair segregates randomly, producing haploid cells containing only one copy of each pair. Fusion of two haploid cells followed by nuclear fusion, a process known as karyogamy, re-establishes the diploid form. It is this alternation between the haploid and diploid generation that is the essence of sexual reproduction in eukaryotic organisms. In most higher eukaryotes the diploid generation of the life cycle predominates. However, in most fungi and algae, the opposite situation exists; the diploid nucleus, once formed by karyogamy, immediately undergoes meiosis to restore the haploid condition. A simple example of this is found in the life cycle of the unicellular alga, *Chlamydomonas reinhardi*. Its free living cells are haploid, and under suitable conditions cell fusion and karyogamy occurs with the resulting diploid nucleus undergoing meiosis to restore the haploid state. In most fungi the life cycle is essentially the same, except the fusion of haploid cells does not at once proceed to karyogamy. Rather, there is a brief (ascomycetes) or prolonged (basidiomycetes) dikaryotic phase, in which the haploid nuclei within the fused cell associate and divide synchronously in pairs. The association of paired haploid nuclei is known as a dikaryon, which Fincham *et al* (1979) refer to as 'surrogate diploidy'.

Regardless of whether the diploid or haploid form predominates, the haploid products of meiosis fuse during sexual reproduction to generate progeny. The result of self fertilization is homozygosity, which ultimately nullifies the ability of recombination to generate new gene combinations. Presumably, it is for this reason that most eukaryotic organisms have evolved barriers to prevent self fertilization. Self infertility is achieved in many ways, ranging from self incompatibility genes in plants to sexual dimorphism in mammals. In this respect, fungi are no different from higher eukaryotes. Sexual dimorphism occurs in some fungi, however the more common approach is by the genetic segregation of self-incompatible and cross-compatible mating type genes. This mating system is known as heterothallism and is opposed to homothallism or self-fertility (see Fincham *et al*, 1979). Much of our present understanding of the genetic basis of heterothallism in fungi can be attributed to the work of John Raper (reviewed by Raper, 1983). The next section describes the life cycle of the maize rust fungus (*Puccinia sorghi* Schw.) as an example of a heterothallic dikaryotic rust fungus.

1.1.1: The rust life cycle.

The life cycle of a rust fungus was first completely described for *Puccinia graminis tritici* (wheat stem rust) by John Craigie in 1927, although this was the culmination of the earlier work of many others. The life cycle of *P. sorghi* (Arthur, 1904; Allen, 1934) is much the same as that of *P. graminis*, and is shown in stages in Figure 1.1 A-M.

The brown uredia of *P. sorghi* are composed of single-celled dikaryotic urediospores, capable of successive rounds of asexual reproduction on the maize plant. These spores germinate on the leaf surface and enter through stomata in the leaf. Towards the end of the growing season when the leaves begin to senesce, black two-celled teliospores are produced.

Figure 1.1. Stages of the life cycle of the maize rust, *Puccinia sorghi*. **A**: A leaf of *Zea mays*, 12 days after inoculation with urediospores of *P. sorghi*. The brown uredia or pustules containing urediospores, have erupted through the leaf epidermis. **B**: An electron micrograph of a uredium showing separate circular urediospores (taken by Stuart Craig, CSIRO). **C**: Individual urediospores germinated on an air/water interface showing the extending germ tube. **D**: A DAPI (4,6-Diamidino-2-phenylindole) stained germinated urediospore showing the two fluorescent nuclei in the germ tube. **E**: A teliospore. The circular body in the centre of each cell, indicated by arrows, is presumably the diploid nucleus derived from karyogamy of the two haploid nuclei of the dikaryon. **F**: A senesced leaf of *Z. mays* showing black telia containing teliospores. **G**: A germinated teliospore showing four basidiophores, from which the basidiospores are released; one basidiospore is still attached. **H**: A DAPI stained germinated teliospore. The stained body is presumably the diploid nucleus which has migrated into the germ tube, prior to meiosis. **I**: Meiosis generates four haploid nuclei, each of which undergoes a post-meiotic mitotic division. Each basidiospore retains one of these haploid nuclei, whereas the other is left behind in the basidiophore. Shown here is a DAPI-stained basidium after the release of the basidiospores and the stained bodies, indicated by arrows, are the haploid nuclei remaining in each basidiophore after the post meiotic mitotic division. **J**: Basidiospores **K**: DAPI stained basidiospores showing two staining bodies, presumably the result of a second mitotic division after meiosis. **L**: A leaf of the alternate host of *P. sorghi*, *Oxalis corniculata*. The arrow indicates a haploid mycelium on the surface of the leaf known as a pycnium (taken by Tony Pryor, CSIRO). **M**: Mixing of inoculum from cross-compatible pycnia produces aecia on the *Oxalis* leaf. Shown here is one aecium and the pycnium from which it was derived is in the middle of the ring of infections. Aecia produce small dikaryotic aeciospores which infect *Z. mays* and produce urediospores (taken by Tony Pryor, CSIRO).

A

1cm

B

1mm

C

30μm

D
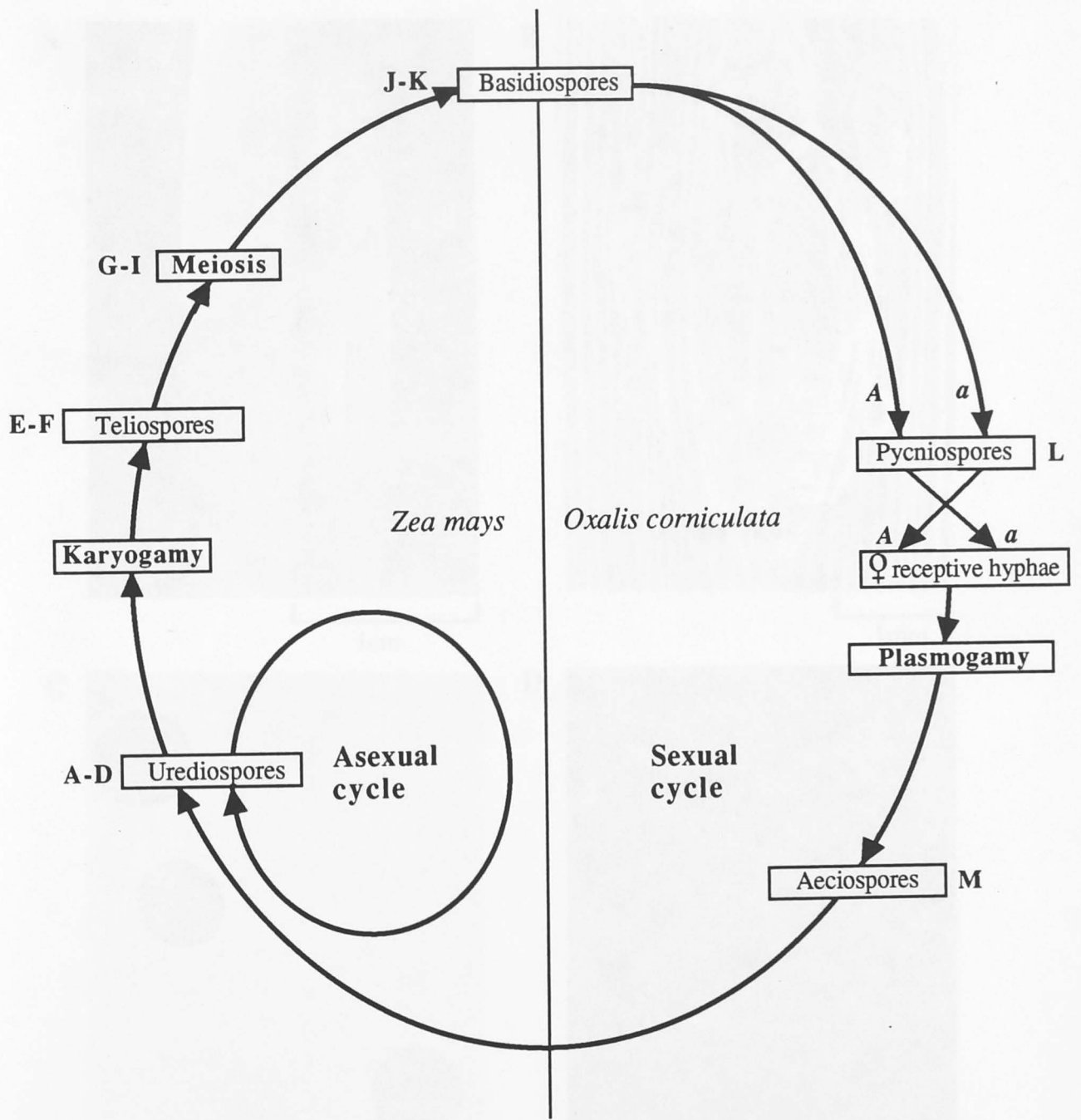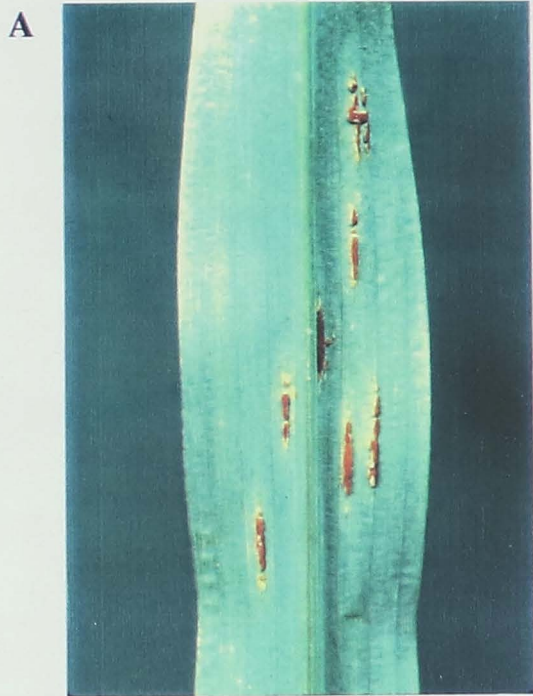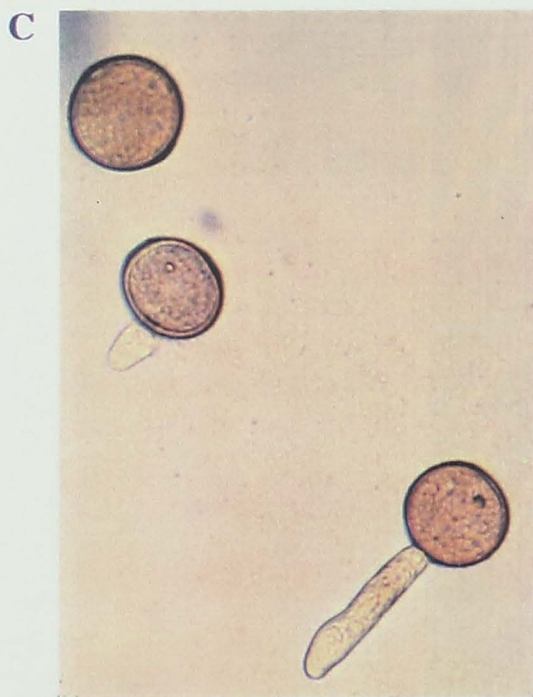
30μm

E

0.5mm

F
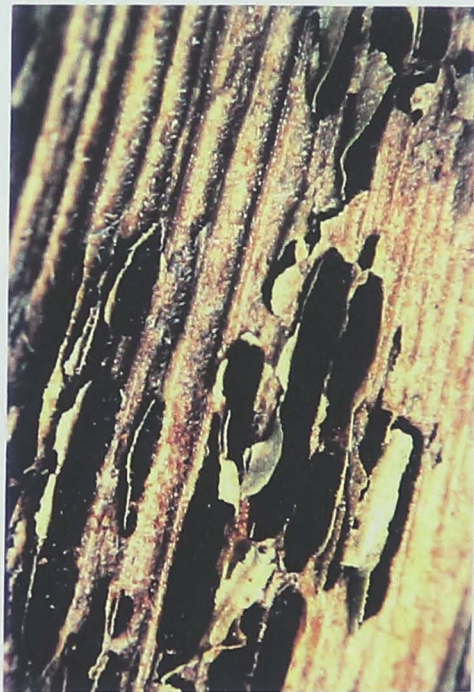
30μm

G

100μm

H

100μm

I
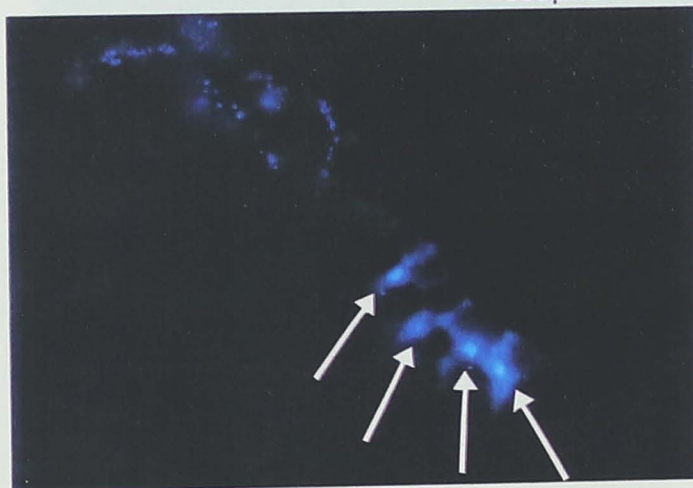
100μm

J
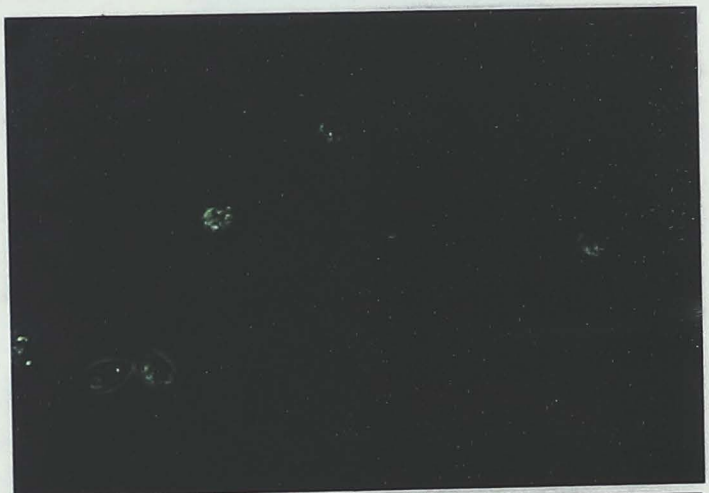
K
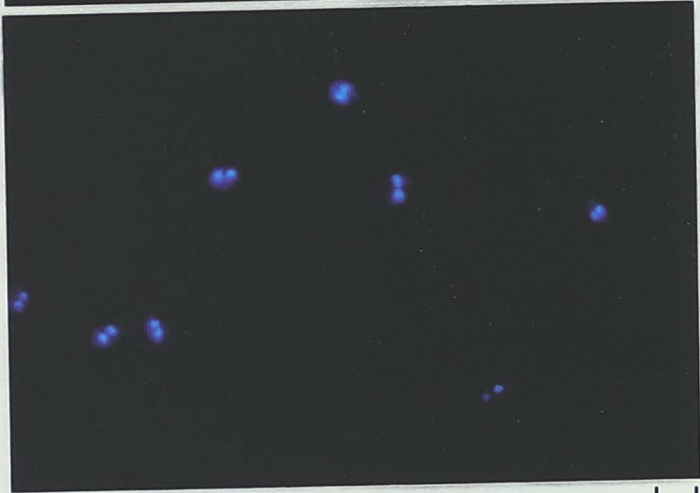
10μm

L
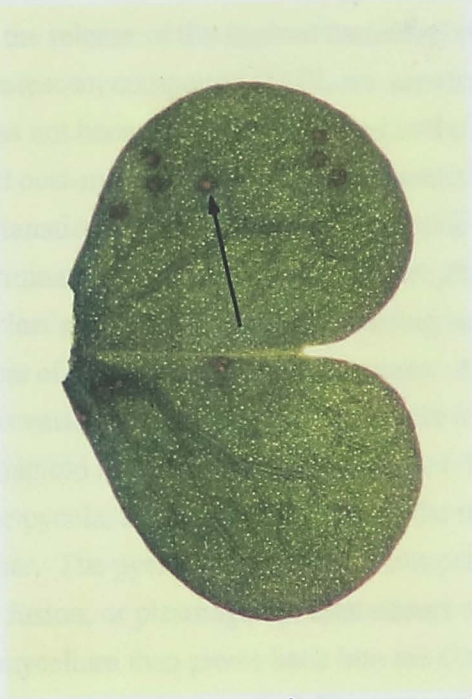
5mm

M
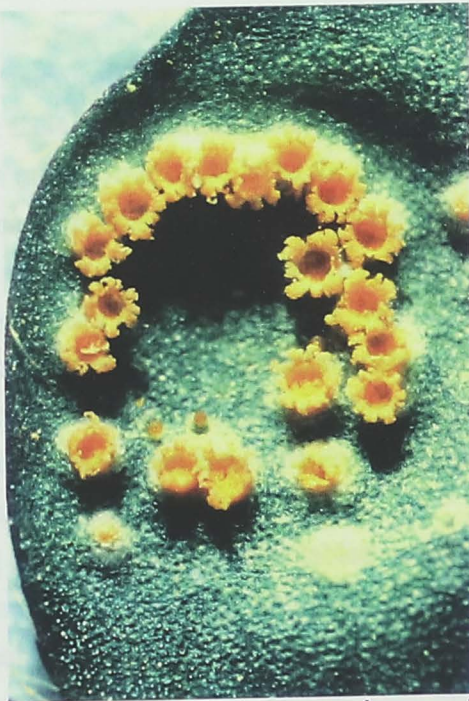
1mm

The teliospore, shown in Figure 1.1F, appears to contain one nucleus in each cell which presumably is the diploid product of karyogamy between the two haploid nuclei of the dikaryon. The teliospore is a dormant over-winter spore and will germinate after several cycles of freezing and thawing. Following germination, the diploid nucleus migrates into the germ tube (Figure 1.1H), where meiosis occurs and a four-celled basidium is produced with each cell containing a haploid nucleus. Each nucleus undergoes a post-meiotic mitotic division in which cytokinesis triggers the release of the haploid basidiospores. Basidiospores of *P. sorghi*, stained with the fluorescent compound DAPI, are seen to contain two nuclei (Figure 1.1K). This observation has not been previously reported in the literature. The most likely explanation is that a second post-meiotic mitotic division occurs in the basidiospores, however the possibility of other explanations such as higher ploidy levels in *P. sorghi* cannot be ruled out. The basidiospores germinate and enter the alternate host plant *Oxalis* spp. by chemical and physical penetration of the leaf surface. Thus the germinating basidiospores enter the plant in a quite different way from that of the germinating urediospores. After entering the leaf, the haploid mycelium develops small pycnia (indicated by an arrow in Figure 1.1L). *P. sorghi* is a heterothallic rust, and the haploid mycelia are of either (+) or (-) mating type. Insects, which are attracted to nectar in the pycnia, are believed to transfer the small haploid pycniospores from one pycnium to another. The pycniospores that are compatible fuse to the female receptive hyphae, and cell fusion, or plasmogamy, then occurs which re-establishes the dikaryon. The dikaryotic mycelium then grows back into the *Oxalis* leaf and an aecium is formed. Each aecium contain yellow single-celled dikaryotic aeciospores which infect the maize plant gaining entry through the stomata, and go on to produce urediospores thus completing the life cycle.

This is known as a macrocyclic life cycle as all five spore states are present; uredial, telial, basidial, pycnial and aecial. By contrast a rust with a demicyclic life cycle lacks the uredial state and one with a microcyclic cycle lacks the uredial and aecial spore states. A further variation exists when both sexual and asexual phases of the life cycle are completed on the one host plant. This is known as an autoecious life cycle, the best studied example of which is the flax rust (*Melampsora lini* Ehrenb. Lev.). When the homokaryotic or sexual phase of the life cycle is completed on one host and the dikaryotic or asexual phase on another, the life cycle is known as heteroecious; *P. sorghi* is a heteroecious rust. Therefore, there are five basic variations on the rust life cycle; heteromacrocyclic, automacrocyclic, heterodemicyclic, autodemicyclic and microcyclic.

The understanding of rust life cycles has enabled genetic study of these organisms to be conducted. Two major gene systems have been used to study sexual and somatic rust genetics. These are mating type genes and avirulence genes.

1.1.2: Mating type genes

Craigie (1927) proposed that pycniospores of either mating type (+ and -) were compatible only with receptive hyphae of the opposite mating type. He concluded that this mating system was controlled by two alleles at a single locus, and crosses were compatible when the alleles were different. With such a system 50% of crosses are expected to be compatible and therefore produce aecia. This explanation was accepted for many years despite aberrant segregation ratios in *M. lini* and *P. recondita*, reported by Flor (1965) and Whitehouse (1949). However, in 1980 Lawrence crossed two unrelated strains of flax rust and found almost 100% aecial formation, whereas when pycnia from the two strains were self-fertilized only 21% and 35% of the matings formed aecia. Lawrence showed that this was statistically different from the 1:1 ratio suggested by Craigie, and also from the ratios that would be expected for two unlinked loci.

A model that does fit the data is the mating type system of another basidiomycete *Schizophyllum commune* (reviewed by Raper, 1983). The mating system in *S. commune* is controlled by four multi-allelic loci, *Aα*, *Aβ*, *Bα* and *Bβ* with 9, 32, 9 and 9 alleles respectively (Raper *et al*, 1958). The four mating type loci are linked in two pairs, *AαAβ* and *BαBβ*, on two separate chromosomes. The mating type of a monokaryon is determined by two factors, one controlled by the *Aα* and *Aβ* genes, the other by the *Bα* and *Bβ* genes. Complete sexual development occurs when two monokaryons with allelic differences in one or both loci controlling the production of each factor, mate. Lawrence (1988) noted that with such a system, 25% of selfings will be compatible if the parent strain is heterozygous at just one of the loci controlling each factor. This proportion would be greatest when all four loci are heterozygous, the value depending on the amount of recombination between the two loci controlling each factor. Although such a system of genetic control cannot be assumed to exist in the rust fungi, it can explain the variation in the frequency of compatible matings recorded in self-fertilization of rust fungi (Lawrence, 1980; Flor, 1965; Whitehouse, 1949).

Recently mating type genes have been cloned from *S. commune* (Giasson *et al*, 1989), *Coprinus cinereus* (Mutasa *et al*, 1990) and the corn smut fungus, *Ustilago maydis* (Konstrad and Leong, 1989; Schulz *et al*, 1990), all basidiomycetes. Although the molecular characterization of these sequences is still in its initial stages, two points are worth noting. (i) Two alleles of the *Aα* locus of *S. commune* have been cloned by complementation for mating type. Although they were found to map to the same region of the genome by Southern analysis, they did not cross-hybridize to one another nor to any of six other *Aα* alleles. The *Aα* locus appears to be a region of substantial DNA sequence variation. (ii) In *U. maydis* the genetic determination of mating type is somewhat simpler than in *S. commune*, and is specified by two genes *a* and *b*. The *b* locus is multi-allelic (25 alleles) and the *a* locus has two alleles. The basidiospores of *U. maydis* are free-living haploid spores that can be cultured axenically and are not pathogenic towards maize. When basidiospores of compatible mating type fuse, a dikaryon is formed which gives rise to filamentous hyphae that infect the maize plant and produce disease symptoms. The initial fusion event occurs only

when each spore has a different allele at the *a* locus (Holliday, 1961), whereas dikaryon formation and the complete pathogenic development of the organism occurs only when each spore has a different allele at the *b* locus. These two events have been separated genetically. When a haploid basidiospore carrying the *b1* allele is transformed with a cloned copy of the *b2* allele, the haploid spore becomes filamentous and pathogenic towards maize (Konstrad and Leong, 1989). Therefore, it seems likely that the *b* locus product alone can control the expression of genes involved in the infection of maize. Four alleles of the *b* locus have been cloned and all were found to contain a long open reading frame capable of encoding a 410 amino acid polypeptide. Sequence comparisons revealed that the 110 amino acids at the N-terminal end of the protein were variable and the rest of the amino acid sequence was conserved. The proposed amino acid sequence of each allele was found to contain a homeobox-like sequence. Homeotic sequences in *Drosophila* and *Saccharomyces* have been found to code for peptide sequences which bind to DNA 'upstream' of the coding region of other genes and are thought to control transcription (reviewed by Scott *et al*, 1989). The likely regulatory influence of the *b* locus is supported by the transformation experiments of Konstrad and Leong (1989).

Characterization of mating type genes, the proteins that they code for, and the homeotic effects that they may be having, are providing important basic knowledge about fungi. It seems likely that the mating type genes in *U. maydis* and *S. commune* act as master regulatory switches controlling a number of differentiation pathways in the fungus, including pathogenesis. These genes may provide not only a step from which genes involved in pathogenesis may be isolated and cloned, but also may lead to an understanding of the genetic system of these and related basidiomycete fungi. If we are to control rust fungi by means other than plant resistance then this basic knowledge is essential.

1.1.3: Avirulence genes.

Studies of avirulence genes have told us almost all we know about rust genetics. However, before giving an overview of this literature it is important to place avirulence in the spectrum of genetic characters involved in microbial pathogenesis. This will require several terms to be defined. There is also a need to discuss the likely evolution of microbial phytopathogenesis, culminating in the interaction between pathogen avirulence and host plant resistance.

A pathogen is an organism capable of causing disease of a particular host. Pathogenicity, or the ability of a pathogen to cause disease symptoms, is under the control of polygenic characters such as germination, spore attachment, penetration, growth rate, as well as single gene characters like avirulence (*Avr*) and the production of toxins and cell wall degrading enzymes. Lamb *et al* (1989) has estimated that up to 100 genes are involved in bacterial phytopathogenesis. Regardless of how disease is caused, all plant pathogens obtain nutrients from their hosts. However, the methods by which this is done divide them into two general categories. Necrotrophs kill living tissue of the host in advance of colonization and

then invade and metabolize the dead cells. They do this by producing relatively non-specific toxins and degradative enzymes. Consequently, in most cases they can be grown in axenic culture and usually have a wide host range. Biotrophs on the other hand do not kill host tissue but rather colonize and draw metabolites from living cells. They rarely produce toxins, cannot usually be grown in axenic culture, and have a narrow host range. Some rust fungi are examples of biotrophic plant pathogens (Dickinson and Lucas, 1982).

The evolutionary interplay between microbial pathogenesis and the plant response has presumably occurred in a step-wise fashion, alternating between the success of the plant and the success of the pathogen. Despite our ignorance about the biochemical nature of plant/pathogen interactions, there have been several suggestions to describe how it occurs (Person and Mayo, 1974; Ellingboe, 1982; Callow, 1984, 1987; Gabriel and Rolfe, 1990). A brief overview is given below.

Plants are continually exposed to many potential pathogenic micro-organisms. Most never develop or even gain entry due to surface topography, cuticle thickness or other such natural barriers. Other micro-organisms are capable of circumventing these barriers, drawing nutrients and colonizing the host tissue, therefore causing disease. It is thought that in the event of pathogen entry, plants have evolved the ability to detect a limited range of common surface components of the majority of pathogens. Upon recognition, a number of anti-microbial compounds such as phytoalexins and pathogen related (PR) proteins are synthesized. This is known as non-specific resistance. This is opposed to the more specific resistance response in which the host directs the death of its own cells around the region of infection, preventing further colonization of the pathogen. This is known as the hypersensitive response (HR), and occurs primarily as a plant response to attack by biotrophic pathogens such as rusts and mildews.

Once faced with non-specific resistance, pathogens presumably are under a selective pressure to overcome this plant response. It is at this point that the modes of pathogenicity have most likely diverged. Many nectrotrophic pathogens have developed the ability to inhibit the effects of anti-microbial compounds. An example of this is the pisatin demethylase enzyme produced by the phytopathogenic fungus, *Nectria haematococca*. Pisatin demethylase inhibits the action of the pea phytoalexin, pisatin (VanEtten *et al*, 1989).

Alternatively, there is some evidence that necrotrophs may produce toxins that interact with specific receptors involved with the recognition of obligate pathogens. An example of this is the Victoria blight of oats which in 1946 destroyed seventy-five percent of the North American oat crop. Victoria blight is caused by the nectrotrophic pathogen *Helminthosporium victorae* which produces the toxin, victorin. Sensitivity of oats to victorin is controlled by a single gene *Vb*. The oat variety Victoria, released in 1942, contained the *Pc2* gene which conferred resistance to the oat stem rust, *Puccinia coronata*. Subsequent genetic studies have revealed that the *Vb* gene and the *Pc2* gene co-segregate. Despite years of effort, the linkage between *Vb* and *Pc2* has not been broken (Wheeler and Luke, 1955; Wallace and Luke, 1961). One explanation for this is that the two characters are controlled by the same gene. That is, a

receptor molecule for the toxin-causing sensitivity may well be the same receptor molecule that recognizes the rust pathogen, causing hypersensitivity and resistance. If this were the case, it seems feasible to hypothesize that *H. victorae* (a nectrotroph) uses the plant's cell-killing ability, or hypersensitivity, to colonize and obtain nutrients from the dead plant cells. This system may shed some light on the evolutionary pathway of pathogen toxin production.

Biotrophs, on the other hand, have presumably circumvented the non-specific resistance mechanism in a different way. If recognition could be suppressed or diverted, the pathogen would be able to grow undetected within the host tissue and cause disease. To invest such an effort in suppressing recognition would be a logical reason why biotrophs commonly interact with only one or a few plant species. The consequence for the plant of such a system of basic compatibility would be a strong selective pressure to restore the ability to recognize the pathogen. The host gene controlling this more specific recognition would be the resistance gene, the response being hypersensitivity, and the pathogen gene controlling the synthesis of the recognized product, the avirulence gene. Cultivar or race specific resistance is seen, therefore, as being superimposed on a system of basic compatibility (Ellingboe, 1982; Person and Mayo, 1974). This specific interaction is known as the 'gene for gene' hypothesis (Flor, 1942) and is thought to describe the interaction between most, if not all, biotrophic pathogens and their host plants.

The biochemical nature of the 'gene for gene' interaction is unknown, and how such a system evolved is still totally speculative. However, the genetic basis of this interaction is well characterized, particularly in the rust fungi. Each product of a single dominant gene in the pathogen controlling avirulence (*Avr*) interacts with a separate and specific product of a single dominant gene in the host controlling resistance (*R*). Put another way, resistance or incompatibility is only expressed when an avirulent gene in the pathogen is balanced by its corresponding resistance gene in the host. A single avirulence/resistance gene combination is sufficient to activate the hypersensitive response, regardless of how many other virulence and susceptibility gene combinations exist. That is, an avirulence/resistance phenotype is independent of gene combinations at other resistance and avirulence loci.

The genetic control of this interaction was first demonstrated in flax and flax rust by Harold Flor in 1942. The 'gene for gene' model has been described in a number of rusts and their host plants, including *Zea mays* and *Puccinia sorghi* (Hooker and Russell, 1962), as well as other host-pathogen interactions (reviewed by Day, 1974). The genetic basis of pathogenicity in *M. lini*, *P. graminis*, *P. recondita* and *P. striiformis* is well reviewed by Sidhu (1988). In order to briefly review the genetics of rust-host interaction, the *M. lini-Linum* system is discussed.

Twenty nine avirulence genes have so far been described in *M. lini* (reviewed by Lawrence, 1988). Data collected from the inheritance of all but two of these genes is consistent with the model that the pathogen possesses avirulence genes which correspond to specific resistance genes in the host. Most avirulence genes are unlinked whereas the corresponding

resistance genes are grouped at several multi-allelic loci. These trends appear to be observed in other rust fungi and their host plants (Day, 1974).

Several cases of apparent deviation from Flor's model have been reported, however these can be explained by added genetic complexity superimposed on the basic 'gene for gene' model. Several anomalous segregation ratios for avirulence have been reported, some of which could be explained by dominant inhibitor genes (I). Flor (1947) crossed two races of *M. lini* both virulent on the flax variety Williston Brown which carries the M^l gene for resistance. The F_1 was virulent on Williston Brown (avr_{Ml}), however the F_2 progeny segregated in the ratio of 17 avirulent to 116 virulent. Shepherd (1963) postulated that a dominant gene capable of inhibiting the expression of the avirulent phenotype was also segregating in the population. Confirmation of this hypothesis came from further crossing experiments by Lawrence *et al* (1981a). In this report, other dominant inhibitor genes Avr_L, Avr_L7, and Avr_L10 were described. No recombination was found between these genes, therefore it is not known whether they represent three closely linked genes or a single gene controlling inhibition against the three avirulence genes. If the latter were true, this would be a notable exception to the 'gene for gene' model. So far inhibitor genes have not been reported for any rust other than *M. lini*.

The generalization that avirulence genes are unlinked is uncertain as it has only been demonstrated in flax rust, and even for this fungus there is strong evidence for tight linkage of some groups of avirulence genes. Flor (1955) reported that the A_P, A_{P1}, A_{P2}, A_{P3} avirulence specificities were controlled by one or several closely linked genes. Lawrence *et al* (1981b) analysed 3160 test-cross progeny and three recombinants between A_P and A_{P1} were isolated, giving an estimated recombination frequency of 0.19%. These results suggest that A_P, A_{P1}, A_{P2}, and A_{P3} are most likely to be separate genes, which is consistent with the 'gene for gene' model, however they certainly appear to be juxtaposed.

There can be no doubt that avirulence genes have been the most commonly used genetic characters in the study of sexual inheritance in rust fungi. Their discovery and characterisation is a tribute to the work of Harold Flor. As the next section will reveal, avirulence genes have also been the primary genetic markers used to elucidate the role of somatic recombination in rust fungi.

1.2: Somatic genetic systems in rust fungi.

Following the discovery that *Berberis vulgaris* was the alternate host of *P. graminis tritici*, it was assumed that removal of the barberry bush would eliminate sexual reproduction, and thereby greatly reduce the potential for genetic recombination and the generation of variation in rust fungi. As it turned out this did not occur. Several genetic studies found that rust fungi have other ways of recombining genes during the somatic phase of the life cycle by exchanging intact nuclei or heterokaryosis, and by somatic recombination or parasexuality.

However, regardless of how recombination takes place, ultimately there must be a source of new genes. This presumably is the result of mutation.

1.2.1: Mutation

One of the notable characteristics of rust fungi is their capacity to generate new virulence specificities. It is not known whether this is the result of mutation or specific somatic mechanisms of gene rearrangement. One estimate of the change of virulence in *P. graminis tritici* was 8.3 per million spores per generation (Schafer and Roelfs, 1985). This mutation rate is significantly greater than 1×10^{-6} to 1×10^{-9} found in most eukaryotic genes. However, the frequency of mutation in rust fungi is difficult to measure because the size of the urediospore population is almost impossible to gauge. In order to test whether mutation is involved in changes in virulence specificity, mutagens have been tested for their ability to induce changes to virulence (reviewed by Day, 1974). These experiments have varied in their success, the major reasons being as follows:

(i) Virulence is a recessive character, therefore if the pathogen is homozygous at an avirulence locus, two simultaneous mutations would be required to cause a phenotypic change.

(ii) Only mutations to virulence of the known *Avr* genes can be tested. There may be many avirulence genes that have not yet been identified.

(iii) The few virulent spores produced by mutagenesis may not be able to express their phenotype when surrounded by avirulent spores which induce a hypersensitive response from the plant.

(iv) It may be impossible to detect mutant urediospores if they already contain an avirulence gene product. That is, if the avirulence gene product is present at the time the mutagen is applied, then mutants will be eliminated by the host's resistance response. This last possibility is unlikely, as if it were true then no mutation study in which the mutagen is applied at the urediospore stage would result in changes of virulence. This is not the case, as the next section will demonstrate.

A genetically well characterised mutation study of the flax rust *Melampsora lini*, was reported by Flor (1960). In three separate experiments Flor irradiated urediospores with ultraviolet radiation, X-rays and thermal neutrons, and assessed changes in virulence at the A_P avirulence gene complex, described in the last section. The race of *M. lini* used in the study had the genotype $Avr_P \; avr_{P1} \; Avr_{P2} \; Avr_{P3}/avr_P \; Avr_{P1} \; avr_{P2} \; avr_{P3}$ and segregated independently of two other loci Avr_M and Avr_{M4}. Mutation to virulence was selected on plants with the P resistance gene which recognizes only the Avr_P avirulence gene. The Avr_M and Avr_{M4} marker genes were used to eliminate contaminants. Two independent mutants lost Avr_P but also lost the closely linked Avr_{P2} and Avr_{P3} avirulence genes. As discussed in the last section, there is no conclusive proof that the A_P, A_{P1}, A_{P2} and A_{P3} avirulence specificities are controlled by separate tightly linked genes. However, the results of Flor's mutation study suggest that mutation to virulence has most likely been the result of a deletion of the Avr_P gene and flanking genes. This is supported by a mutation study by Schwinghamer (1959) who demonstrated that the frequency of mutation to virulence is proportional to the square of the dose of ionizing radiation, so that it is probably a two-hit process. Therefore, it appears that virulence can result from the loss of an avirulence gene.

To summarise, these studies have revealed that in general mutation is contributing to the frequency of changes in rust avirulence. It is not clear how often mutation occurs at avirulence loci, compared with other genes. A precise and well-defined genetic map containing many neutral genetic markers would allow further investigation of this question, and focus on what mechanisms may control avirulence mutagenesis.

1.2.2: Heterokaryosis

The majority of fungi are haploid. As the hyphae elongate, nuclear division occurs and all the nuclei share a coenocytic cytoplasm. In the hyphae of lower fungi such as the phycomycetes, the cytoplasm is continuous. Whereas in ascomycetes, the hyphae are divided by septa and each compartment contains one or more nuclei. The septa of ascomycetes generally have a central pore through which nuclei can pass. In the higher fungi (basidiomycetes), the cellular organization more closely resembles that of plants and animals; nuclear division is synchronised with the formation of the septum and specialized structures known as clamp connections ensure that each hyphal compartment receives one copy of each nucleus of the dikaryon.

However, this arrangement of nuclei is not always strictly observed. Hyphal fusion, a process known as anastomosis, can result in the mixing of genetically different nuclei in the same mycelium. Such a mycelium is described as a heterokaryon, and the process as heterokaryosis. Heterokaryosis is particularly common and important in coenocytic fungi (Burnett, 1976).

In the basidiomycete fungi two genetically different nuclei exist in a stable situation in the one cytoplasm. This dikaryotic state could be described as a stable heterokaryon. Despite this extra complication several genetic studies suggest that heterokaryosis may occur in rust fungi. The extra complication for dikaryotic fungi is that heterokaryosis would involves the mixing of nuclei of opposite mating types, and therefore must ensure the re-establishment of a compatible dikaryon.

Flor (1964) designed an experiment which provides strong evidence for the occurrence of heterokaryosis in rust fungi. Flor mixed two races of *M. lini* which were heterozygous at four separate avirulence loci and inoculated a plant susceptible to both races. He knew that in the progenitor races the virulent alleles resided in one nucleus and the avirulent alleles in the other. As in the mutation study described in the last section, independent avirulence specificities were monitored to check whether contamination had occurred. Spores were retrieved from the doubly inoculated plant that were homozygous for virulence at all four loci. Flor concluded that the combination of virulence genes must have originated by the reassortment of nuclei of the two parental races. However, Flor was also able to determine the combination of mating type and virulence genotype of each nucleus in the parent races. He found that the reassortment of nuclei may be controlled by mating type, so that only compatible nuclei combined in the resulting hybrid cytoplasm. Such a situation does exist in the

basidiomycete, *Schizophyllum commune*, where only compatible nuclei migrate into one cytoplasm to form a new dikaryon (Buller, 1941; Ellingboe and Raper, 1962).

A number of examples of potential heterokaryosis and nuclear reassortment have been described in other rust fungi, in particular the wheat stripe rust fungus *P. striiformis* (reviewed by Manners, 1988). Variants have been recovered after joint inoculation of different races on susceptible host lines, and screening the harvested spores on plants resistant to both parent races (Little and Manners, 1969a,b, Goddard, 1976a, Wright and Lennard, 1980, Taylor, 1983). In each case, no more than two recombinant types were observed. This is an important difference from recombination of sister chromatids where a number of different recombinants are obtained. As rust fungi are dikaryotic, only two recombinant types would result from the reassortment of nuclei of compatible mating type. It is of interest to note that no alternate host for *P. striiformis* has been identified, therefore it is not known whether stripe rust can undergo sexual reproduction and recombination. However, there seems to be as much genetic variability in stripe rust as any of the other wheat rusts.

The results described above suggest that heterokaryosis and nuclear reassortment may be important sources of genetic variation in rust fungi in nature. However, in many genetic studies of rust fungi, conclusive evidence for heterokaryosis has not been obtained and so studies involving the joint reassortment of neutral genetic markers are needed.

1.2.3 : The parasexual cycle

In a heterokaryon, genetically different haploid nuclei mostly remain separate. However, occasionally they fuse to produce a diploid nucleus. The subsequent behaviour of this diploid nucleus may proceed along two paths. Either, haploidization may occur and the homologous chromosomes segregate to restore the haploid state. In this fashion, unlinked genes will segregate and generate new gene combinations. Alternatively and less commonly, haploidization is accompanied by mitotic recombination between homologous chromosomes. This process is analogous to that which produces recombinant gametes by meiosis, and thus has been termed parasexual recombination. Parasexuality was first described for *Aspergillus nidulans* by Pontecorvo in 1956. The sequence of nuclear fusion, mitotic crossing over and haploidization occurs rather infrequently according to Pontecorvo: 1 in 10^6 or 10^7 mitoses for nuclear fusion, 1 in 500 for mitotic crossing over and 1 in 1000 for haploidization. These small frequencies suggest that the parasexual cycle would be of little importance to species producing spores constantly by meiosis. However, it may be quite significant in generating variation in fungi that lack a sexual cycle (imperfect fungi), or in heterothallic species like rusts, where sexual mating may be relatively infrequent.

Subsequent work on somatic recombination in *Schizophyllum commune* by Ellingboe (1964) and Ellingboe and Raper (1962) have challenged the sequence of events in parasexual recombination described by Pontecorvo. Haploidization in the parasexual cycle proceeds via stages of chromosome loss. However, Ellingboe (1964) argues that this reduction step in *S. commune* is indistinguishable from meiosis. Ellingboe stated that there was no evidence that

haploidization and crossing over were separated in space and time, as described by Pontecorvo.

Regardless of exactly how parasexuality occurs there is no doubt that such a phenomenon exists in fungi. Watson and Luig (1958) co-inoculated a susceptible wheat plant with two races of *P. graminis tritici*, Red 111 and Orange NR-2. Eleven recombinant types were isolated from the harvested spores and therefore could not have resulted from simple nuclear reassortment as described in the last section. It was observed that one recombinant, clearly not a contaminant, was identical in virulence specificity to a prevalent field isolate. This gave some support to the suggestion that parasexual recombination was occurring in rust fungi in nature. A similar study on *P. graminis tritici* and *P. coronata* (oat stem rust) has given results that indicate that parasexual recombination occurs in these rust fungi (Ellingboe, 1961; Bartos *et al*, 1969).

Other explanations have been offered as to why more than two recombinant types are produced from mixed inoculations. Hartley and Williams (1971) propose that dikaryotic cultures may contain more than two haploid nuclear genotypes. Alternatively, mitotic non-disjunction of whole chromosomes may lead to the production of additional genotypes. Furthermore, Nelson *et al* (1955) suggested that recombinants that were derived from mixed inoculations of *P. graminis* f. sp. *tritici*, resulted from heterokaryosis in which stable tri-nucleate mycelia were formed.

There are obviously many permutations of somatic recombination of nuclei, chromosomes and genes that are available to fungi. The challenge for geneticists is to establish in what form and how often somatic recombination events occur. A further question plant pathologists would like to answer is how important these events are in contributing to new virulence specificities in the field. Day (1974) concluded from his review of somatic recombination in fungi, that reassociation and recombination of nuclei, chromosomes and genes during vegetative growth of urediospore mixtures contributes to genetic variability in rusts.

The genetic studies of rust fungi have done much to enhance our understanding of the biology of these organisms and the management of disease by breeding for resistance. However, one consistent limitation of these studies is the paucity of genetic markers in rust fungi. The confirmation of suggested genetic mechanisms in rusts could be argued more conclusively if segregating markers other than virulence were used.

The next section deals with genetic markers that have been used in genetic studies of rust fungi to date. This section also discusses the use of molecular probes to define restriction fragment length polymorphisms and their potential application to rust fungi and other phytopathogens.

1.3: Genetic markers in rust fungi

Markers used in genetic studies can be defined as phenotypic differences that occur at certain positions (loci) on chromosomes which make it possible to follow the transmission of those loci through successive generations. Genetic markers are essential for studying population structure and in constructing linkage maps of eukaryotic genomes. Furthermore, for plant pathologists they provide important practical information about the spread of epidemics and the origin of new virulent forms in the pathogen population. This type of information can greatly facilitate the control of plant diseases and allow predictive plant breeding.

To date, avirulence markers have most commonly been used in genetic studies of rust fungi. Rust species are divided into physiological races determined by their virulence on a set of host lines each carrying different resistance genes which collectively are called the host 'differentials'. However, as discussed earlier, avirulence is a very mutable character. It is not known whether this stems from a large pool of genetic variation in the pathogen population, or specific mechanisms exist by which changes of virulence occur. The variability of virulence specificity may differ greatly from that of phenotypically neutral markers.

The following section describes markers, other than those involved with virulence, that have been used for studying rust fungi. Also, a description is given of the molecular basis of Restriction Fragment Length Polymorphisms (RFLPs), and their use in studying the genetics of phytopathogenic fungi.

1.3.1: Spore pigmentation.

The cytoplasm of a urediospore of *P. graminis tritici* normally contains an orange carotenoid and the spore wall is brown. The two colours together produce a wild type urediospore that is reddish brown. The spontaneous 'orange' mutant has a colourless spore wall, whereas another mutant ,'grayish brown', has a colourless cytoplasm (Newton and Johnson, 1927). Both mutants are recessive and when crossed give an F_1 hybrid with wild-type spores. Selfing the F_1 produces the double mutant named, 'white spores', which has colourless walls and cytoplasm (Newton *et al*, 1930). Watson and Luig (1959) and Ellingboe (1961) used this spore colour phenotype, in combination with other avirulence genes, to study somatic hybridization in *P. graminis tritici*.

There have been many reports of changes in spore pigmentation by spontaneous and induced mutation in other rusts including *M. lini*, *P. hordei*, *P. helianthi* and *P. graminis avenae* (reviewed by Day, 1974).

1.3.2: Isozymes

Isozymes are electrophoretically distinguishable forms of an enzyme and are most frequently caused by amino acid changes to the protein resulting in charge differences. The changes usually have no effect on function and, therefore, are selectively neutral. Isozymes are inherited as codominant variants and hence can be used as genetic markers.

Isozymes have been used in genetic studies of a number of plant pathogenic fungi (Newton, 1987) including the rusts *P. graminis tritici* and *P. recondita* (Burdon *et al*, 1983; Burdon and Roelfs, 1986) and *P. striiformis* and *P. hordei* (Newton *et al*, 1985). All of these studies concluded that, despite variation in virulence specificity, very little isozyme variation exists. Burdon and Roelfs (1986) looked at 65 races of *P. graminis tritici* from 13 countries and detected 13 loci with 10 enzyme systems. Five loci showed no variation, whereas 2 to 4 alleles were detected at the remaining loci. Linde *et al* (1990) compared isozyme and virulence diversity patterns in the bean rust fungus *Uromyces appendiculatus*. In 27 geographically diverse isolates of the fungus, 15 phenotypic isozymes markers were reported. However, greater diversity was found for virulence than for isozymes.

One of the few examples where isozymes have been used to study genetic systems in rust fungi was reported by Burdon *et al* (1981). Previous work by Watson and Luig (1959) and Luig and Watson (1972) suggested that a somatic hybridization event had occurred between *P. graminis tritici* (wheat stem rust) and *P. graminis secalis* (rye stem rust) on the rough wheat grass, *Agropyron scabrum*. The hybrid, known as the *scabrum* rust, was virulent on neither wheat nor rye but virulent on *A. scabrum* and the barley grass *Hordeum leporinum*. This hypothesis was supported by differences in the virulence characteristics of the hybrid and the putative parental races. Burdon *et al* (1981) showed that isozyme variants of two independent enzyme systems were inherited in a way that was consistent with Luig and Watson's hypothesis. Futhermore, they concluded that the hybrid probably arose by exchange of nuclei.

Although these studies reveal that isozymes are simple and reliable genetic markers, they are limited by the number of enzyme detection systems available and the relatively low degree of variability detected. This appears consistent with comparisons of total protein profiles separated by two-dimensional isoelectric focusing and polyacrylamide gel electrophoresis (Kim *et al*, 1985). Out of 280 polypeptides detected in two races of *P. graminis secalis* only 5-7 polypeptides differed, and there were 17 differences between rye and wheat stem rust and 92 between rye and oat stem rust.

1.3.3 : Double stranded RNA.

Mycoviruses with double stranded RNA (dsRNA) genomes have been isolated from many fungal species and they are particularly widespread among the uredinales. Pryor *et al* (1990) have found dsRNAs in 33 of 38 collections of rust fungi representing 15 species from four genera. In most fungi the dsRNAs are encapsidated in isometric virus-like particles (Dickinson and Pryor, 1989), however their presence appears to have no phenotypic effect on the fungus (Pryor *et al*, 1990).

Despite the lack of knowledge about the function of dsRNAs they have been used as markers in the rust fungi (Newton *et al*, 1985). Pryor *et al* (1990) could identify isolates of *P. striiformis*, *P. recondita*, *P. graminis*, *P. sorghi*, *P. menthae*, *P. helianthi*, *P. coronata* and *Melampsora lini* on the basis of the size and number of dsRNAs they contained. Furthermore,

Pryor and Boelen (1987) were able to differentiate between two races of *P. sorghi* on their dsRNAs. However, Lawrence *et al* (1988) showed that dsRNAs in flax rust are inherited in a non-Mendelian manner, which raises considerable doubt as to their usefulness as genetic markers in rust fungi.

1.3.4 : Restriction Fragment Length Polymorphisms (RFLPs)

RFLPs were first used by Botstein *et al* (1980) as genetic markers in the human genome. Since then they have been rapidly applied to many different organisms. Several plants including maize, tomato, pepper, *Arabidopsis* and lettuce have RFLP maps covering most of their genomes (Helentjaris *et al*, 1986; Tanksley *et al*, 1988; Chang *et al*, 1988 and Landry *et al*, 1987). As with many other systems, the application of RFLP mapping to phytopathogenic fungi has lagged behind, with the notable exception of the downy mildew of lettuce, *Bremia lactucae* (Hulbert and Michelmore, 1988).

The basic principle underlying RFLPs is that when total genomic DNA from an organism is digested with a restriction endonuclease a unique array of DNA fragments of different lengths are produced. Differences between individuals in the lengths of the fragments could result from a number of molecular changes. One or more individual base pairs could differ resulting in the loss of a restriction site, or the formation of a new one. Alternatively, gross chromosomal changes such as insertion, deletion or inversion of DNA could alter the size of the fragments. These molecular changes can be recognised by fractionating the mixture of fragments by electrophoresis in an agarose gel and seeking fragments with altered mobility. A specific fragment or fragments can be selected from a large genomic population by the blotting and hybridization technique developed by Southern (1975). Observed molecular differences, even if caused by a single base mutation, represent a heritable change giving rise to a phenotype expressed in terms of a restriction fragment length. Therefore, RFLPs can be used like any other genetic marker. The principle of RFLP analysis is shown in Figure 1.2.

RFLP analysis has several advantages over other genetic markers.
(i) The phenotype is not the result of gene expression, therefore neutral mutations can be detected.
(ii) The number of potential markers is limited only by the size of the genome as the technique is capable of detecting single base changes anywhere in the genome.
(iii) Most isozyme or morphological phenotypes require gene expression of that locus, and this may be tissue specific. Detection of RFLPs requires genomic DNA and this can be extracted from any part of the organism.
(iv) RFLPs are codominant, therefore heterozygotes can be unequivocally distinguished from either homozygote in an F_2 progeny test.

To date, few examples of RFLPs in fungi have been reported. This probably can be attributed to the fact that in the more commonly studied saprophytic fungi the trend still remains
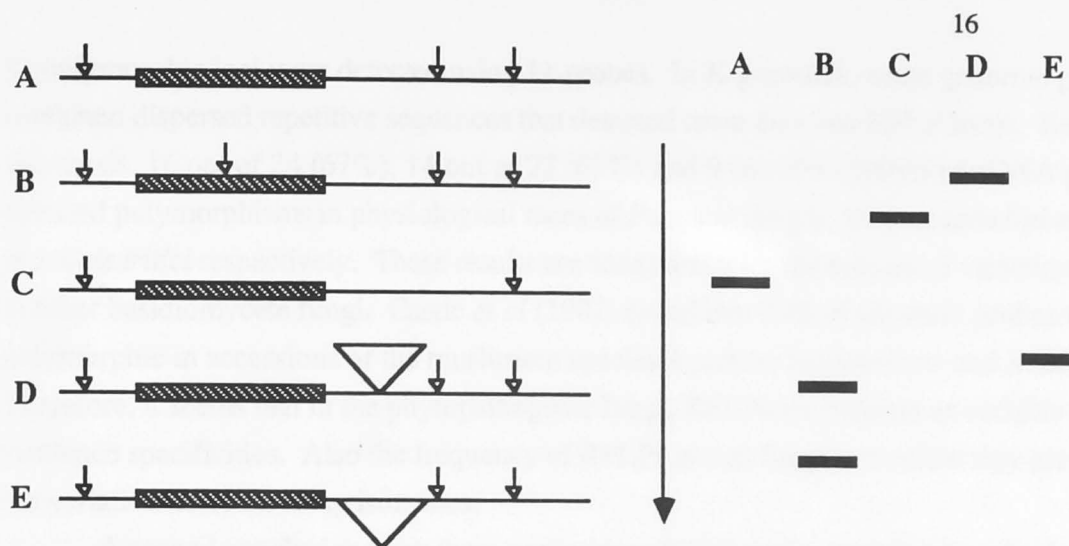
Figure 1.2. Ways in which molecular changes result in restriction fragment length polymorphisms. (i) Shaded region is homologous to a cloned sequence used as a probe. Arrows indicate restriction endonuclease sites. (ii) Banding pattern when genomic DNA from the 5 lines is restricted with a restriction enzyme, separated on an agarose gel, blotted and hybridized to a radioactively labelled probe from the shaded region. **A.** Wild type pattern. **B.** Base mutation creating a new restriction site within the probe which generates two genomic fragments that hybridize to the probe. **C.** Base mutation causing the loss of a restriction site. **D.** Insertion of DNA within the wild type fragment. **E.** Deletion of DNA from within the wild type fragment. (Adapted from Michelmore and Hulbert, 1987)

to use auxotrophic, morphological and drug resistant mutants as genetic markers. These have been sufficient, given the small genome size of most fungi, to establish quite complete genetic maps (O'Brien, 1990). In most phytopathogenic fungi such mutants are not available, therefore the use of RFLP markers may become important. Of the RFLPs so far identified in fungi the majority have been in mitochondrial DNA. They have been used to identify strains and construct phylogenetic trees (Taylor, 1986; Forster *et al*, 1988; Kistler *et al*, 1987). There has only been several reports of RFLPs in nuclear DNA of phytopathogenic fungi, including *Bremia lactucae* (downy mildew of lettuce; Hulbert and Michelmore, 1988); *Septoria tritici* (wheat leaf spot; McDonald and Martinez, 1990); *Erysiphe graminis hordei* (powdery mildew of barley; Christiansen and Giese, 1990; O'Dell *et al*, 1989); and *Colletotrichum gloeosporioides* (the cause of anthracnose on *Stylosanthes*; Braithwaite *et al*, 1990).

In these studies random genomic and ribosomal DNA clones have been used as probes to identify RFLPs in isolates of different virulence and geographic origin. In both mildews of lettuce and barley, RFLP and avirulence loci have been positioned on a genetic map. Although the maps of these fungal genomes are far from complete, close linkage between an RFLP and an avirulence locus will enable the avirulence gene to be cloned by chromosome walking (Young, 1990).

Although these reports are preliminary, several conclusions can be drawn from the data. Firstly, there is the observation of how frequently RFLPs detect variation in intraspecific comparisons. In *S. tritici*, 33 out of 34 probes (97%) revealed polymorphisms in different isolates. In *B. lactucae* 38% (76/201) of the probes were polymorphic whereas in *E. graminis*,

31 polymorphic loci were detected using 11 probes. In *E. graminis*, some genomic probes contained dispersed repetitive sequences that detected more than one RFLP locus. Reported in this thesis, 16 out of 24 (67%), 14 out of 22 (63%) and 9 out of 9 (100%) of cDNA probes detected polymorphisms in physiological races of *Puccinia sorghi*, *Melampsora lini* and *P. graminis tritici* respectively. These results are consistent with the amount of variation observed in other basidiomycete fungi. Castle *et al* (1987) found that 70% of genomic probes were polymorphic in accessions of the mushroom species *Agaricus brunnescens* and *A. bitorquis*. Therefore, it seems that in the phytopathogenic fungi, RFLPs are perhaps as variable as virulence specificities. Also the frequency of RFLPs in rust fungi is considerably greater than the variation detected using isozymes.

A second conclusion from these preliminary RFLP studies is that genomic clones frequently contained repetitive sequences. Genomic DNA from the majority of fungi, mainly saprophytic laboratory strains, contain between 2-10% repetitive DNA (refer to Table 1.1). It has always been assumed that lower eukaryotes like fungi contain less repetitive DNA than higher eukaryotes. However, recent reports suggest that *Bremia lactucae*, *Fusarium graminearum* and *Puccinia sorghi* (refer to chapter 2) contain much more repetitive DNA in their genomes. Francis *et al* (1990) found from reassociation analysis and reconstruction experiments that 65% of the genome of *Bremia lactucae* is repeated, and almost 90% of genomic clones in a 1.0-5.0kbp size range contained repeated DNA sequences. Similar observations, although not as pronounced, were found using genomic probes from *S. tritici* and *E. graminis*. Furthermore, chapter 6 of this thesis describes an RFLP in *P. sorghi* caused by the insertion of a highly repeated sequence. Therefore, it appears that a body of data is accumulating to suggest that the genomes of phytopathogenic fungi may be more complex than their saprophytic counterparts.

Whether this generalization is true or not remains to be seen, however these examples serve to emphasize the multifaceted applications of RFLPs. In the few reports sighted so far in the literature, RFLPs have already provided an important tool to dissect phytopathogenic fungi at the population, species, race and genome level. However, RFLPs should not be looked at as just genetic markers. They provide an insight into the genetic system and phylogeny of an organism, as well as providing information about the structure of the genome. It is an aim of this thesis to use RFLPs to study rust fungi.

1.4: Fungal genomes

The total genetic information stored in the chromosomes of an organism is said to constitute its genome. The fungal genome is composed of three major parts within the cell - nuclear DNA, mitochondrial DNA and extrachromosomal DNA. Chronologically, fungal genomes were studied firstly by cytology, then by measurements of DNA base composition and genome size and complexity, and more recently at the gene structure level using molecular techniques. When the studies described in this thesis were started, information about the genomes of rust fungi had only been obtained at the cytological level. No measurements of

base composition or genome size and complexity had been reported for any rust fungi, and no gene had been cloned and characterised.

The majority of information on the genetics of fungi comes from a few species, mainly *Saccharomyces*, *Neurospora* and *Aspergillus*. Therefore, generalizations about genome structure within the fungal kingdom are considerably biased.

1.4.1: Karyotype

Despite the small size of fungal mitotic chromosomes, haploid chromosome counts have been recorded for a number of fungi, including several rust species. A haploid chromosome number of 6 has been reported in *P. graminis tritici*, *P. helianthi*, *P. recondita*, *P. striiformis* and *P. sorghi*. (M^{C}Ginnis 1953, 1954, 1956; Valkoun and Bartos, 1974; Goddard, 1976b). McGinnis (1954) reported a chromosome number for *P. coronata* of 3, and *Melampsora lini* probably has 5 or 6 chromosomes (Kapoorie, 1973). M^{C}Ginnis (1956) suggested that the haploid chromosome number of grass rusts is 3, and that rusts with a haploid chromosome number of 6 may be tetraploids. This is supported by Burnett (1976) whose mitotic preparation from *P. graminis* showed evidence of residual terminal attraction of chromosomes. In early stages of mitosis, chromosomes appear to be joined at the ends which may reflect residual homology. Burnett suggested that the cereal rust fungi that have a haploid chromosome number of 6 may be allopolyploids. No further investigation of this observation was found in the literature.

Recently several techniques have been developed that may assist in the analysis of fungal genomes. Firstly, confocal microscopy enables the genomic chromatin within the nucleus to be seen in 3 dimensions. This has led to an estimate of the chromosome number in *P. graminis* to be 18 (Szabo, personal communication). Whether this is the haploid or diploid number is unknown. The second technical development is the separation of small but intact chromosomes from simple eukaryotic organisms like fungi using pulsed-field agarose gel electrophoresis. This technique was first described in 1984 by Schwartz and Cantor who separated all 16 chromosomes of *Saccharomyces cerevisiae* (bakers yeast). Since then several modifications to the initial apparatus have been described which are capable of separating DNA molecules up to 7000-9000 kbp (7-9 Mb) in size. The basic technique involves migrating chromosomal DNA molecules through an agarose gel by an electric field which alternates in direction through 120° over a set pulse time. Increasing the pulse time increases the size of molecules separated (Southern *et al*, 1987; Chu *et al*, 1986; Birren *et al*, 1988). This method has been used successfully to separate chromosomes from many fungi in particular *Saccharomyces cerevisiae* (Schwartz and Cantor, 1984; Carle and Olson, 1985), *S. pombe* (Smith *et al*, 1987), *Candida albicans* (Snell and Wilkins, 1986), *Neurospora crassa* (Orbach *et al*, 1988), *Aspergillus niger* (Debets *et al*, 1990) and several plant pathogenic fungi, including *Phytophthora* (Howlett, 1989), *Bremia lactucae* (Michelmore, personal communication), *Ustilago maydis* (Kinscherf and Leong, 1988), *Magnaporthe grisae* (Hamer *et al*, 1989), *Cochliobolus heterostrophus* (Tzeng *et al*, 1989), *Colletotrichum gloeosporioides* (Masel *et al*,

1990) and *Septoria nodorum* (May and Anderson, unpublished). Two notable observations have come from the electrophoretic studies of fungal chromosomes. The first was the large extent of variability in the length of chromosomes from fungal strains thought to be closely related. This has been observed in yeast (Steensma *et al*, 1988; Ono and Ishino-Arao, 1988) and *Ustilago maydis* (Kinscherf and Leong, 1988). The second was the observation of populations of small 'mini chromosomes', 100-1000kbp in length, observed in *Bremia lactucae* (Michelmore, personal communication), *Colletotrichum gloeosporioides* (Masel *et al*, 1990) and *Septoria nodorum* (May and Anderson, unpublished). They are inherited in a non-Mendelian fashion in *B. lactucae* (Michelmore, personal communication), and in all reports differ in size and number between closely related strains. The function of these 'mini chromosomes' is unknown.

1.4.2: Genome organization
Nuclear DNA

One of the first attempts to analyse the genomic DNA of an organism was by measuring base composition, recorded as G+C content (%GC). The %GC content of a DNA molecule directly influences its buoyant density in a CsCl gradient, which can be measured by analytical ultracentrifugation. Storck and Alexopoulos (1970) measured the %GC content of 322 fungal species and related this information to fungal phylogeny. Their basic conclusion was that fungal evolution is accompanied by an increase in %GC content, a trend also observed among bacteria of the actinomycetes (DeLey, 1968). Average measurements of the fungal taxa revealed that basidiomycetes have %GC content greater than 50%, ascomycetes approximately equal to 50% and oomycetes and zygomycetes less than 50%.

Studies of genomic DNA base composition have also led to the discovery of satellite DNAs. These satellites are identified by analytical ultracentrifugation as they have different buoyant densities from the major part of the genomic DNA. A 2μm long plasmid in yeast was first observed as a small satellite peak distinct from the major nuclear DNA peak on CsCl gradients (Stevens and Moustacchi, 1971). The nature and frequency of these satellite sequences among fungi will be discussed later.

The measurement of the kinetics of DNA/DNA reassociation has provided much information about the organization of eukaryotic genomes. The technique is reviewed by Britten *et al* (1974). The average size of the fungal haploid genome is about 2.5×10^7bp or 0.05pg of DNA. This is 5 times the average size of a bacterial genome and 1% that of human beings. Table 1.1 presents the results of genome size estimates of a number of fungal species measured by reassociation kinetics. In general it appears that the size of the genome is larger in the more evolutionary advanced fungi, like the basidiomycetes.

In most fungi, 5-10% of the genome is comprised of repeated sequence DNA compared to 10-90% in higher eukaryotes. There are several exceptions to this rule, in particular the oomycete *Bremia lactucae* (downy mildew of lettuce), in which 65% of the genome is

Table 1.1. Fungal nuclear DNA content and organization

Fungal taxa	Nuclear DNA content	Single copy fraction	Repetitive DNA fraction			Reference
	bp x 10^7	Percentage of genome	Percentage of genome	Number of copies /diploid genome	Average size of repeated unit	
MYXOMYCOTA (slime moulds)						
Physarum	56	58	42	1.5x10^4	1.6x10^5	Fouquet *et al*, 1974
Dictyostelium	4.5	70	30	113	1.2x10^5	Firtel and Kindle, 1975
EUMYCOTA (true fungi)						
Oomycetes						
Achlya	4.3	82	fraction I 14	80	7.6x10^4	Hudspeth *et al*, 1977
			fraction II 2	2200	380	
Bremia	5	34	fraction I 21	1200	9.6x10^3	Francis *et al*, 1990
			fraction II 38	40	4.9x10^4	
Chytridiomycetes						
Allomyces	2.6	75	10	320	1.1x10^4	Ojha *et al*, 1977
Zygomycetes						
Phycomyces	6.6	50-55	30-35	1300	1.6x10^4	Harshey *et al*, 1979
Ascomycetes						
Penicillium	4.3	78	10	5828	9.5x10^3	Sahasrabudhe *et al*, 1985
Saccharomyces	1.4	87	fraction I 11	63	1.6x10^4	Lauer *et al*, 1977
			fraction II 1.7	71	2.0x10^3	
Aspergillus	2.7	97-98	2-3	60	1.1x10^4	Timberlake, 1978
Neurospora	2.7	90	8	140	1.5x10^4	Krumlauf and Marzluf, 1979
Fusarium	2.4	77-79	21-23	500	4.2x10^4	Szecsi, 1981
Basidiomycetes						
Coprinus	4.5	87	10-12	110	4.8x10^4	Dutta *et al*, 1972
Schizophyllum	3.6	90	7	49	5.0x10^4	Dons *et al*, 1979
Agaricus	3.4	83	16	50	6.8x10^5	Arthur *et al*, 1982

composed of repeated sequences, and *Fusarium graminearum* which contains repeated DNA in 22% of its genome (Szecsi, 1981).

Another technique to measure genome size involves the use of homologous cloned DNA sequences in genomic reconstruction experiments (Francis *et al*, 1990). This technique has been used to measure the genome size of a number of fungal species including *Bremia* (Francis *et al*, 1990); *Phanerochaete* and *Sporotrichum* (Raeder and Broda, 1984). The genome size of the lignin-degrading basidiomycete fungi, *Phanerochaete* and *Sporotrichum*, was measured to be 4-5x10^{7}bp.

The only other technique which has been used to measure genome size is the staining and fluorescent densitometry of intact nuclei. Murrin *et al* (1986) reported the genome size of the zygomycete, *Entomophaga aulicae*, to be 0.8pg of DNA. This is over an order of magnitude larger than the average size of other fungal genomes. Such techniques as fluorescent densitometry do not measure genome complexity and it is possible that the *Entomophaga* genome is polyploid.

Mitochondrial DNA

Mitochondrial DNA (mtDNA) constitutes between 1-20% of the total cellular DNA in fungi. In a yeast cell there are about 10-20 mitochondria, each containing 4 copies of the genome. In most cases fungal mtDNA is a closed covalently linked circular molecule (Evans, 1983). Two exceptions are the linear mitochondrial genomes of the yeast *Hansenula marku* (Wesolowski and Fukuhara, 1981) and the slime mould *Physarum polycephalum* (Kawano *et al*, 1982). The content of genes in mitochondrial genomes has remained relatively constant despite the differences in length of mtDNA in eukaryotic organisms. In higher animals the mitochondrial genome varies between 16-17kbp, which is close to the estimated size of the coding sequences (Wallace, 1982). In contrast, there is extensive variation in the size of mitochondrial genomes of plants and fungi (Ward *et al*, 1981). In fungi the length of mtDNA varies from about 19kbp in the yeast *Torulopsis glabrata*, to about 176kbp in the mushroom *Agaracis bitorquis* (Clark-Walker and Sriprakash, 1981; Hintz *et al*, 1985). The mitochondrial genomes of basidiomycetes such as *Schizophyllum* and *Agaricus* fall within the upper end of this scale. Furthermore, species of a single genus may vary in the sizes of their mtDNA. Hintz *et al* (1985) reported that mtDNA from *Agaricus brunnescens* ranges from 95.5 to 100kbp, whereas in *A. bitorquis* the mtDNA ranges from 148.5 to 176.3kbp.

The reason for this variability in size of fungal mitochondrial genomes in unknown. Turner *et al* (1982) postulates that the variability is the result of duplications or deletions of regions within basically similar genomes. Alternatively, large AT rich non-coding sequences scattered throughout the genomes of several fungal mitochondria may contribute to length variation. The function of these regions is unknown.

Regardless of what has caused the variability in mtDNA length, this character has provided a useful tool for understanding fungal phylogeny. Insufficient data exists on mtDNA from phytopathogenic fungi to comment on the significance of structure and function in relation

to the lifestyle of these organisms. However, there are several examples of restriction fragment length polymorphisms in mtDNA reported in *Fusarium* and *Phytophthora* that could be used in strain identification (Kistler *et al*, 1987; Forster *et al*, 1988).

Extra chromosomal DNA

Circular and linear DNA plasmids have been found in the mitochondria and nuclei of many fungi. However, unlike bacterial plasmids, many of which are known to confer a selective advantage on the host, most fungal plasmids are not known to have any phenotypic effect. Most fungal plasmids are found within the mitochondria and some show homology to mtDNA (reviewed by Nargang, 1985; Böckelmann *et al*, 1985; Lambowitz *et al*, 1986).

Of those plasmids that show homology to mtDNA, the mitochondrial plasmid associated with the senescence phenotype in *Podospora anserina* is the most thoroughly understood. Isolates with this plasmid grow normally for 20 days, but then the rate of growth decreases, and eventually they die. Senescence is caused by the replication of a circular 2539bp DNA molecule. The sequence of this molecule is located in the mitochondrial genome of normal isolates, but in some isolates it becomes excised and replicates autonomously as a plasmid. Its excision is under nuclear control and it has been reported to transpose and integrate into the nuclear genome. Kuck *et al* (1985) has proposed that this DNA molecule is the intron I of the cytochrome-C oxidase gene (*COI*). DNA sequencing of the plasmid has revealed a long open reading frame thought to code for an RNA maturase involved in processing the *COI* gene transcript (Karsch *et al*, 1987). Once the plasmid is excised from *COI*, or transcribed into DNA from a pre mRNA, ligation and autonomous replication can occur. The plasmid DNA is thought to block essential functions in the mitochondria or nucleus which leads to senescence.

Other mutants associated with structural changes in mtDNA caused by excision and insertion of extra chromosomal DNA, are the *rho⁻* mutant in yeast (Evans, 1983); *poky* and *stopper* mutants in *Neurospora crassa* (Akins and Lambowitz, 1984; De Vries and De Jonge, 1984) and the *ragged* mutant of *Aspergillus anserina* (Cummings *et al*, 1985).

In contrast to the senescence-inducing defective mitochondrial DNAs, the true mitochondrial plasmids or those independent of mtDNA, appear to have neither a positive or negative effect on the cell. The first report of such a plasmid by Collins *et al* (1981) was in *Neurospora crassa*. Eight different plasmids were reported in eight different wild strains and, as yet, no plasmids have been found in any laboratory strain. The 'Mauriceville' plasmid found in a Texas strain of *N. crassa* by Natvig *et al* (1984) has been well characterized. DNA sequence data revealed a scattered arrangement of five 18bp palindromic elements within this plasmid. Furthermore, a long open reading frame capable of encoding a 710 amino acid polypeptide was found. This protein has not been identified and its function, if any, remains unknown. However, blocks of sequence homology to viral reverse transcriptase genes and to group I mitochondrial introns have been found. Kuiper and Lambowitz (1988) demonstrated reverse transcriptase activity from strains containing the plasmid, whereas no activity was

found in strains that did not contain the plasmid. On the basis of this evidence, it is possible that these plasmids are related to transposable elements described in other organisms (Nargang *et al*, 1984).

Recently, mitochondrial plasmids have been found in several plant pathogenic fungi including *Claviceps*, (Tudzynski *et al*, 1983); *Cochliobolus*, (Garber *et al*, 1984); *Fusarium*, (Kistler and Leong, 1986, Samac and Leong, 1988); *Ceratocystis*, (Giasson and Lalonde, 1987) and *Gaeumannomyces*, (Honeyman and Currier, 1986). These are all linear mitochondrial plasmids and contain sequences that appear related to those of transposable elements and viruses. They contain terminal inverted repeats and 5' terminal covalently attached proteins (Duvell *et al*, 1988). It has been proposed by Meinhardt *et al* (1986) that the terminal inverted repeats and the terminal proteins function in plasmid replication.

Despite the structural and sequence information of these plasmids, no definite physiological role has been demonstrated for them. Particular mitochondrial plasmids from *Fusarium oxysporum* have been correlated with the host range of the fungus. However, the evidence was not conclusive because the correlation was not established in an isogenic background (Kislter and Leong, 1986; Kistler *et al*, 1987). A strain of *Nectria haematococca* (*Fusarium-solani* f. sp. *cucurbitae*) from which the plasmids had been removed was less pathogenic (Samac and Leong, 1988). However, the process of removing the plasmid appears to have created mutations that caused a decrease in pathogenicity (Samac and Leong, 1989a). Samac and Leong (1989b) suggest that mitochondrial plasmids may represent 'selfish DNA' that has no adaptive value for the cell but can replicate efficiently in host mitochondria.

The remaining category of fungal plasmids includes those found to reside in the nucleus. Although not as common as mitochondrial plasmids, one representative, the 2μm plasmid from *Saccharomyces cerevisiae*, was the first reported and most extensively studied of all eukaryotic plasmids (reviewed by Broach, 1982). Most strains of *Saccharomyces cerevisiae* contain about 30-100 copies of the plasmid in the cell nucleus. The DNA is associated with nuclear histone proteins and folded into chromatin, however it has never been found integrated into the chromosomes. The plasmid is a covalently linked circular molecule 6318bp long, and contains two 599bp inverted repeats, separating unique sequences 2346bp and 2774bp in length. The inverted repeats are sites of intra-molecular recombination which may allow oligomeric plasmids to form. The plasmid contains an origin of replication and four coding regions, three of which are involved in DNA replication and the fourth in intra-molecular recombination. Despite considerable research effort, the role of the 2μm plasmid still remains obscure, and may represent another example of 'selfish' DNA.

Other nuclear plasmids have been described in the slime mould *Dictyostelium discoideum* (Metz *et al*, 1983), the mycorrhiza forming fungus *Morchella* (Meinhardt and Esser, 1984) and the plant pathogenic fungus *Rhizoctonia* (Hashiba *et al*, 1984). Hashiba *et al* reported that the presence of the plasmid has been correlated with low phytopathogenicity or hypovirulence, however whether it is the causative agent still remains to be determined.

1.5.3: Gene structure

The fungal genes that have been isolated and cloned have almost all come from the ascomycetes, *Saccharomyces*, *Neurospora* and *Aspergillus*. The basidiomycetes are poorly represented, although this is changing with the development of genetic systems in *Schizophyllum* and *Ustilago* (Wang *et al*, 1988; Froeliger *et al*, 1987). Similarly, very few genes have been cloned and sequenced from phytopathogenic fungi. Genes that have been isolated are a mating type gene from *Ustilago maydis* (Konstrad and Leong, 1989) and cutinase and pisatin demethylase (*PAI*) genes from *Nectria haematococca* (Van Etten *et al*, 1989). Mating type genes control the selection of compatible haploid nuclei during somatic and sexual hybridization, however these genes may have a role in pathogenesis. The proteins encoded by these genes show sequence similarity to transcription factors implicated in homeotic development in other eukaryotes. This has already been discussed in section 1.1.2. Cutinase is a plant cell wall degrading enzyme involved in fungal penetration, and pisatin demethylase is an enzyme involved in overcoming the anti-fungal activity of the pea phytoalexin, pisatin. The structure of these genes in relation to gene expression and function is not well understood.

Collectively, the data on fungal gene structure yield two major conclusions. Firstly, it appears that ascomycetes and 'lower fungi' use different signal sequences in gene expression from those used by the higher fungi (basidiomycetes) (Turner and Ballance, 1987). A consequence of this is that *Aspergillus* spp. are able to express genes from many fungal species, including the zygomycete *Mucor* (Cullen and Leong, 1986). However, gene expression in the basidiomycete *Coprinus cinerius* seems more specific and is limited to genes from other basidiomycete species. A *trp-2* mutant *C. cinerius* strain was complemented by heterologous tryptophan synthetase genes from two other basidiomycete fungi, *Schizophyllum commune* (*trp1*) and *Phanerochaete chrysosporium* (*trpC*), but not by a gene from the ascomycete *A. nidulans* (Casselton and Herce, 1989). Furthermore, attempts to express genes from *Ustilago maydis* (corn smut) in *A. nidulans* have so far failed (John Clarkson, personal communication).

The second point concerns the structure of messenger RNA (mRNA) and the sites of polyadenylation, origin of translation, and the splicing of intervening sequences. Genes in fungi, as in higher eukaryotes, are transcribed as individual mRNAs. They are polyadenylated, although the length of the poly-A tails that have been reported are smaller than in higher eukaryotes (Rambosek and Leach, 1987). Of the few fungal genes where polyadenylation sites have been identified no consensus sequence exists. The initiation of translation of fungal genes appears, as in higher eukaryotes, to be associated with the methionine codon ATG (Rambosek and Leach, 1987). Introns have been found in most but not all fungal genes that have been sequenced. The typical intron is between 49 and 85bp, although a larger class between 127 and 280bp has been described. No intron has yet been found that exceeds 280bp, and this is considerably shorter than most mammalian and plant introns which are frequently greater than 1kbp. In yeast nuclear genes, introns contain an internal conserved sequence and a consensus sequence at the 5' and 3' splice sites. This is also

the case in filamentous fungi, although the consistency of the sequence is not as great as it is in
the yeasts. The consensus sequence of the splice sites in yeast introns is:

5' GTPuNGT ...... NPuCTPuACN ...... PyAG 3'   (Rambosek and Leach, 1987).

In general, too few fungal genes have been cloned and sequenced for any confident
generalisations to be made about gene structure in relation to expression and function. The data
at present would suggest that no striking differences exist between fungal and higher
eukaryotic genes.

1.4.4: Transposable elements

Many of the rearrangements observed in the mitochondrial genomes of fungi can be
attributed to DNA molecules which resemble, at the sequence level, mobile genetic elements.
In addition, many group I introns described in fungal mitochondrial genes, contain coding
sequences homologous to reverse transcriptases and RNase H enzymes. It has been proposed
that these introns were once mobile elements that have retained self-splicing activity but lost the
ability to replicate autonomously (Samac and Leong, 1989b).

Despite these findings, there have been few reports of true mobile elements in fungi.
One exception is the Ty elements in the yeast *Saccharomyces cerevisiae* (reviewed by Boeke,
1989). The Ty or 'Transposon yeast' elements comprise a family of dispersed and divergent
repetitive DNA sequences. Most members of the Ty family so far described are located in the
nucleus except for one which is located in the mitochondria. On the basis of sequence data the
elements can be divided into two classes, known as Ty1 and Ty917. Each element is 6.3kbp
long with the last 330bp at each end constituting a direct repeat sequence, called delta (δ).
There are approximately 30 copies of the Ty1 type and 6 of the Ty917 type in a typical yeast
genome with another 100 dispersed δ elements, called solo δ's. The Ty DNA is transcribed
into two polyA$^+$ RNA species, both initiated 95bp from the right hand end of the element. One
terminates after 5kbp, the other after 5.7kbp, which is within 40bp of the left boundary. This
longer RNA therefore starts and stops within the δ sequences and as a result has repeats at its
ends. From the analysis of the nucleotide sequence of the Ty element, two open reading
frames can be found orientated in the same direction. One sequence may code for a DNA
binding protein, whereas the other encodes proteins with regions of significant similarity to the
reverse transcriptases, proteases, integrases and RNase H sequences of retroviruses.

Because Ty and retroviral sequences are so similar, Ty transposition has been proposed
to occur in a retroviral manner through an RNA intermediate, possibly transcribed by a host
encoded RNA polymerase II enzyme. The RNA transcript would then act as a template for
reverse transcription into DNA, primed by a host methionine tRNA molecule. The RNA
template is degraded by the RNase H enzyme and the second strand DNA synthesised again by
reverse transcriptase. It is not known what primes the synthesis of the second strand, however
it is suggested that a purine-rich sequence in the δ region may act as a primer binding site. The

25

Ty element, along with the full length RNA, integrase and reverse transcriptase activity is then packaged into virus-like particles which accumulate within the cell. Unlike retroviruses which then infect other cells, these particles remain within the host cell and the Ty DNA can re-insert into the host genome.

The movement of transposable-like elements in filamentous fungi has not yet been strictly demonstrated. The most likely candidate so far is the Tad element found in a wild strain of *Neurospora crassa* (Kinsey and Helber, 1989; Kinsey, 1990). The element was discovered as a 7kbp insertion in two independently isolated spontaneous mutants of the glutamate dehydrogenase gene (*am*). Mutation occurred when crossing the wild strain containing the Tad element with laboratory strains that did not contain the element. Insertion of Tad at the *am* gene in the two mutants was accompanied by duplication of 14 and 17bp 'target' sequences. Although no terminal repeats have been found, and the precise excision of the Tad element has not yet been demonstrated, the structure and nature of the insertion is indicative of other mobile elements, such as the I element of *Drosophila melanogaster* and the Long Interspersed Repeated Sequences (LINES) of mammals (Fawcett *et al*, 1986). Both the I element and LINES are thought to be retrotransposons because they contain open reading frames similar to those of retroviruses.

Other examples of fungal sequences that resemble true autonomous mobile elements have only been determined by sequence similarity. None of these sequences have been shown to be mobile. One significant report is that of the MGR, or *Magnaporthe grisea* repeat sequence, described by Hamer *et al* (1989). *M. grisea* is a fungal plant pathogen responsible for the disease known as rice blast. Hamer *et al* identified a family of repetitive DNA sequences within the *M. grisea* genome which they termed MGR sequences. Analysis of five MGR clones demonstrated that these sequences were related but variable. Also two methods were used to show that they were dispersed amongst the *M. grisea* chromosomes. Firstly, MGR sequences segregated independently when isolates of *M. grisea* were crossed. Secondly, an MGR probe hybridized to all the chromosome-sized molecules extracted from *M. grisea* which were separated by pulsed field gel electrophoresis. MGR probes hybridized to a large poly A+ RNA (7.3kbp) as well as smaller poly A+ RNAs (2.5 and 3.0kbp). Sequence data so far collected has revealed nucleotide sequence homology to reverse transcriptase genes and to LINES, described in other eukaryotic genomes (Mark Orbach, personal communication). Hamer *et al* (1989) suggest that MGR sequences bear some resemblance to families of transposable elements found in other eukaryotic organisms like the Ty element of *S. cerevisiae*. However, mobility of individual MGR sequences has not yet been demonstrated. Many isolates of *M. grisea* that are not pathogens of rice do not contain MGR sequences, however there are some strains which contain MGR sequences and are not pathogens of rice. Hamer *et al* suggest MGR sequences are not sufficient to confer pathogenicity toward rice, but simply represent a product of the evolutionary separation between strains that can and cannot infect rice.

The MGR sequences are another example of the complexity and repetitive nature of the genomes of phytopathogenic fungi. Whether MGR sequences are mobile remains to be proven, however it is tempting to speculate on the likely involvement of such sequences in generating mutations throughout the genome. It has been suggested that most spontaneous mutations observed in *Drosophila melanogaster* are the result of the insertion of mobile genetic elements (Harada *et al*, 1990; Green, 1980; Spradling and Rubin, 1981).

1.5: Aims of the Thesis

This review has summarised the extensive research efforts that have been invested in attempts to understand the genetic basis of rust pathogenicity. Years of arduous research have revealed the contribution of both sexual and somatic recombination to the generation of new gene combinations. However, this review has emphasized that in the past, genetic studies of rust fungi have been limited by the the lack of genetic characters and the difficulties associated with working with a biotrophic plant pathogen.

Therefore, the primary aim of this thesis was to study the genetic organization of a rust fungus using techniques unrelated to conventional virulence markers. This was done firstly, by measuring the size, complexity and base composition of genomic DNA from *Puccinia sorghi* and relating this to data from other fungi. Secondly, the application of techniques in molecular biology were used to develop a set of probes identifying restriction fragment length polymorphisms (RFLPs) in genomic DNA. Such probes were used in inheritance studies to measure the degree of genetic heterogeneity in genomic DNA. The further applications of RFLPs to rust research were discussed. These included the characterization of repeated DNA sequences, the phylogeny of rust fungi, the epidemiology of rust pathogens, and the relative importance of sexual and somatic genetic systems in generating variation in natural populations.

Chapter 2: Size and complexity of genomic DNA from the maize rust fungus, *Puccinia sorghi*

2.1 : Abstract

The base composition, size and complexity of genomic DNA from *Puccinia sorghi* has been determined by thermal denaturation, analytical ultracentrifugation and reassociation kinetics. The buoyant density of DNA in CsCl was found to be 1.7021gm/ml, which corresponds to a G+C content of 43%. From thermal denaturation curves the G+C content was estimated to be 48%. The haploid genome size of *P. sorghi* was found to be 4.5×10^7 basepairs of which 21.8% represented a moderately repetitive fraction. The size of the *P. sorghi* genome is similar to that of other basidiomycete fungi, however the amount of repetitive DNA is greater than that which has been reported for most other fungi.

2.2 : Introduction

Puccinia sorghi Schw. is the causal agent of a rust disease of *Zea mays* L. It is a basidiomycete fungus belonging to the order uredinales, some members of which are extremely destructive plant pathogenic fungi of agricultural crop plants. Previous genetic studies of rust fungi have concentrated primarily on pathogenicity and resistance characters involved in the rust-host interactions (Day, 1974; Sidhu, 1988). Flangas and Dickson (1961) and Hooker and Russell (1962) studied the genetics of the interaction between *P. sorghi* and *Z. mays* and reported the Mendelian inheritance of virulence genes. However, little is known about the size and complexity of genomic DNA of *P. sorghi*, or of other rust fungi for that matter. Such information is of basic importance for further molecular genetic studies of rust pathogens. The reason for this limited information can be attributed, in part, to the difficulty of working with organisms that are obligate parasites. Some success has been acheived in maintaining rusts in axenic culture, however growth is generally very slow (reviewed by Maclean, 1982). Axenic culture of *P. sorghi* has not been reported.

The average size of a fungal genome is 2.5×10^7bp which is about 6 times the size of the *Escherichia coli* genome and 1% of that of human beings. In general, fungi have relatively little repetitive DNA (Van Etten *et al*, 1981). Most of these measurements have been made from saprophytic fungi and there is little data from phytopathogenic fungal species. An exception is the work of Francis *et al* (1990) who reported an unusually high proportion of repetitive DNA in the obligate pathogen *Bremia lactucae*, the causal agent of the downy mildew of lettuce.

Following the development of methods for extracting nucleic acids from *P. sorghi* (Pryor and Boelen, 1987) characterization of this rust genome has become possible. Reported here is the measurement of the base composition of *P. sorghi* genomic DNA by thermal denaturation and analytical ultracentrifugation, and the measurement of genome size and complexity by reassociation kinetics.

2.3 : Materials and Methods

<u>Urediospore culture</u>. *Puccinia sorghi* was maintained asexually on glasshouse grown seedlings of the universally susceptible maize line, P133. Seedlings were grown in shallow trays (500 seeds/m^2) in a glasshouse for 10-12 days. Plants were inoculated by spraying with a 0.1mg/ml solution of spores containing a 0.05% dilution of polyoxyethylene sorbitan mono-oleate (Sigma) to facilitate spore suspension. The plants were kept at 100% humidity in a chamber overnight and then returned to the glasshouse. Urediospores were collected 10-15 days after inoculation using a vacuum cleaner fitted with a spore trap.

<u>Extraction of DNA</u>. Urediospores were germinated as described by Pryor and Boelen (1987). Germinated spores were harvested, drained of excess water using a Buchner funnel, snap frozen in liquid N$_2$ and stored indefinitely at -80°C. DNA was extracted from germinated urediospores as described by Pryor and Boelen (1987) with some modification. Material was ground to a powder in liquid N$_2$ mixed with DNA extraction buffer (2% sodium dodecyl sulphate, 250mM NaCl, 200mM Tris-HCl pH 8.0, 25mM EDTA and 1% β-2,mercaptoethanol; 20mls per gram of ungerminated urediospores). An equal volume of phenol-chloroform (1:1) was added, and after thorough mixing the two phases were separated by centrifugating at 5000rpm for 10 minutes. The aqueous phase was collected and the total nucleic acids precipitated with an equal volume of isopropanol. The precipitate was collected by centrifugation, resuspended in 10mM Tris-HCl pH 8.0, 1mM EDTA (100µl per gram of ungerminated spores), and treated with ribonuclease A (Sigma) at 37°C for 30 minutes (1µg/ul final concentration). After RNase treatment, self-digested proteinase K (Boehringer) was added to give a final concentration of 1µg/µl, and incubated at 37°C for 1 hour. An equal volume of phenol-chloroform (1:1) was mixed, the phases separated by centrifugation and the DNA precipitated with ethanol. The DNA was centrifuged to equilibrium in 1.7 gm/ml CsCl and 0.25mg/ml ethidium bromide in a Ti50 rotor (Beckmann) at 40,000rpm for 40 hours and purified according to Sambrook *et al* (1989).

DNA from *E. coli* (strain *JM101*) and *Microccocus lysodeikticus* was extracted according to Marmur (1961). *Neurospora crassa* DNA was a gift from Dr. Brett Tyler (Australian National University, Canberra).

<u>Thermal denaturation</u>. DNA from each species (at 1mg/ml) was sheared in a cup sonicator (Branson model L) to an average length of 300bp. This required three 15 second bursts at 70-80 watts. The average length of the sonicated DNA was determined by agarose gel electrophoresis. The sheared DNA was dissolved in a solution of 0.2 x SSC and 1mM EDTA to give a final concentration of 0.1mg/ml. Aliquots of 10µl were removed to 0.5ml Eppendorf tubes and overlayed with a drop of paraffin oil to prevent evaporation during the experiment. The tubes were placed in a water bath and gradually heated. At 2°C intervals duplicate tubes were removed and immediately mixed with 170µl of S1 nuclease buffer (200mM NaCl, 50mM NaAcetate pH 4.5, 1mM ZnSO$_4$, 0.5% glycerol) to which 1µl of 50 units/µl S1 nuclease

(Sigma) was added. The tubes were incubated at 37°C for 15 minutes to allow digestion of single stranded DNA and the reaction stopped by adding 20µl of 1M Tris-HCl pH 9.0, 100mM EDTA. The tubes could then be stored at -20°C.

Reassociation Kinetics. The methods for estimating genome size and complexity by reassociation kinetics and S1 nuclease treatment is outlined by Britten *et al* (1974). DNA of each species was sheared to approximately 300bp, and 10µl aliquots overlayed with paraffin oil. The DNA was adjusted to a concentration of 0.1mg/ml using the same buffer as in the thermal denaturation experiment, and several aliquots were adjusted to 0.5mg/ml DNA. The higher concentrations were necessary in order to measure the higher C_0t values. Tubes were heated to 100°C for 2 minutes in a heating block, then placed immediately into a water bath at 66°C and removed at time intervals corresponding to each C_0t point. C_0t values were calculated for each sample by multiplying the DNA concentration (mol. nucleotides/litre) by time (seconds). Following treatment with S1 nuclease to remove non-reassociated DNA, the tubes were stored at -20°C.

Measurements of double-stranded DNA concentration. Fluorimetry was used to measure the amount of DNA melted by thermal denaturation or the rate of DNA reassociation. Fluorimetry measures both single and double-stranded DNA, however the products of S1 nuclease digestion of single-stranded DNA are too small to contribute to the reading. DNA samples taken from -20°C were thoroughly dissolved and a 20µl sub-sample was added to 1ml of Hoechst dye for fluorometric measurements. The proportion of double-stranded DNA at each temperature or C_0t point could be calculated relative to the fluorescence of a control that had not been denatured before being treated with S1 nuclease. In the reassociation experiment the stability of denatured single-stranded DNA was estimated after approximately 7 days at 66°C (C_0t 100). This was done by denaturing the sample after 7 days and measuring the fluorescence of single-stranded DNA. This value was compared to the fluorescence of a sample immediately after denaturation (C_0t 0).

Buoyant density. *P. sorghi* genomic DNA was centrifuged to equilibrium (44,000rpm, 25°C, 24 hrs) in a 1.7gm/ml CsCl gradient in a Spinco Model E analytical ultracentrifuge equipped with absorption optics and a photoelectric scanner (Beckman). The buoyant density of *P. sorghi* DNA was calculated relative to that of *M. lysodeikticus* DNA (ρ=1.72409 gm/ml) used as an internal reference marker (Sober, 1968). Base composition was calculated from the buoyant density by the method of Szybalski (1968).

2.4 : Results

The isolation of DNA from *P. sorghi* described here results in the recovery of 25-30µg of DNA for each gram of ungerminated urediospores. This yield is similar to that obtained from another basidiomycete fungus (Arthur *et al*, 1982).

The thermal denaturation curves for *E. coli*, *N. crassa* and *P. sorghi* are shown in Figure 2.1. The Tm values, or the temperatures at which half the DNA is denatured, are 81.5°C, 79°C and 78°C respectively. Using the relationship between Tm and base composition of Marmur and Doty (1962), the base composition of *E. coli*, *N. crassa* and *P. sorghi* was determined to be 57%, 51% and 48% G+C respectively. The *E. coli* and *N. crassa* measurements agreed with previously published estimates (Storck and Alexopoulos, 1970). The shoulder on the *N. crassa* curve representing 30% of the total DNA and with a base composition of 30% G+C is probably mitochondrial DNA (Luck and Reich, 1964).

The CsCl equilibrium gradient profile of *P. sorghi* and *M. lysodeikticus* genomic DNA is shown in Figure 2.2. The buoyant density of *P. sorghi* DNA was calculated to be 1.7021gm/ml by comparison to the known buoyant density of *M. lysodeikticus* DNA. This density is equivalent to a base composition of 43% G+C.

The reassociation kinetics of genomic DNA from *E. coli*, *N. crassa* and *P. sorghi* are shown in Figure 2.3A. The estimates of the genome sizes of *E. coli* and *N. crassa* are 4.2×10^6bp and 3.2×10^7bp respectively, and agree with previously published estimates (Krumlauf and Marzluf, 1979). The size of the *P. sorghi* genome was estimated to be 4.5×10^7bp. Using a log/log transformation of the reassociation data (shown in Figure 2.3B), it was possible to resolve two components within the genome of *P. sorghi*. The kinetics of a moderately repetitive component and a unique component are shown in Table 2.1. The repetitive fraction, comprising 21.8% of the genome, was composed of around 220 copies of an average repeat length of 56kbp. The unique fraction represented 59.7% of the genome and had a complexity of 3.3×10^7bp. The remaining 18% of the DNA did not hybridize. It was determined that approximately 15% of single-stranded DNA degraded during the 7 day incubation period to reach C_ot 100 (see methods). This could explain the proportion of unhybridized DNA.

Table 2.1. Genome size and complexity of genomic DNA from *P. sorghi*.

Component	Percentage of genome	$C_ot_{1/2}$	Average number of copies	Size (bp)
Moderate repeat	21.8	0.105	220	5.6×10^4
Single copy	59.7	22.7	1	3.3×10^7
did not hybridize	18.5			

Total genome size 4.5×10^7bp

Figure 2.1. The thermal denaturation profiles of *E. coli*, *N. crassa* and *P. sorghi* genomic DNA. The respective Tm values of 81.5, 79 and 78°C are indicated.

Figure 2.2. A 260nm absorbance scan of an ultracentrifuge cell containing *P. sorghi* and *Micrococcus lysodeikticus* DNA centifuged to equilibrium at 44,000rpm at 25°C for 24 hours. **T**: top of the gradient. **B**: bottom of the gradient. Peak (i) is *P. sorghi* DNA (ρ=1.7012gm/ml or 43% G+C). Peak (ii) is *M. lysodeikticus* DNA (ρ=1.7241 gm/ml or 71% G+C) which was used as a reference. The other peak is the air/liquid interface at the top of the ultracentrifuge cell.

1

% single stranded DNA

■ — E. coli
○ — N. crassa
□ — P. sorghi

Temperature °C

2

T   i   ii   B
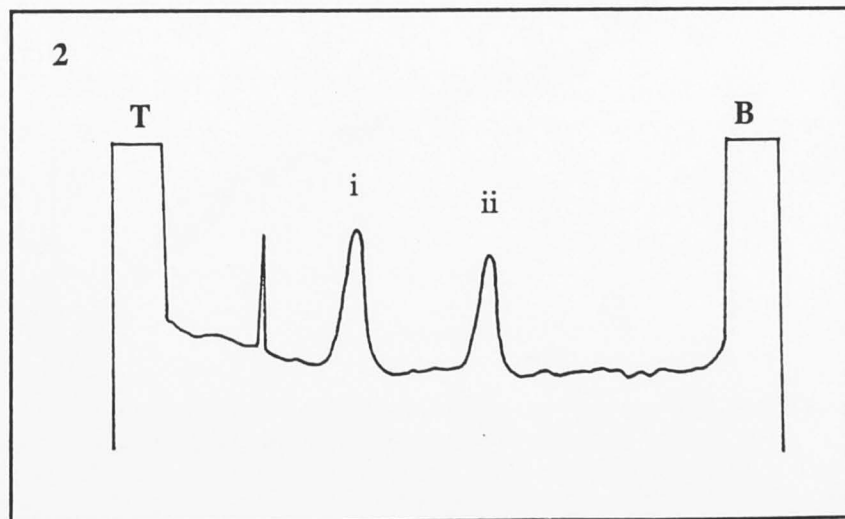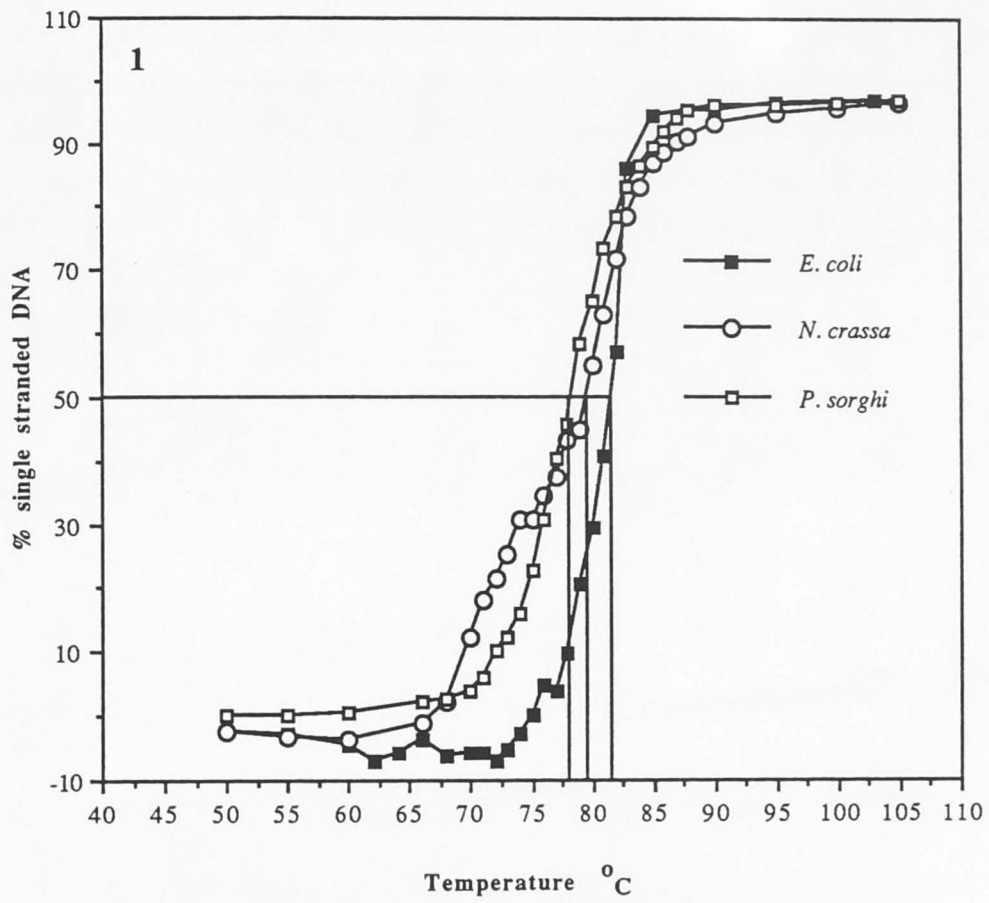
Figure **2.3A**. The renaturation profiles of *E. coli*, *N. crassa* and *P. sorghi* genomic DNA. Each point is the average of a duplicate measurement. The $C_0t_{1/2}$ of the *E. coli* and *N. crassa* genomes was measured to be 5.5 and 42 respectively. Assuming the genome size of *E. coli* is 4.2×10^6 bp, then an estimate of the size of the *N. crassa* genome is 3.2×10^7 bp. This is in agreement with a previously published measurement of 2.7×10^7 bp (Krumlauf and Marzluf, 1979). **2.3B** The relationship between the log C_0t and the log of the fraction of reannealed genomic DNA of *P. sorghi* ($\log C_0/C_{ss}-1$). The curve can be divided into two components representing a moderately repeated fraction ($C_0t_{1/2}=0.105$) and a unique fraction ($C_0t_{1/2}=22.7$).

2.5 : Discussion.

Reported here is the isolation and characterisation of DNA from germinated uredospores of the maize rust fungus *P. sorghi*. Reassociation measurements at equilibrium based on thermal denaturation and Cot behaviour demonstrate... uredospore derived DNA has a G+C content of between 45-48%. Shapiro and ... predict that assuming the G+C content of the non-DNA fungal fraction, and that total DNA from uredospores been consistent between... the G+C content would be expected to give rise to the character of genomic DNA from *P. sorghi* ... the magnitude of this change... there is no change to the relationship between G+C content and the melting of an organism's genomic substances.

Calculation of the haploid genome size from reassociation kinetics at renaturation kinetics gave an estimate of 4.5x10⁷bp. This value is comparable to those reported for the basidiomycetes *Coprinus* (Dutta et al. 1974) and *Ustilago* (Garber et al., 1975, Ullrich et al., 1980). Therefore, at least in the case of *P. sorghi* which is a pathogen there is a highly specialised relationship with its host plant, there are no obvious genome changes related to the size of the genome in comparison to say free living saprophytic relatives.

A moderately repeated fraction of an average kinetic complexity contributes 22% to the total genome of *P. sorghi*. In general, repetitive DNA in the genome comprises between 2-10% of the genome - *Coprinus* 10-20% (Dutta et al., 1974), *Schizophyllum* (Dutta et al., 1974, Ullrich et al., 1980), *Pyrcularia* 10% (Dickinson and Hanley, 1985), *Aspergillus* 2% (Timberlake, 1978) and *N. crassa* 10% (Krumlauf and Marzluf, 1979). Therefore, it would appear that *P. sorghi* contains more repetitive DNA than other fungi. This also appears to be true for other plant pathogenic fungi like *Fusarium graminearum*, which has 31-33% of the genome reported (Sracol, 1983). Pennis et al (1990) reported that genomic DNA of the pathogen *Bremia lactucae* (downy mildew of lettuce) contained a number of components of repetitive DNA, that together comprised 65% of the genome. Thus, it would appear that this represents a significant difference between ... duration of the characterised fungal genomes in the analysis. Whether this is a general feature of plant pathogenic fungi reflecting some aspect of the nature of symbiosis, or other nature of the pathogenic life style, is unknown.

Except the most repetitive sequence components, the DNA reassociation curve of *P. sorghi* shows ... DNA to reassociate faster than those for *E. coli* and *N. crassa*. This slow reassociation, has been observed where DNA from different fungal species within a genus is compared at stringent (Dd et al et al, 1976). Delay argues that the slow kinetics of cross species DNA reassociation is a consequence of the sequence variation between genomic DNA of the two species, involving nucleotide base pair mismatches and/or heterozygosity at various loci (Laird et al., 1969, Bonner and Britten, 1973, Bonner and Watson, 1965; Sutton and Dyer, 1969... chapter et al., 1971). As expected, in a PhD study of genomic DNA of *P. sorghi* using uredinal DNA sequences of those revealed a high degree of sequence heterogeneity. This sequence heterogeneity has been attributed to the allelic differences at the cDNA level (see chapters 3 and 4). At last being any substantive differences between alleles implies that sequence differences are between two copies of the

3 A

3 B

31

2.5 : Discussion.

Reported here is the isolation and characterization of DNA from germinated urediospores of the maize rust fungus *P. sorghi*. Independent measurements of G+C content based on thermal denaturation and CsCl buoyant density techniques, indicate that *P. sorghi* DNA has a G+C content of between 43-48%. Storck and Alexopoulos (1970) measured the G+C content of DNA from 322 fungal species, and found that DNA from basidiomycete fungi contained between 49-61% G+C. This value is somewhat higher than the G+C content of genomic DNA from *P. sorghi*, but the significance of this difference is difficult to gauge as the relationship between G+C content and the biology of an organism is not well understood.

Calculation of the haploid genome size of *P. sorghi* measured by reassociation kinetics gave an estimate of 4.5×10^7bp. This value is comparable to those reported for the basidiomycetes, *Coprinus* (Dutta *et al*, 1972) and *Schizophyllum* (Dons *et al*, 1979, Ullrich *et al*, 1980). Therefore, at least in the case of *P. sorghi* which is a pathogen living in a highly specialized relationship with its host plant, there have been no apparent gross changes in the size of the genome in comparison to any free living saprophytic relatives.

A moderately repeated fraction of an average length of 56kbp contributes 22% to the total genome of *P. sorghi*. In general, repetitive DNA in the Eumycota represents between 2-10% of the genome - *Coprinus* 10-12% (Dutta *et al*, 1972), *Schizophyllum* 7% (Dons *et al*, 1979, Ullrich *et al*, 1980), *Penicillium* 10% (Sahasrabudhe and Ranjekar, 1985), *Aspergillus* 2% (Timberlake, 1978) and *N. crassa* 10% (Krumlauf and Marzluf, 1979). Therefore, it would appear that *P. sorghi* contains more repetitive DNA than other fungi. This also appears to be true for other plant pathogenic fungi like *Fusarium graminearum* which has 21-23% of the genome repeated (Szecsi, 1981). Francis *et al* (1990) reported that genomic DNA of the pathogen *Bremia lactucae* (downy mildew of lettuce) contained a number of components of repetitive DNA that together constituted 65% of the genome. Francis *et al* point out that this represents a significant difference between *B. lactucae* and most other fungal genomes so far analysed. Whether this is a general feature of phytopathogenic fungi reflecting some aspect of the mode of nutrition, or other aspects of the pathogenic life style, is unknown.

Despite the consistency of duplicate measurements, the DNA reassociation curve of *P. sorghi* genomic DNA is significantly flatter than those for *E. coli* and *N. crassa*. This same phenomenon has been observed when DNA from different but closely related bacterial species is denatured and reassociated (DeLey *et al*, 1970). DeLey argues that the altered kinetics of cross species DNA reassociation is a consequence of the sequence variation between genomic DNA of the two species. Genetic studies of rust fungi have reported a high level of heterozygosity at avirulence loci (Johnson, 1954; Wilcoxson and Paharia, 1958; Luig and Watson, 1961; Samborski and Dyck, 1968,1976; Haggag *et al*, 1973). In addition, an RFLP study of genomic DNA of *P. sorghi* using random cDNA sequences as probes, revealed a high degree of sequence heterogeneity. This sequence heterogeneity has been attributed in part to allelic differences at the cDNA loci (see chapters 3 and 5). As rust fungi are dikaryotic, differences between alleles implies that sequence differences are between the two nuclei of the

dikaryon. If there are many sequence differences between the two nuclei, then presumably a reassociation curve of total genomic DNA may resemble that of a comparison of two species, like that of DeLey *et al* (1970).

In conclusion, the size and base composition of rust genomic DNA appears not to differ considerably from that of other basidiomycete fungi. The larger amount of repetitive DNA observed in the *P. sorghi* genome is similar to that found in other plant pathogenic fungi, however the significance of this to the biology of these organisms is unknown.

Chapter 3 : DNA Restriction Fragment Length Polymorphisms and genetic heterogeneity in the maize rust, *Puccinia sorghi.*

3.1 : Abstract

A cDNA library was synthesized from poly A$^+$ RNA extracted from germinated urediospores of *Puccinia sorghi*. The library was used to develop a set of probes for detecting restriction fragment length polymorphisms (RFLPs). Twenty four probes, chosen at random from the cDNA library, detected sixteen RFLPs between an Australian and a North American isolate of *P. sorghi*. Hybridization of these cDNA sequences to blots containing genomic DNA of *Puccinia recondita* (wheat leaf rust) and *Melampsora lini* (flax rust) revealed a low level of interspecific hybridization and no intergeneric hybridization. Thus cDNA probes from *P. sorghi* seem only to be useful for detecting intraspecific variation. When a range of restriction enzyme digests of *P. sorghi* were probed with the various cDNAs, multiple hybridizing fragments were detected in most tracks. This complexity in the hybridization pattern was shown not to result from internal restriction sites in the cDNA, and was unlikely to result from cloning artifacts or restriction sites within intervening sequences in the genes that corresponded to the cDNAs. The basis of the multiple banding pattern is unknown, however the most likely explanation is that some form of genetic heterogeneity such as heterozygosity and/or gene duplication exists within the genome of *P. sorghi*.

3.2 : Introduction

Despite the economic and agricultural significance of rust fungi, little is known about the genetic system of these organisms. Previous studies have concentrated primarily on the genetics of the rust/host interaction which has been well characterized in the flax rust (*Melampsora lini*) and its host plant *Linum usitatissimum* (Flor, 1971; Lawrence, 1988). The pathogenic behaviour of the fungus is controlled by dominant avirulence genes (*Avr*). To date, *Avr* genes have been the most commonly used markers in genetic studies of rust fungi, demonstrating Mendelian inheritance and sexual recombination, as well as the likely occurrence of somatic events such as heterokaryosis, nuclear exchange and parasexual recombination (reviewed by Day, 1974). However, changes from avirulence to virulence, presumably by mutation of *Avr* genes, occur frequently in rust fungi (Luig, 1979; Flor, 1960; Schwinghamer, 1959; Schafer and Roelfs, 1985). It is not known whether mechanisms exist for specifically mutating the *Avr* loci, or if mutations occur uniformly throughout the genome. A further limitation of using *Avr* genes as genetic markers is the time-consuming and arduous task of screening many isolates for their virulence genotype. For these reasons, Burdon *et al* (1983, 1986) used phenotypically neutral isozyme markers to study genetic variability in the rust fungi. However, these studies are limited because there are few enzyme systems available for analysis, and little variability is found. It is clear that to study variability, population structure and the genetic systems of rust fungi, a larger number of selectively neutral, easily scored

genetic markers are required. Restriction fragment length polymorphisms (RFLPs) fit these requirements.

RFLPs, although used successfully to construct linkage maps of the human genome and several plant genomes (Botstein *et al*, 1980; Helentjaris *et al* 1986; Tanksley *et al*, 1988; Chang *et al*, 1988 and Landry *et al*, 1987), have not been reported widely in fungi. There has been no report of the application of RFLP analysis to rust fungi, and there are only a few examples of its application to phytopathogenic fungi (Michelmore and Hulbert, 1987). These include *Bremia lactucae* (lettuce downy mildew; Hulbert and Michelmore, 1988), *Septoria tritici* (wheat leaf spot fungus; McDonald and Martinez, 1990), *Erysiphe graminis* (wheat powdery mildew; O'Dell *et al*, 1989, Christiansen and Giese, 1990) and *Colletotrichum gloeosporioides* (the cause of anthracnose on *Stylosanthes*; Braithwaite *et al*, 1990). These studies emphasized the potential uses of RFLPs in the genetic study of plant pathogenic fungi. In each case a high level of within-species variation was detected, and there were several reports of probes containing dispersed repetitive sequences possibly capable of 'finger printing' many different races. In addition, RFLP analysis has uncovered a likely somatic hybridization event between two field isolates of *Bremia lactucae*. This provides experimental evidence of a mechanism that is capable of generating genetic variability in fungi, but which is rarely observed in nature (Hulbert and Michelmore, 1988).

Puccinia sorghi is a rust fungal pathogen of *Zea mays*. Harvesting and germinating urediospores in amounts necessary for nucleic acid extraction has made the molecular genetic study of this organism difficult. However, methods for rust culture and extraction of nucleic acids from *P. sorghi* reported by Pryor and Boelen (1987) have enabled molecular techniques to be applied to rust fungi. Two races of *P. sorghi*, maintained in our laboratory, were isolated from different geographic locations and have a very different spectrum of virulence genes (Table 3.1). Therefore, it was anticipated that RFLPs would be detected between the two races.

The aim of this study was to develop a set of probes from a cDNA library of *P. sorghi*, to assess the feasibility of using RFLPs to measure genetic variability, and to examine genetic systems in rust fungi. DNA from another *Puccinia* species and from another rust genus was included to provide a measure of variability and sequence similarity in the order uredinales, and also to see if probes from *P. sorghi* could be used in RFLP analysis of other rust fungi.

3.3 : Materials and Methods
Rust races

The origins of the rusts used in the study are listed in Table 3.1. Two races of *P. sorghi*, race 1 and race 2, are differentiated according to their virulence towards alleles of the *Rp1* locus of maize which confers resistance to infection by *P. sorghi* (Hooker and Russell, 1962). The virulence specificity of the two races is shown in Table 3.1. The CH5 race of *Melampsora lini* (flax rust) was an F_1 hybrid between two races, 228 and P1C (Lawrence *et al*, 1981b). An isolate of the wheat leaf rust fungus, *P. recondita* (76-0), collected from the field,

was typed by the Plant Breeding Institute at the University of Sydney and amplified on the susceptible wheat variety, 'Isis'.

Pathogen	Origin	Reaction of *P. sorghi* races to alleles of the *Rp1* locus of *Z. mays*							
		b	*j*	*c*	*m*	*d*	*f*	*td*	*a*
P. sorghi race 1	Australia	+	+	+	+	-	-	-	-
P. sorghi race 2	USA	-	-	-	-	+	+	+	+
M. lini race CH5	Lawrence *et al* (1981b)								
P. recondita race 76-0	PBI Sydney Uni.								

Table 3.1. The origin and virulence specificities of the rust races used in this RFLP study. The isogenic set of differential maize plants used to type the two races of *P. sorghi* was developed by Hooker and Russell, (1962). (+) susceptible, (-) resistant ; PBI stands for Plant BreedingInstitute.

Extraction of DNA

Urediospores were germinated and total DNA extracted as described in chapter 2, except that the final CsCl density gradient purification step was omitted. The concentration of DNA was measured by agarose gel electrophoresis and ethidium bromide staining using λ bacteriophage DNA as a standard (Sambrook *et al*, 1989).

Extraction of poly A⁺ RNA

Germinated spores were ground to a powder in liquid N_2 and added to RNA extraction buffer (20mls of 100mM Na Acetate pH 4.6, 10mM EDTA, 4% sodium dodecyl sulphate (SDS) for every gram of ungerminated urediospores). An equal volume of phenol/chloroform (1:1) was added and mixed thoroughly and the aqueous phase separated by centrifugation at 5000rpm for 5 minutes. Total nucleic acids were precipitated from the aqueous phase with 1/10th volume of 3M sodium acetate (pH 4.6) and two volumes of ethanol. The pellet was collected by centrifugation at 10,000rpm for 10 minutes, dried and resuspended in 8mls of buffer A (10mM Tris-HCl pH 7.4, 10mM EDTA, 0.5% SDS). Self-digested proteinase K (Sigma) was added to a final concentration of 100µg/ml and incubated at 37°C for 5 minutes. NaCl (4M) was added immediately to a final concentration of 0.5M, and the tube rapidly cooled to room temperature. Oligo-dT (type 7, Pharmacia) equilibrated in buffer B (10mM Tris-HCl pH 7.4, 10mM EDTA, 0.5% SDS, 0.5M NaCl) was added (0.2gms per gram of ungerminated spores) and mixed for 30 minutes on a rotating wheel at room temperature. The total mixture was centrifuged gently at 1000rpm for 1 minute to pellet the oligo-dT. The supernatant was removed and the pellet resuspended in 10mls of buffer B and poured into a small column fitted with a glass wool plug. After the buffer had passed through, 10mls of buffer A containing 0.25M NaCl were washed through the column. The poly A⁺ RNA was eluted by adding 2mls of 10mM Tris-HCl pH 7.4, 1mM EDTA. The RNA was precipitated with 1/10th volume 3M Na Acetate pH 4.6 and two volumes of ethanol and collected by centrifugation at 80,000g for 30 minutes. The pellet was dried and resuspended in 200µl of H_2O and the concentration determined by measuring the absorbance at 260nm. The quality of the RNA was determined

by measuring the absorbance at 260nm. The quality of the RNA was determined by electrophoresis in a formaldehyde gel and by invitro translation in a rabbit reticulocyte lysate system (Amersham) and then analysing the products in a SDS-polyacrylamide gel (Sambrook *et al*, 1989) (refer to appendix A).

cDNA synthesis

cDNA was synthesized from 5µg of mRNA template using a kit supplied by Amersham and following the method described in the manual provided. The cDNA was ligated to *Eco RI* linkers (Boehringer), cut to completion with *Eco RI*, and size fractionated using a sepharose 4B column (Pharmacia). cDNA molecules smaller than 300bp were excluded. The cDNA was ligated into the pUC118 bacterial plasmid vector (Vieira and Messing, 1987) and cloned into the *E. coli* strain *JM101*. Recombinant colonies were detected using a β-galactosidase reporter system and individual clones were stored as bacterial cells in 7% DMSO at -80°C.

Restriction endonuclease digestion, blotting and hydridization

The restriction endonucleases *Eco RI*, *Bam HI*, *Hind III*, *Bgl II*, *Pst I*, *Sac I*, and *Sal I* were used to cut genomic DNA from the various rust races. Restriction enzyme reactions were carried out according to the specifications of the manufacturer (Pharmacia) with the addition of spermidine (Sigma) to a final concentration of 5mM. Digested DNA was separated by electrophoresis through a 1% agarose gel and blotted onto filters (Hybond-N). Radioactively labelled probes were hybridized to the filters in 50% formamide at 42°C and after 16 hours the filters were washed twice for 15 minutes in 2xSSC (0.3M NaCl, 0.03M Na Citrate) and 0.1% SDS, at 42°C. The filters were air dried and exposed to X-ray film (Fuji) at -80°C for 1-4 days. Before using another probe, the filters were stripped of labelled DNA by washing 3-4 times in distilled water at 95°C. Methods for electrophoresis, Southern blotting, hybridization, and autoradiography are described by Sambrook *et al* (1989). Electrophoresis of RNA and northern blotting was done as described by Higgins and Spencer (1991), and the filters were hybridized with probes using the same method as for Southern blots.

Isolation and labelling of cDNA probes

Plasmid DNA was extracted from bacteria grown in 2mls of LB broth for 8-16 hours (Sambrook *et al*, 1989). Recombinant plasmid DNA was cut with *Eco RI* and the cloned cDNA insert separated from the vector DNA by electrophoresis on a 1% agarose gel. The gel strip containing the cDNA insert was placed in a 1ml syringe and frozen at -20°C. The buffer and DNA was squeezed from the frozen gel strip through an 18 gauge needle. This extract was mixed twice with phenol/chloroform (1:1), centrifuged and the DNA in the aqueous phase was precipitated in ethanol. The DNA precipitate was collected by centrifugation and resuspended in 10µl of 10mM Tris-HCl pH 7.4, 1mM EDTA and 2µl (approximately 0.2µg) labelled with 32P-dCTP using a nick translation kit (Bresatec).

3.4 : Results

Twenty-eight randomly selected cDNA clones were used as probes and hybridized to Southern blots of *P. sorghi* genomic DNA. Out of this sub-sample, one probe was

represented three times and two others were represented twice. The data from the hybridization of 24 different cDNA probes is summarised in Table 3.2, and the hybridization pattern of three of these probes, pPSOc26, pPSOc25 and pPSOc22, is shown in Figure 3.1a,b,c. The results showed a lack of hybridization of the cDNA sequences to genomic DNA of *M. lini*, and some faint hybridization to genomic DNA from *P. recondita*. A number of RFLPs were detected between race 1 and race 2 of *P. sorghi*. One example is the hybridization pattern of pPSOc26 (Figure 1b), in which the race 1 and 2 patterns are identical for all 7 enzymes except for *Pst I*. It was observed that all the probes in almost all the restriction enzyme digests hybridized to two or more bands of *P. sorghi*.

Out of 24 probes, 16 (67%) detected RFLPs between race 1 and race 2 of *P. sorghi*. This was not unexpected given the different virulence specificities and geographic origins of the two races (Table 3.1). Fifteen of the 16 RFLPs could be explained by a modification in a restriction enzyme site causing a change in the length of a genomic restriction fragment hybridizing to the probe. One probe (pPSOc24) hybridized to genomic fragments from race 1 and race 2 that were different in length by 500bp in all the restriction enzymes tested. This difference was subsequently shown to be due to the insertion of a 496bp element into the race 2 genome (refer to chapter 6).

Hybridization of *P. sorghi* cDNA probes to genomic digests of *Melampsora lini* and *Puccinia recondita* DNA indicated the extent of interspecific and intergeneric sequence similarity. None of the 18 clones that were tested hybridized to *M. lini* genomic DNA, and 6 showed very faint hybridization to *P. recondita* genomic DNA (indicated by arrows in Figure 3.1b).

The restriction enzyme *Sal I* does not cut when the CpG doublet of its restriction site is methylated. The genomic DNA of *P. sorghi* digested with *Sal I* remained mostly uncut and migrated to limiting size in the agarose gel. This can be seen by the hybridization pattern of the pPSOc22 probe in Figure 3.1c. This could be because the genomic DNA of *P. sorghi* is methylated, or for some reason the *Sal I* enzyme was inactive. However, in 7 out of 19 clones, discrete *Sal I* hybridization bands were observed suggesting the enzyme was active (note the *Sal I* lanes of Figure 3.1a). Therefore, it would appear that genomic DNA extracted from germinated urediospores of *P. sorghi* is methylated.

As shown in Table 3.2, most cDNA probes hybridized to more than one restriction fragment. Probes pPSOc25 and pPSOc26 hybridized to 2 bands and pPSOc22 hybridized to 3 or 4 bands (Figure 3.1a,b,c). While several probes hybridized to a single fragment with one restriction enzyme, there was no case of a probe hybridizing to a single fragment for all restriction enzymes tested. Because the probes were derived from gene transcripts, it was expected that at least some sequences would hybridize to a single genomic fragment. However, this was not the case. The average length of the cDNA probes was 900bp, the largest being 2100bp, and yet the genomic fragments to which they hybridized were 5-20kbp in length. Possible explanations for the multiple banding patterns could be the result of:
(i) internal restriction sites within the genomic sequence hybridizing to the probe,

Figure 3.1: Autoradiographs of a genomic blot hybridized sequentially to three different *P. sorghi* cDNA clones, pPSOc26 (a), pPSOc25 (b) and pPSOc22 (c). After each hybridization the blots were stripped of the probe and reused. Lanes 1 and 2 contain genomic DNA of *M. lini* and *P. recondita* respectively, digested with *Eco RI*. The remaining 12 lanes, 3 to 16, are a pair-wise comparison of genomic DNA from race 1 and race 2 of *P. sorghi*, digested with 7 different restriction enzymes. In each comparison, race 1 DNA is loaded first. The enzymes used are: lanes 3, 4 *Eco RI*; lanes 5, 6 *Hind III*; lanes 7, 8 *Bam HI*; lanes 9, 10 *Bgl II*; lanes 11, 12 *Pst I*; lanes 13, 14 *Sac I*; lanes 15, 16 *Sal I*.

1a

1b

1c
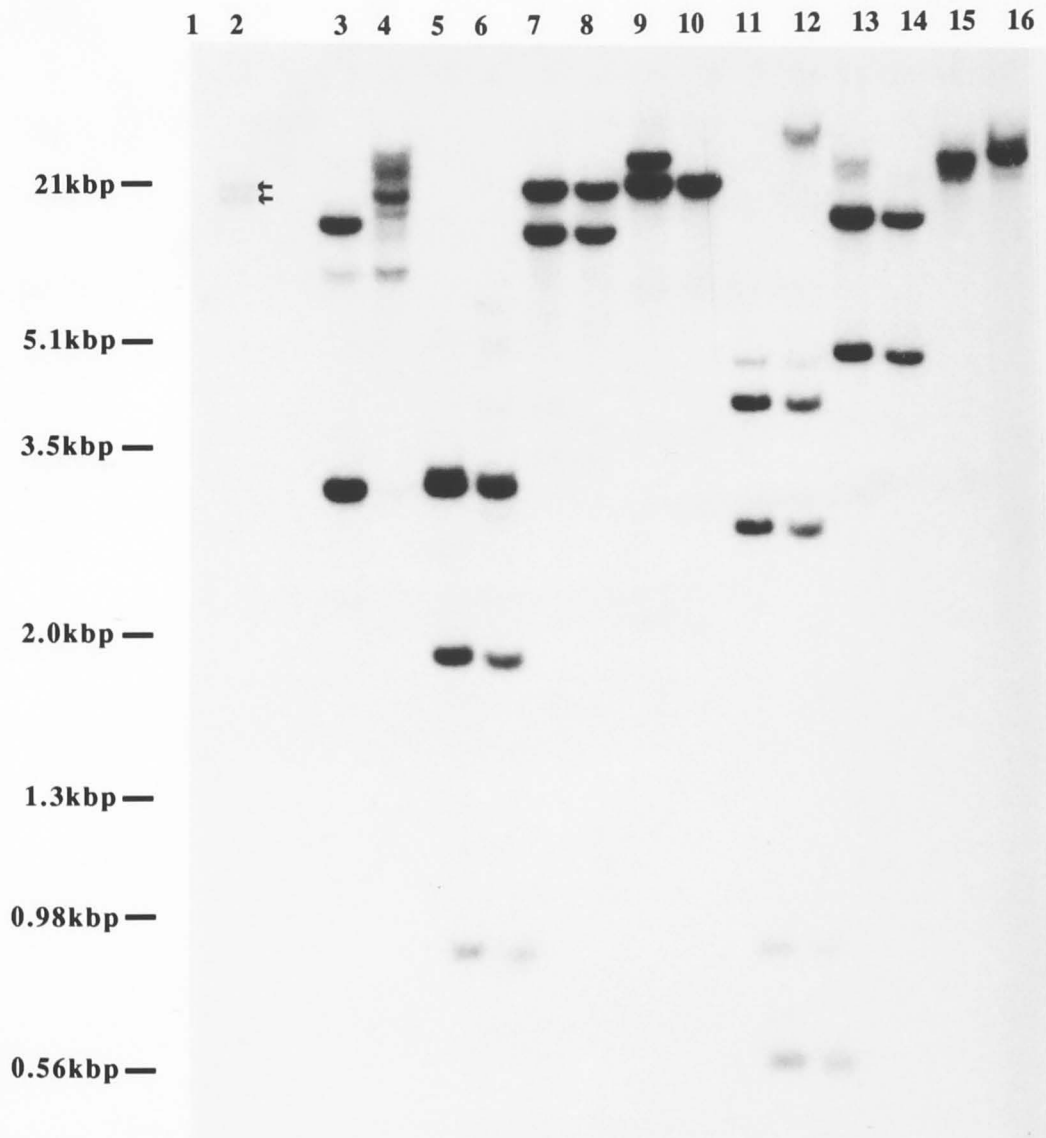
Enzyme		Eco RI	Eco RI		Hind III		Bam HI		Bgl II		Pst I		Sac I		Sal I	
								Restriction enzyme/DNA combination								
DNA		M.l.	P.r.	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
pPSOc	Size															
1	1550	a	a	3	2*	2	2	2	2	2	2	a	a	1	1	2	2
7	1600	a	1	3	3	3	3*	3	3	a	a	a	a	a	a	a	a
9	900	a	a	3	2*	2	2*	2	2	4	3*	a	a	2	2	1	1
10	2000	a	a	3	3	2	4*	1	1	2	2	a	a	2	2	1	1
16	1900	a	a	3	3	2	4*	1	1	2	2	a	a	2	2	1	1
18	750	a	a	3	3*	1	1*	4	4	4	4*	a	a	1	1	b	b
20	1000	c	c	3	3	2	2	2	2	a	a	a	a	a	a	a	a
21	600	c	c	2	2	2	2	2	2	a	a	a	a	a	a	a	a
22	700	c	2	3	d	4	4*	4	4*	4	4	3	3	4	4	b	b
23	600	c	c	3	d	2	2	2	2	1	1	2	2	3	3	b	b
24	1100	c	1	1	d	2	2*	4	4*	2	2*	2	2*	2	2*	b	b
25	600	c	2	2	d	3	3	2	2	2	1*	4	4	2	2	b	b
26	1300	c	c	1	d	2	2	2	2	2	2	2	2*	2	2	2	2
29	400	c	2	2	d	2	2	2	2	2	2*	2	2	4	4*	b	b
30	1300	c	c	2	d	3	4*	2	3*	2	3*	3	4*	2	2	2	1*
35	400	c	1	2	d	2	2	2	2	2	2	2	2	2	2	b	b
38A	2000	c	c	2	d	3	3	4	4	2	2	4	4	1	1	2	2
38B	500	c	c	3	3	1	1	3	3	a	a	a	a	a	a	a	a
45	700	c	c	3	3	4	4*	3	3	a	a	a	a	a	a	a	a
47	900	c	c	2	d	2	2*	2	2*	2	2*	2	2*	2	3*	b	b
48	1400	c	c	2	d	2	2*	2	2	1	1	2	2	2	2	b	b
49	500	c	c	2	d	2	2	2	2	2	2	4	2*	2	2	1	1
50	600	c	c	1	d	2	2*	2	2	2	2*	2	2*	2	2*	b	b
55	1200	c	c	2	d	2	2	1	1	2	2	2	2	2	2	b	b

Table 3.2. Results of the RFLP analysis of race 1 and race 2 of *P. sorghi*. Numbers refer to the number of genomic bands hybridizing to a particular probe. The underlined data is shown in Figure 3.1 a,b,c.

Size: size estimate of the cDNA insert in basepairs (bp).
a: enzyme not tested.
b: only undigested DNA hybridizing to the probe.
c: no hybridization detected.
d: partial genomic digest.
M.l.: *Melampsora lini*.
P.r.: *Puccinia recondita*.
R1: race 1 of *P. sorghi*.
R2: race 2 of *P. sorghi*.
*: RFLP between race 1 and race 2 of *P. sorghi*.
38A and 38B: pPSOc38 contained two different *Eco RI* fragments, 38A and 38B.

(ii) restriction sites within introns in the genomic sequence corresponding to the cDNA probes,

(iii) cloning artifacts such as ligation of different cDNA molecules into the one clone,

(iv) some form of genetic heterogeneity in the rust genomic DNA.

The following experiments provide some evidence against the first three of these possible explanations.

If internal restriction sites in the cDNA probes were responsible for the multiple banding patterns, then in the majority of cases all of the test enzymes should have cut within the cloned insert. Five cDNA clones were cut with five different restriction enzymes. Clone pPSOc22 contained sites for only two enzymes, *Hind III* and *Sac I*; pPSOc24, 32 and 26 each contained a single site for one of the enzymes tested, while pPSOc50 did not contain any sites (Figure 3.2). It was concluded that the presence of internal restriction sites within the cDNA clones could not explain the degree of multiple hybridization that is observed.

Another explanation for multiple bands could be the presence of the restriction sites in introns within the genes specifying the mRNA from which the cDNA was made. In this case, probes specific to the 5' and 3' ends of the cDNA clone should hybridize to a reduced number of restriction fragments. Subclones of the ends of two cDNA clones (pPSOc22 and pPSOc30) were made. Figure 3.3 is a restriction map of the two cDNA clones showing the sites used to subclone the ends. When either end of the pPSOc30 clone was used to probe genomic blots of *P. sorghi* DNA, the same hybridization pattern was observed, arguing that extra genomic bands hybridizing to this probe are not the result of intervening sequences (Figure 3.4A).

The results for the other clone pPSOc22, are more complicated. The right end (probe II) gave the same hybridization pattern as the whole pPSOc22 sequence, whereas the left end (probe I) produced half the pattern (Figure 3.4b). This could be explained by the ligation of two unrelated cDNA sequences into one clone, with the junction point being within the right-hand subclone. However, hybridization of either end to a northern blot of total RNA from urediospores of race 1 and race 2 of *P. sorghi* revealed the same 1.3kbp RNA (Figure 3.5), which argues against a cloning artifact being responsible for the hybridization result. Alternatively, an intron within the region spanned by the right-hand end of the clone containing *Bam HI* and *Eco RI* sites would explain the hybridization result shown in Figure 3.4B. Other more likely explanations are that the pPSOc22 coding region has been duplicated and the right-hand end has been conserved while the left-hand end diverged, or the right-hand end of pPSOc22 may contain a conserved motif present in another separate unrelated gene.

3.5 : Discussion

The results reported here demonstrate the use of cDNA clones as probes to detect RFLPs in *Puccinia sorghi*. However, the probes showed little homology to another species of *Puccinia* and no homology to a representative of another rust genus. The successful application of the *P. sorghi* cDNA probes to other rust fungi may be possible by lowering the stringency of hybridization, however at this stage it appears likely that homologous probes will be required for RFLP studies of the uredinales fungi.

Figure 3.2 An ethidium bromide stained agarose gel of plasmid DNA of 5 different *P. sorghi* cDNA clones digested with; lane 1, *Eco RI*; lane 2, *Eco RI + Hind III*; lane 3, *Eco RI + Bam HI*; lane 4, *Eco RI + Bgl II*; lane 5, *Eco RI + Pst I*; lane 6, *Eco RI + Sac I*. Note the internal restriction sites in pPSOc22 (*Hind III* and *Sac I*), pPSOc24 (*Bam HI*), pPSOc32 (*Hind III*), pPSOc26 (*Hind III*) and none found in pPSOc50. All the enzymes except for *Bgl II*, which does not have a restriction site in the plasmid, have one site within the polylinker region of the plasmid (pUC118). The unmarked lanes contain a *Eco RI/Hind III* digest of λ bacteriophage DNA which was used as a molecular size standard.
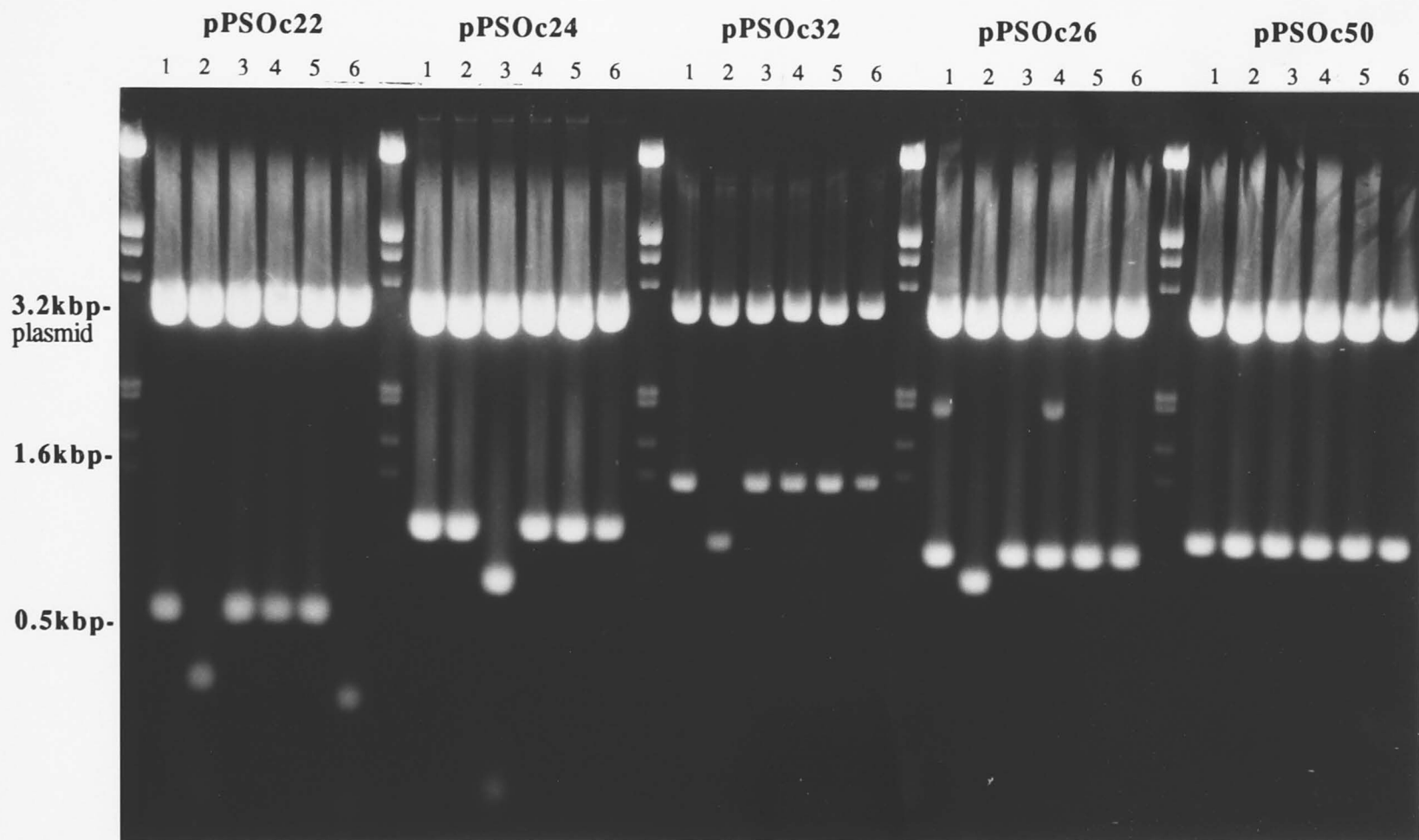
Figure 3.3 A map showing restriction enzyme sites within the pPSOc30 and pPSOc22 *P. sorghi* cDNA clones. The shaded boxes represent subcloned fragments from either end of both clones that were used as probes to genomic blots of *P. sorghi* DNA. The autoradiographs of the hybridizations using these probes is shown in Figure 3.4. The subcloning of the *Sac I/Eco RI* fragment indicated by probe I was the result of a partial *Sac I* digest.
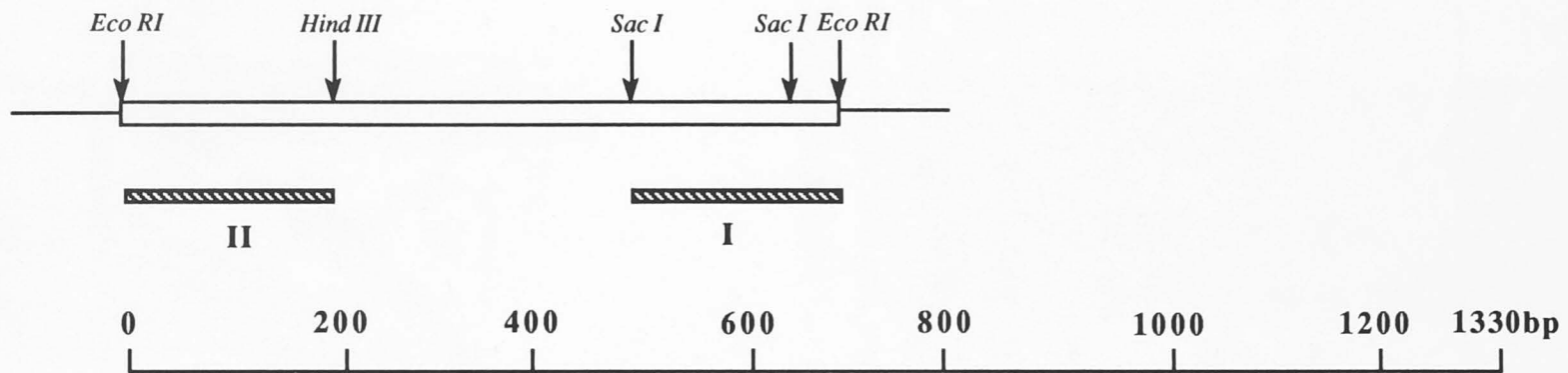
pPSOc30

Eco RI   Sac I   Sal I   Eco RI

Y   X

pPSOc22

Eco RI   Hind III   Sac I   Sac I Eco RI

II   I

0   200   400   600   800   1000   1200 1330bp
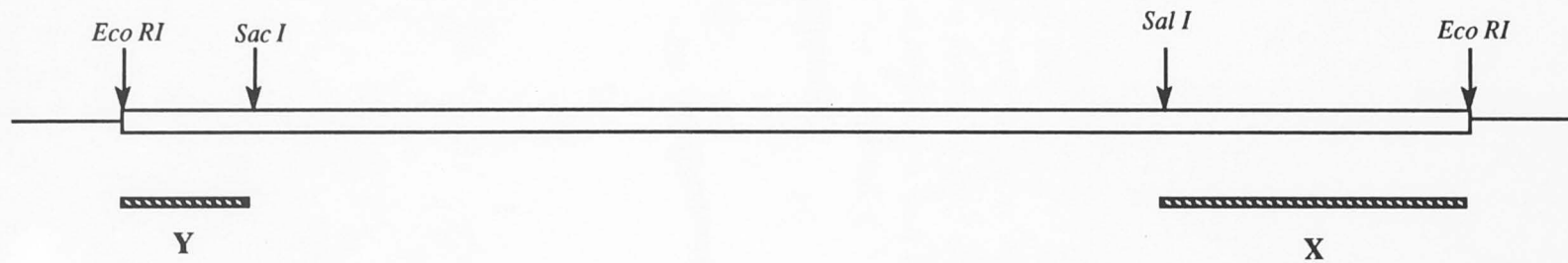
Figure 3.4

A: Autoradiographs of Southern blots of genomic DNA from race 1 and race 2 of *P. sorghi* cut with *Bam HI* and hybridized to either end (fragments X and Y, Figure 3.3) of pPSOc30.
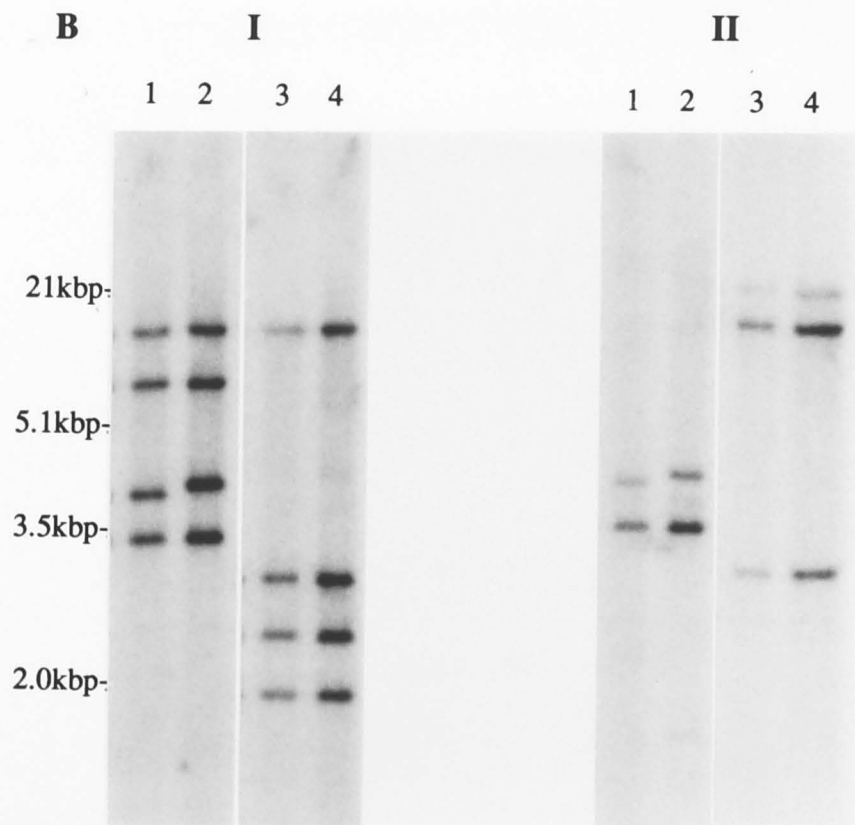
Figure 3.4

B: Autoradiographs of Southern blots of genomic DNA from race 1 and race 2 of *P. sorghi*, hybridized to either end (fragments I and II, Figure 3.3) of pPSOc22. Lanes 1 and 2 contains genomic DNA from race 1 and race 2 respectively, digested with *Bam HI*. Lanes 3 and 4 contain genomic DNA from race 1 and 2 respectively, digested with *Eco RI*.

A

X   Y

1 2   1 2

21kbp-

5.1kbp-

3.5kbp-

B   I   II

1 2   3 4   1 2   3 4
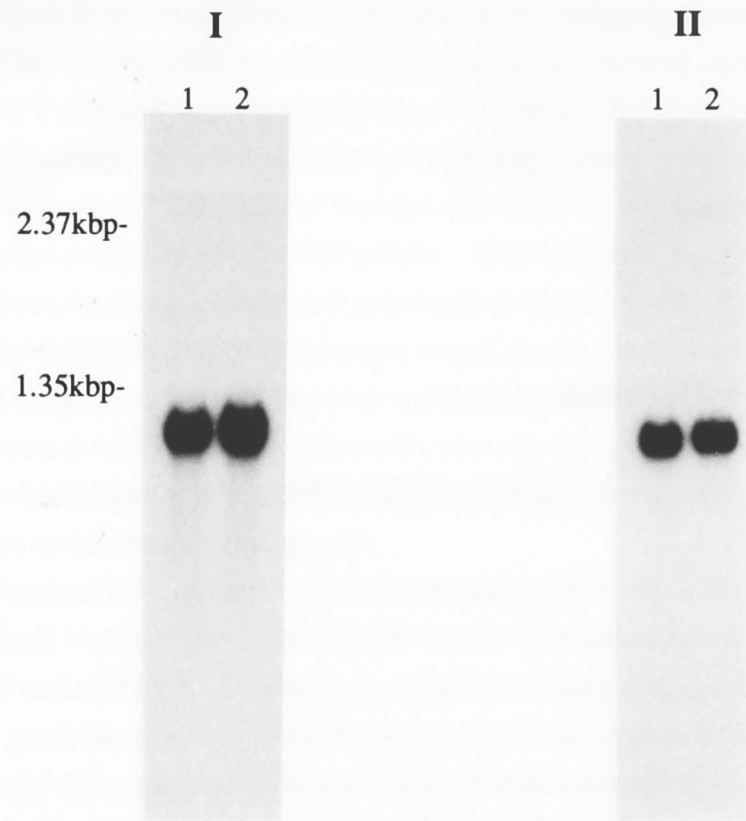
21kbp-

5.1kbp-

3.5kbp-

2.0kbp-
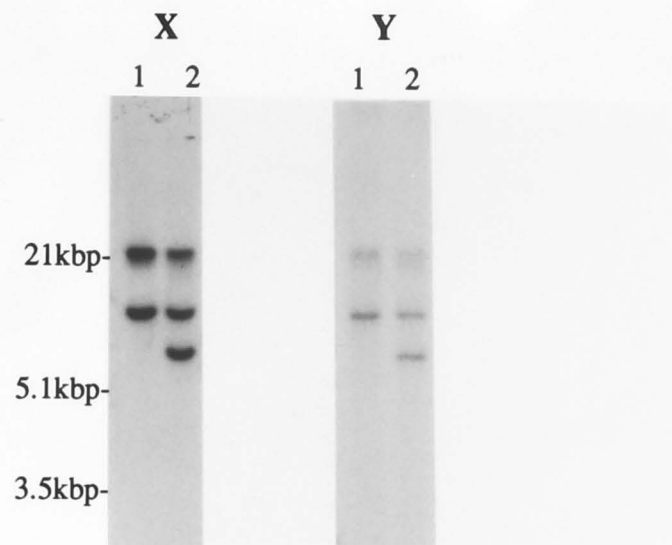
Figure 3.5 Autoradiographs of northern blots of total RNA from race 1 (lane 1) and race 2 (lane 2) of *P. sorghi*, hybridized to either end (fragments I and II, Figure 3.3) of pPSOc22.

I   II
1   2   1   2

2.37kbp-

1.35kbp-

The use of the methyl sensitive restriction enzyme *Sal I* indicates that genomic DNA from *P. sorghi* may be methylated. The role of methylation in eukaryotic genomic DNA is unknown, however it is believed to be associated with chromatin actively involved in RNA transcription (Bird, 1986). In general, fungal DNA is not highly methylated, however in the basidiomycete *Coprinus cinereus*, there is extensive DNA methylation at the nucleotide doublet CpG (Zolan and Pukkila, 1985). In another basidiomycete *Schizophyllum commune*, Specht *et al* (1988) and Buckner *et al* (1988) observed a high degree of methylation in ribosomal DNA, and reported that the methylation is developmentally controlled and may be involved in regulating RNA transcription. The existence of regions of unmethylated DNA associated with gene transcription would be supported by the hybridization pattern of pPSOc26 and several other clones. It would be interesting to know if this pattern is developmentally regulated.

The collective results of the RFLP analysis showed that out of 24 cDNA clones tested with up to 7 different restriction enzymes, 67% detected RFLPs between two physiological races of *P. sorghi*. This was perhaps not surprising given the very different geographical origin and virulence specificity of the two selected races. However, what was surprising was the general complexity of the RFLP pattern. All probes hybridized to two or more restriction fragments in almost all of the restriction enzymes tested. Since the probes were cDNA sequences and likely to mostly represent unique genes, and were much smaller than the genomic fragments to which they were hybridizing, this result begged an explanation. Furthermore, it was shown experimentally that internal restriction sites within the genomic sequence homologous to the cDNA probes, or artifacts in the cDNA cloning, were unlikely to contribute to the observed complexity.

Alternatively, multiple bands may be the result of restriction sites within introns in the genes which corresponded to the cDNA clones. This was shown to be unlikely for two clones, pPSOc22 and pPSOc30. Furthermore, based on existing data, the intron explanation is unlikely given the relatively large size of the restriction fragments (5-20kbp) and the general infrequency and small size of fungal introns (Rambosek and Leach, 1987). Introns reported in fungal genes are between 49 and 85bp. Thus while the occurrence of introns to explain the multiple bands cannot be absolutely ruled out, it appears that some other explanation is needed.

The most likely explanation is that some form of heterogeneity exists in the genomic DNA of *P. sorghi*. Heterogeneity may take the form of duplicate copies of the hybridizing sequence in the genome. It is possible that genes involved in germination may be preferentially duplicated, however there is no evidence for such a suggestion. McGinnis (1953, 1954 and 1956) reported the haploid chromosome number of *Puccinia* species to range from 3 to 6. In mitotic preparations from rust fungi containing 6 chromosomes, McGinnis (1956) observed secondary association of mitotic chromosomes at anaphase. This residual terminal attraction between mitotic chromosomes has been argued to be the consequence of allopolyploidy (Burnett, 1976). Therefore, it is possible that multiple bands could result from the duplication of genetically dissimilar genomes.

Another form of genomic heterogeneity that could explain the multiple bands is gene heterozygosity. Rust fungi are dikaryotic, which means that unlike most other fungi which are haploid, they contain two separate haploid nuclei within the one cytoplasm, a state known as the dikaryon. The dikaryon is effectively the same as a diploid nucleus. Therefore, multiple bands may be explained by each band representing an allele at a single locus, and therefore in a separate nucleus. If heterozygosity is responsible for the multiple bands then 24 out of the 24 cDNA loci tested are likely to be heterozygous, and thus the two nuclei of the dikaryon are extremely heterogeneous. Furthermore, if the two isolates from race 1 and race 2 were derived from a random mating population then one would expect some loci to have become fixed in the homozygous state. This however does not appear to be the case. Therefore, it is likely that sexual or somatic recombination occurs rarely, or some mechanism of fixed heterozygosity exists in *P. sorghi*.

In the event that heterozygosity is responsible for the consistency of the multiple banding patterns, it cannot explain the occurrence of more than two bands. Therefore, it is likely that the number of bands are a combination of allelic differences and gene duplication. In order to differentiate between gene duplication and heterozygosity as the source of multiple bands, segregating progeny from a genetic cross between race 1 and race 2 would be required. This cross is being conducted at present.

This report provides clear evidence of the value of molecular probes and neutral RFLP markers to study the genetic heterogeneity in rust fungi. The integration of RFLPs into genetic, population and evolutionary studies of rust fungi will undoubtedly lead to a greater understanding of these organisms.

Chapter 4 : DNA restriction fragment length polymorphisms in the wheat stem rust fungus, *Puccinia graminis tritici*.

4.1 : Abstract

A cDNA library was synthesized from poly A^+ RNA extracted from germinated urediospores of the wheat stem rust fungus, *Puccinia graminis tritici* (race 343-1,2,3,5,6). The library was used as a source of probes to detect RFLPs in genomic DNA from three major races of *P. graminis tritici* in Australia, as well as two *formae speciales* of *P. graminis*. DNA extracted from another *Puccinia* species infecting wheat, *P. recondita tritici* (wheat leaf rust), was included in the analysis. Nine different cDNA probes were analysed and all detected polymorphisms between the races and *formae speciales* of *P. graminis* that were tested. Seven detected polymorphisms between *P. graminis* and *P. recondita*, and the remaining two showed no detectable homology to genomic DNA of the two rust species. The potential applications of RFLP markers to study the origin of genetic variability in *P. graminis tritici* are discussed.

4.2 : Introduction

Puccinia graminis Pers. f. sp. *tritici* Eriks. & Henn. the causal agent of stem rust of wheat (*Triticum aestivum* L.), is one of the world's more destructive and economically significant pathogens. The reasons for this are that the host is a genetically uniform agricultural crop plant which is widely grown compared to wild progenitors, and *P. g. tritici* is a highly specialized and aggressive pathogen. Furthermore, surveys indicate a large pool of variation in virulence against host resistance in populations of *P. g. tritici* (Roelfs and Groth, 1988; Luig and Watson, 1970). The existence of this variation has been attributed to the combined effects of mutation and sexual recombination, including the likely influences of somatic recombination events (Watson and Luig, 1958; Ellingboe, 1961; reviewed by Watson, 1981). The relative contribution of these mechanisms in generating variation in nature is not known.

In the Pacific north west of the United States, *P. g. tritici* undergoes a complete life cycle with the teliospore stage existing as a vital over-wintering form. Further to the east, eradication about 50 years ago of *Berberis vulgaris*, the alternate host of *P. g. tritici*, has prevented sexual reproduction leaving the pathogen dependent on the asexual stage of its life cycle. A survey of virulence, as well as independent genetic markers such as isozyme variants, revealed a greater degree of genetic variation in the sexually reproducing populations (Burdon and Roelfs, 1985a,b). In Australia, the absence of the alternate host and/or the conditions for teliospore germination has meant that sexual reproduction of *P. g. tritici* has most probably never occurred. Since 1920 Australian isolates of *P. g. tritici* have been typed for virulence against major genes for resistance

in wheat. Watson (1981) analysed this data to outline the probable origin of the major races of *P. g. tritici* in Australia from 1920-1980. He suggested that the majority of Australian *P. g. tritici* rust races have arisen as mutants of a limited number of introduced strains, several of which appear to have come from Africa. In 1981-82, 16 virulent forms were reported in Australia and yet Burdon *et al* (1983) could find no variation in these isolates at 11 different isozyme loci. They suggest that the variation in virulence is the result of the introduction of several foreign races, followed by selective step-wise mutation to new virulent forms. Such a situation might explain the variation in virulence and the lack of variation in isozyme patterns.

However, in combination with mutation, somatic hybridization events are likely to contribute to the generation of new virulence gene combinations of *P. g. tritici*. There are a number of putative somatic hybridization events documented in the literature, one being between two *formae speciales* of *P. graminis*, *P. g. tritici* and *P. g. secalis* (Watson and Luig, 1959; Luig and Watson, 1972). Both *P. g. tritici* and *P. g. secalis* infect the rough wheat grass, *Agropyron scabrum*, where the hybridization event is believed to have taken place. The putative hybrid is pathogenic on *A. scabrum* and the barley grass *Hordeum leporinum*, but non-pathogenic on wheat or rye. Isozyme analysis of the hybrid supported the hypothesis of its origin, and suggested that exchange of whole nuclei between the presumptive parents was responsible for the formation of the new rust (Burdon *et al*, 1981).

Our current knowledge of the population structure of *P. g. tritici* in Australia and North America can be attributed to the use of virulence and isozyme markers. However, population studies of *P. g. tritici* have been limited by the number and sensitivity of the genetic characters that can be assessed. Recently, molecular probes detecting RFLPs in genomic DNA of eukaryotic organisms (Botstein *et al*, 1980) have been developed as genetic markers. The successful use of RFLPs as markers in a number of phytopathogenic fungi has been reported (Hulbert and Michelmore, 1988; McDonald and Martinez, 1990; Christiansen and Giese, 1990; Braithwaite *et al*, 1990; for review see Michelmore and Hulbert, 1987). RFLP markers have also been reported in other rust fungi including *P. sorghi* (maize rust) and *Melampsora lini* (flax rust) (see chapters 3 and 5). An important conclusion from these studies was that while molecular probes derived from cDNA libraries were very successful in detecting RFLPs in rust genomic DNA, there was little or no cross homology of probes between rust species. This indicated the need for species-specific cDNA probes.

The aim of the work presented in this chapter is to synthesize a cDNA library from poly A^+ RNA, extracted from germinated urediospores, and develop a set of probes capable of detecting RFLPs between physiological races and *formae speciales* of *P. graminis*. Such probes could provide genetic markers of sufficient sensitivity to thoroughly document the extent and nature of putative somatic hybridization events.

4.3 : Material and Methods

Rust strains

The origin of the races and *formae speciales* of *P. graminis* and *P. recondita tritici* used in the RFLP analysis is listed in Table 4.1. The urediospores of race 343, from which mRNA was extracted for cDNA synthesis, were typed for virulence specificities at the Plant Breeding Institute, and multiplied from a single pustule on the wheat cultivar 'Transfer'. 'Transfer' is susceptible to *P. graminis tritici* and resistant to *P. recondita tritici*, eliminating the chance of any potential contamination. Nucleic acids were extracted from germinated urediospores as described in chapter 2. Urediospores were germinated on an air/water interface to which several drops of 1-nonanol (Aldrich) were added to stimulate germination (Burdon and Roelfs, 1985a).

Rust races	Host plant	origin
Puccinia graminis tritici		
34-2,12,13 (accession no. 84552=427	wheat	PBI
126-5,6,7,11 (accession no. 334=427)	wheat	PBI
343-1,2,3,5,6	wheat	field isolate
P. graminis secalis (accession no. 57241B=915)	rye	PBI
scabrum rust (accession no. 71406=107)	*Agropyron scabrum*	PBI
P. recondita tritici (race 76-0)	wheat	PBI

Table 4.1. Origin and description of the rust races used in the RFLP study. PBI stands for the Plant Breeding Institute, University of Sydney.

DNA and RNA extraction and cDNA synthesis

All nucleic acid extraction and purification steps were conducted according to the methods described in chapters 2 and 3. Complementary DNA synthesis was performed using a kit provided by Amersham, and the cDNA sequences cloned, with *Eco RI* linkers (Boehringer), into the *Eco RI* site of the pGEM 7Zf(+) bacterial plasmid vector (Promega). Approximately 8.0×10^4 recombinant colonies were obtained and the library was stored as bacterial cells and as purified plasmid DNA at -80°C.

Southern blot and hybridization

Genomic DNA (2μg) from germinated urediospores was digested with the restriction endonucleases, *Eco RI*, *Hind III* or *Bam HI* (Pharmacia) and separated on a 1% agarose gel. Electrophoresis, Southern blots, hybridization and labelling of probes were all done according to the methods described in chapter 3.

4.4 : Results

A cDNA library of 8.0×10^4 recombinant clones was constructed from poly A$^+$ RNA extracted from germinated urediospores of the wheat stem rust *P. graminis tritici*. Fifty-eight colonies were selected at random from the library and 41 were found to contain recombinant DNA ranging in size from 200bp to 2.2kbp. Nine clones were selected and hybridized to Southern blots containing digests of genomic DNA from *P. g. tritici*, *P. g. secalis*, *scabrum* rust and *P. recondita tritici*. The autoradiographs of three of these hybridizations using the cDNA clones pPGtc10, pPGtc26 and pPGtc36 are shown in Figure 4.1 a,b,c, and the data from all the hybridizations is summarised in Table 4.2.

The three physiological races of *P. g. tritici*, the *formae speciales* of *P. g. tritici* and *P. r. tritici* could be distinguished from one another by all the probes using at least one of the restriction enzymes tested. Two out of the nine clones did not show any homology to the wheat rust *P. r. tritici*, (for example refer to Figure 4.1b, lane 6).

The variation between the three physiological races of *P. g. tritici* indicated that the races 34 and 343 were more closely related to each other than to 126. Twenty three out of 27 probe/enyzme combinations detected polymorphisms between race 343 or race 34 and race 126, whereas only three probe/enzyme combinations detected a clear RFLP between race 343 and race 34 (for example refer to Figure 4.1a, *Hind III*, lanes 1 and 3).
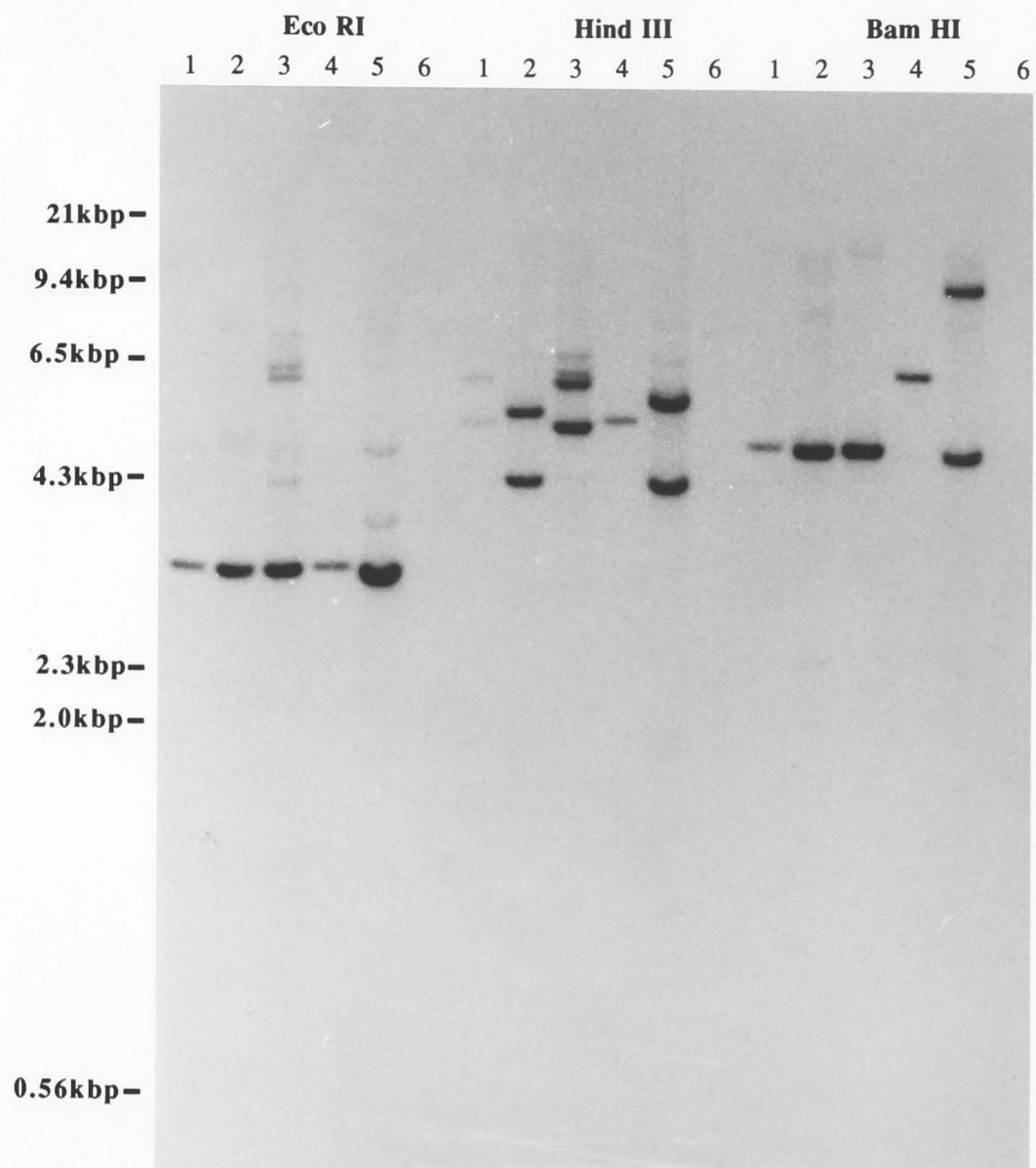
The results of the RFLP analysis of the *scabrum* rust support the hypothesis that this rust originated from a somatic hybridization event between *P. g. tritici* and *P. g. secalis*. In 22 out of 27 probe/enzyme combinations the banding pattern of the *scabrum* rust could be identified from the addition of bands from *P. g. tritici* and *P. g. secalis* (for example refer to Figure 4.1a, *Hind III*, lanes 1 to 5). In the other 5 cases the *scabrum* rust contained bands that were not present in either of the putative parent rusts (for example refer to Figure 4.1b, *Bam HI*, lane 5). Out of the 22 cases where the *scabrum* bands could be identified in the parental races, the race of *P. g. tritici* from which the *scabrum* band originated could be clearly identified on 6 occasions. In one instance the band could be traced to either race 34 or 343, and in the other 5 cases to race 126 (for example refer to Figure 4.1b, *Hind III*, lanes 2 and 5). Therefore, if the *scabrum* rust did originate from a somatic hybridization event between *P. g. tritici* and *P. g. secalis*, our evidence suggests that the *P. g. tritici* parent is likely to be race 126 or a derivative.

Figure 4.1: Autoradiographs of a Southern blot containing genomic DNA of *P. graminis tritici* digested with the restriction endonucleases *Eco RI*, *Hind III* and *Bam HI*, and hybridized to the cDNA probes pPGtc10 (figure 1a), pPGtc26 (figure 1b) and pPGtc36 (figure 1c). The order of the loadings are: lane 1, race 34; lane 2, race 126; lane 3, race 343; lane 4, *P. g. secalis*; lane 5, *scabrum* rust; lane 6, *P. recondita tritici*. The loading of genomic DNA is not even throughout the gel with race 34 being less and the *scabrum* rust being more than in the other lanes. Genomic DNA from race 343 did not cut to completion in each of the digestion reactions, generating several faint partial bands hybridizing to the probes. These were disregarded in the RFLP analysis.
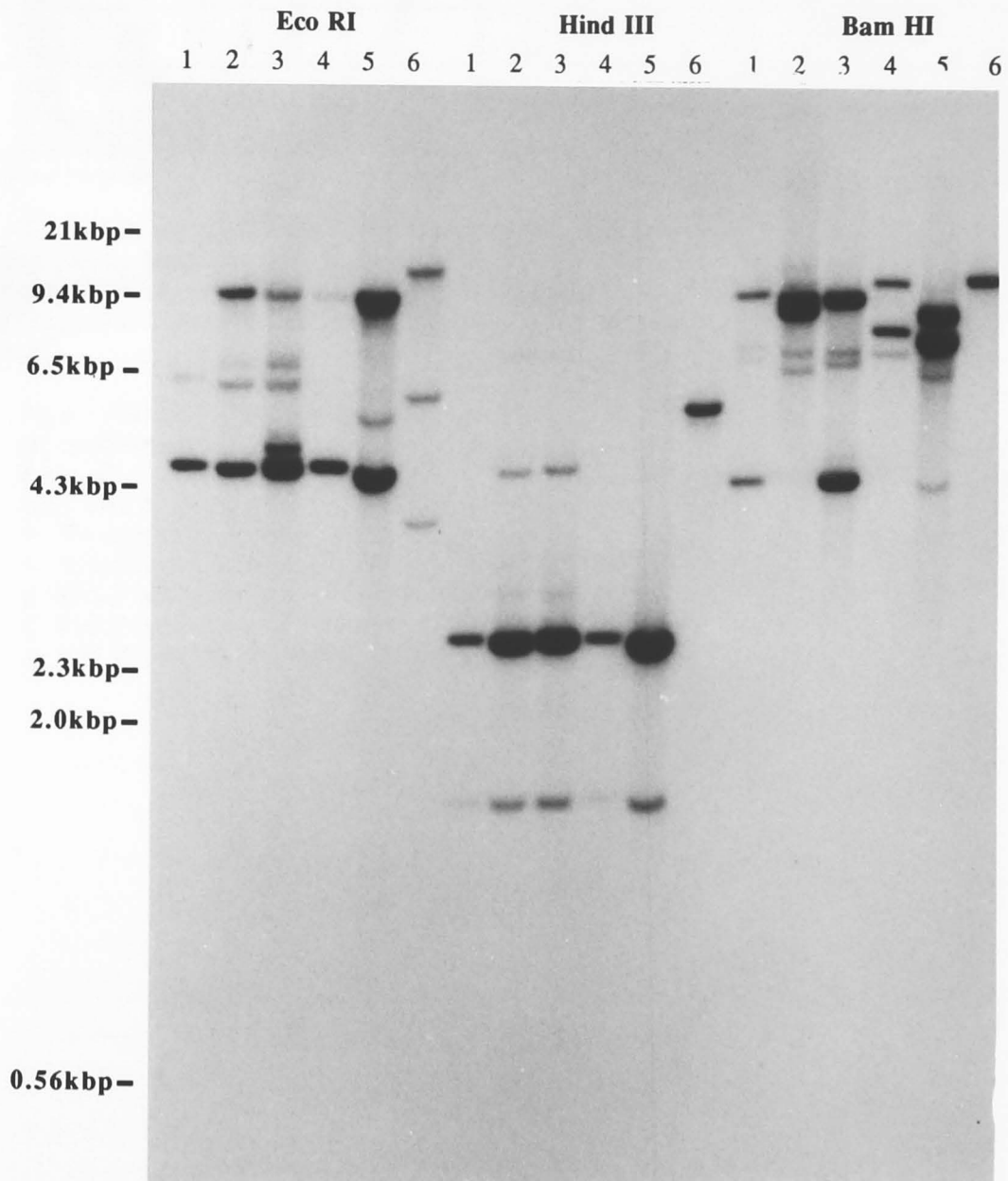
1a

Eco RI   Hind III   Bam HI

1 2 3 4 5 6   1 2 3 4 5 6   1 2 3 4 5 6

21kbp—
9.4kbp—
6.5kbp —
4.3kbp—
2.3kbp—
2.0kbp—
0.56kbp—

1b

1c

		Eco RI						Hind III						Bam HI					

Restriction enzyme/DNA combination

Enzyme		Eco RI						Hind III						Bam HI					
DNA		34	126	343	P.g.s	sc	P.r.t	34	126	343	P.g.s	sc	P.r.t	34	126	343	P.g.s	sc	P.r.t
pPGtc	Size																		
5	800	1	1	1	1	1	1#	2†	1¶	2	2	2	1#	1	1¶	1	2	1	1#
7	800	2†	2¶	2	2	2	1#	2†	2¶	2§	2	2	2#	1†	2¶	1	2	2	2#
8	500	2†	1¶	2	2	1	1#	2†	2	3	1	2	1#	2†	1¶	2	2	2	1#
10	900	2†	1¶	2	2	2	2#	4†	4¶	7§	4	4	1#	4†	2¶	4	3	2	2#
15	650	2†	2¶	2	1	2	1	2	2	2	1	2	1#	2†	1¶	2	1	2	1#
26	1000	1	1	1	1	1	*	2†	2¶	2	1	2	*	1	1	1	1	2	*
29	1900	4†	3¶	4	2	3	4#	3†	1¶	3	1	2	3#	2†	2¶	2	1	2	4#
35	2100	2†	2¶	3§	1	2	*	2†	1¶	2	1	4	*	2†	2¶	2	1	2	*
36	800	1†	2¶	1	1	2	3#	1	1	1	1	1	1#	2†	2¶	2	2	2	1#

Table 4.2: Results of the RFLP screen of race and *formae speciales* of *P. graminis* and *P. recondita tritici*. Numbers refer to the number of bands hybridizing to the particular probe. Due to the unequal loading of DNA, particularly race 34 relative to races 126 and 343, some faint bands were disregarded in the compiling of this table. Results that are underlined are shown in Figure 4.1 a,b,c.

P.g.s. : *Puccinia graminis secalis*.
sc: *scabrum* rust.
P.r.t.: *P. r. tritici*
Size: size estimate of the insert DNA in basepairs (bp).
*: No detectable hybridization
†: RFLP between race 34 and race 126 of *P. g. tritici*.
§: RFLP between race 34 and race 343 of *P. g. tritici*.
¶: RFLP between race 126 and race 343 of *P. g. tritici*.
#: RFLP between *P. g. tritici* and *P. r. tritici*.

4.5 : Discussion

The results reported here demonstrate the successful application of RFLP analysis of genomic DNA extracted from the wheat stem rust fungus, *Puccinia graminis tritici*. The probes were derived from a cDNA library of 8×10^4 recombinant clones. Johnson and Lovett (1984) have shown by hybridization kinetics that zoospores of the zygomycete *Blastocladiella emersonii* contain approximately 4500 to 6000 different RNA sequences. Therefore, it is likely that the cDNA library from *P. g. tritici* is a source of many different probes for RFLP analysis.

Nine different cDNA sequences were used in an RFLP analysis of *P. g. tritici*. Two of the nine sequences did not hybridize to genomic DNA from wheat leaf rust (*P. recondita tritici*) and several others showed reduced homology. This supports the results described in chapter 3 in which none of the cDNA sequences from the maize rust *P. sorghi* showed intergeneric homology, and 25% showed interspecific homology. The phylogenetic significance of these results are more difficult to assess. Baum and Savile (1985) suggest, primarily on the basis of spore morphology, that of the *Puccinia* species infecting cereal hosts, *P. graminis* and *P. recondita* were quite distantly related. However, as the relationship between sequence similarity and *Puccinia* speciation is unknown, the significance of the hybridization results awaits further analysis.

The origin of new virulent races of *P. g. tritici* in Australia has been attributed to the combined influences of selection by step-wise mutation, introduction of exotic races and somatic hybridization. Race 126 was a major race in Australia 70 years ago when virulence typing began, but died out in the 1960's, whereas races 34 and 343 represented 80% of all the isolates of *P. g. tritici* tested in the 1989/90 season (Park, personal communication). Race 343 is thought to be derived from race 326 which was first detected in Australia in 1969. Burdon *et al* (1982) suggest on the basis of isozyme data, and data provided by Luig (unpublished) and de Sousa (1975) that race 326 originated from Africa. Race 34-2,12,13 is believed to have originated from 34-2, which in turn originated from 21-0 (Watson, 1981). Race 21-0 appeared in Australia for the first time in 1954, and Burdon *et al* (1982) postulate that its origin was also from Africa. Step-wise mutation of 34-2 to virulence against Sr_{27} and Sr_{Satu} has resulted in the formation of 34-2,12,13 (Singh and McIntosh, 1990).

The results of the RFLP analysis showed that variation between race 126 and either 34 or 343 is greater than between 34 and 343. This supports the results of the isozyme study of Burdon *et al* (1982). The similarity in the banding pattern of race 34 and 343 is more difficult to assess. Burdon *et al* (1982) detected no variation between the two races, so together with the RFLP results, it appears that race 34 and 343 may have a common African ancestor. The hybridization pattern of *P. g. tritici*, *P. g. secalis* and the *scabrum* rust strengthen the existing hypothesis based on virulence and isozyme studies that the *scabrum* rust is a somatic hybrid between *P. g. tritici* and *P. g. secalis*.

The results reported here suggest that the *P. g. tritici* parent may have been race 126 or a derivative.

In conclusion it should be noted that this is a preliminary study aimed primarily at assessing the applicability of RFLPs to study the population structure of *P. g. tritici* in Australia and elsewhere. Previous reports using isozymes were limited by the lack of variation that was detected, however the results reported here suggest that the potential application of RFLPs should be viewed with more optimism. A large survey of isolates of many different races needs to be done before the significance of the segregation of individual hybridization patterns can be assessed in terms of race origin. This report indicates that such a survey is feasible and would be informative.

Chapter 5 : The inheritance of Restriction Fragment Length Polymorphisms in the flax rust *Melampsora lini*

5.1 : Abstract

Random cDNA sequences synthesized from poly A$^+$ RNA extracted from germinated urediospores of the flax rust fungus, *Melampsora lini*, were used as probes to detect RFLPs between races of *M. lini* infecting cultivated flax and an isolate infecting Australian native flax. Out of 22 different probes, about 50% detected RFLPs between the races of *M. lini* that infect cultivated flax, and 80% of the probes detected polymorphisms between the rusts of cultivated flax and a rust infecting Australian native flax. The inheritance of seven RFLP loci was followed in 3 hybrids and 19 F$_2$ progeny derived from a cross between two of the *M. lini* races. In most cases the inheritance of RFLP loci could be explained by the segregation of alleles at a single locus. However, in one case, parental bands were not transmitted to the progeny and it was postulated that this was due to the existence of null alleles.

5.2 : Introduction

Genetic studies of most phytopathogenic fungi, including rusts, have been restricted to an analysis of virulence and isozyme markers. In rust fungi these markers have studied the sexual transmission of genes (Johnson, 1954; Wilcoxson and Paharia, 1958; Luig and Watson, 1961; Samborski and Dyck, 1968,1976; Haggag *et al*, 1973; Burdon *et al*, 1986), as well as somatic events, such as heterokaryosis and nuclear exchange (Flor 1964; Watson and Luig, 1959; Burdon *et al*, 1981) and parasexual recombination (Watson and Luig, 1958; Ellingboe, 1961). The lack of other markers, however, has prevented a more comprehensive genetic analysis from being carried out.

Restriction fragment length polymorphisms present an alternative, almost unlimited supply of selectively neutral genetic markers which could be used in genetic studies of phytopathogenic fungi (Michelmore and Hulbert, 1987). The inheritance of RFLP markers and the construction of linkage maps have been reported for the downy mildew of lettuce, *Bremia lactucae* (Hulbert and Michelmore, 1988) and the powdery mildew of wheat *Erysiphe graminis* (Christiansen and Giese, 1990). Furthermore, in the preceding chapters the use of random cDNA probes to detect RFLPs in the maize rust fungus (*Puccinia sorghi*) and the wheat stem rust fungus (*P. graminis tritici*) has been described. In the studies of both maize and wheat rusts cDNA sequences were used as probes. Since cDNA sequences are mostly derived from mRNA templates of single or small gene families they are likely to give simple hybridization patterns. In most RFLP studies, particularly of animal systems, these expectations have been met (Balazs *et al*, 1984). However, in *P. sorghi* the hybridization of random cDNA probes to genomic blots gave multiple banding patterns in all 24 cDNAs that were analysed. It was concluded that the multiple banding pattern most probably represented some form of genetic heterogeneity in the maize rust genome, and two possible explanations were suggested. Either the different bands represented different alleles in the nuclei of the dikaryon (heterozygosity), or

the extra bands reflected duplicate copies of the cDNA sequence. Heterozygosity alone could not explain patterns of greater than two bands suggesting that both explanations may apply. A distinction between these two possibilities could be made by observing the transmission of the multiple bands in progeny of genetic crosses. It was not experimentally possible to generate sexual progeny from *P. sorghi*, but such material for flax rust was available from Lawrence *et al* (1981b) who had successfully crossed two races of *Melampsora lini* (Ehrenb.) Lev. One of the progeny was selfed to produce an F_2 population. This cross segregated for 9 unlinked avirulence loci.

The aim of the experiments described here was first to determine if the same degree of multiple banding is observed using random cDNA probes hybridized to genomic blots of *M. lini* DNA as was observed in *P. sorghi*, and secondly, to observe how these bands were transmitted in a sexual cross in order to establish the basis of the multiple banding patterns. Since previous work had indicated that the *P. sorghi* cDNA clones did not cross-hybridize to *M. lini* genomic DNA, a cDNA library was synthesized from poly A^+ RNA from germinated urediospores of *M. lini*.

5.3 : Materials and Methods

Rust races

Four races of *M. lini* have been used in the RFLP study, 271, 228, P1C and LMS. The first three are rusts of cultivated flax, *Linum usitatissimum*, whereas LMS occurs on Australian native flax, *L. marginale*. The origin of the races is described by Lawrence *et al* (1981b), but in brief, 271 is of South American origin, 228 is a North American isolate and P1C is derived from self-fertilization of a New Zealand isolate. The races 228 and P1C were crossed to produce the hybrids CH4, CH5 and CH6. CH5 was self-fertilized to produce the F_2 progeny. The method of crossing *M. lini*, and the maintenance of the races is also described by Lawrence *et al* (1981b). All the races of *M. lini*, including the hybrids and the F_2 progeny, were individually amplified on the universal susceptible flax variety, Hoshangabad.

Extraction of DNA, poly A^+ RNA and cDNA synthesis

Rust germination and collection, DNA and RNA extraction, poly A^+ RNA purification and cDNA synthesis were all carried out according to the methods described for the maize rust, *Puccinia sorghi* in chapters 2 and 3. The cDNA library was synthesized from 5μg of polyA$^+$ RNA extracted from race CH5 and cloned with *Eco RI* linkers into the bacterial plasmid vector pGEM 3Zf(+) (Promega). The total library was stored as aliquots of bacterial cells in 7% DMSO at -80°C.

Identification of RFLP loci

Gel electrophoresis of digested DNAs, blotting, hybridization and labelling of DNA probes were all conducted as previously described in chapter 3. Genomic DNA from the races 228, P1C, CH5, 271 and LMS was digested with the restriction endonucleases *Eco RI*, *Hind III* and *Bam HI* (Pharmacia). Those restriction enzymes which detected RFLPs in the parent

races 228 and P1C were used to digest the hybrids and F_2 progeny DNAs for segregation analysis.

<u>Analysis of data</u>

To compare the complexity of the hybridization patterns of *P. sorghi* and *M. lini*, the number of digests which contained 1, 2, 3 and more than 3 bands were pooled into classes and expressed as a percentage of the total number of probe/enzyme combinations that were tested. The inheritance of 7 RFLP loci detected between P1C and 228 was determined by the segregation of bands in the hybrids and F_2 progeny. The genotype of each of the F_2 progeny at the 7 RFLP loci, and at the 9 avirulence loci also segregating in the cross, was analysed by the mapmaker program (Lander *et al*, 1987) to detect possible genetic linkage relationships.

5.4 : Results

Of sixty colonies selected from the *M. lini* cDNA library, 44 were found to contain inserts, ranging in size from 300 to 2500bp. Twenty-four cDNAs were randomly selected and, of these, 22 gave distinctive hybridization patterns when hybridized to genomic blots of *M. lini* DNA. Two cDNA clones were represented twice. Figure 5.1 a,b and c shows the autoradiographs of Southern blots hybridized with three different cDNA probes demonstrating increasing levels of complexity. The data collected from the 22 different hybridization patterns is summarized in Table 5.1 in which the number of hybridizing bands in each probe/restriction enzyme combination is recorded. This data was pooled and standardized as described in the methods, and compared with the equivalent data from the hybridization patterns of *Puccinia sorghi* genomic DNA reported in chapter 3. From this comparison (see Table 5.2) it can be seen that the incidence of multiple bands, and hence the genomic complexity, is greater in *P. sorghi* than in *M. lini*.

Table 5.3 summarizes the frequency with which probes detected RFLPs between the races of *M. lini*. Fifty to 55% of probes detected RFLPs between the three races infecting cultivated flax, whereas over 80% of the probes detected RFLPs between the rusts of cultivated flax and race LMS which infects native Australian flax.

Eleven RFLPs were detected between race 228 and race P1C which were the parents of a genetic cross performed by Lawrence *et al* (1981b). Seven of these RFLPs were selected and the inheritance of the polymorphic bands was observed in the hybrids and F_2 progeny of the cross between the two races. The hybridization patterns of 3 of the 7 RFLP probes to Southern blots containing genomic digests of the two parental races, the 3 hybrid rusts and 19 F_2 progeny are shown in Figure 5.2 a,b,c. In 5 of the 7 RFLPs the inheritance of bands could be explained by the segregation of alleles at a single locus. An example of this is the inheritance of the RFLP loci defined by probe pMLc8. The pMLc8 probe hybridized to two bands in both parents (Figure 5.2a). All four parental bands were of different length. Each hybrid contained two bands, one inherited from each parent. In the F_2 progeny, the bands segregated in a 1:2:1 ratio for

Figure 5.1. Autoradiographs of blots containing genomic DNA from races of *M. lini* hybridized to cDNA clones pMLc37 (1a), pMLc15 (1b) and pMLc35A (1c). Lanes 1 to 5 contain genomic DNA from races LMS, CH5, 271, 228 and P1C respectively, cut with *Eco RI*, *Hind III* and *Bam HI* as shown.
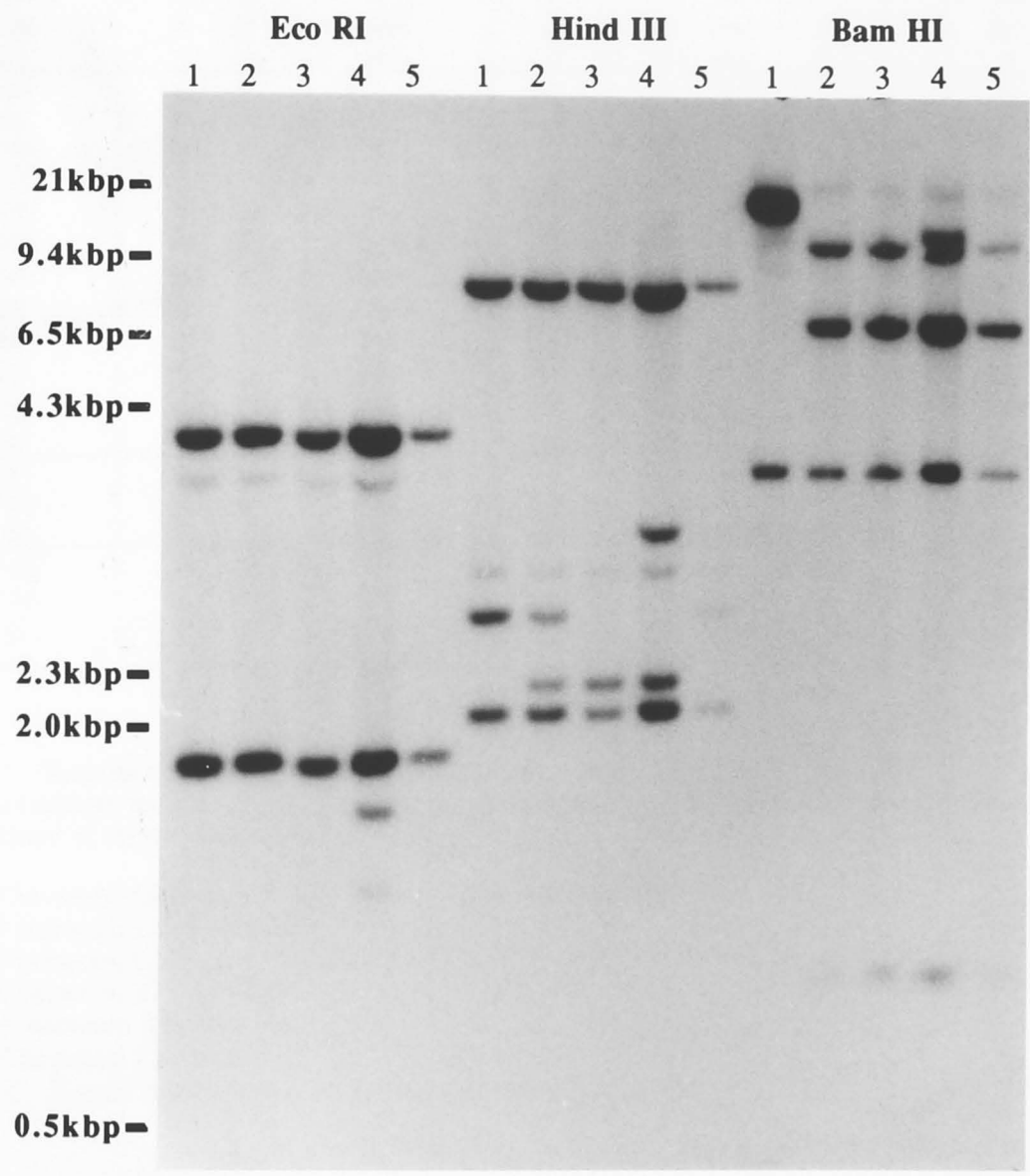
1a
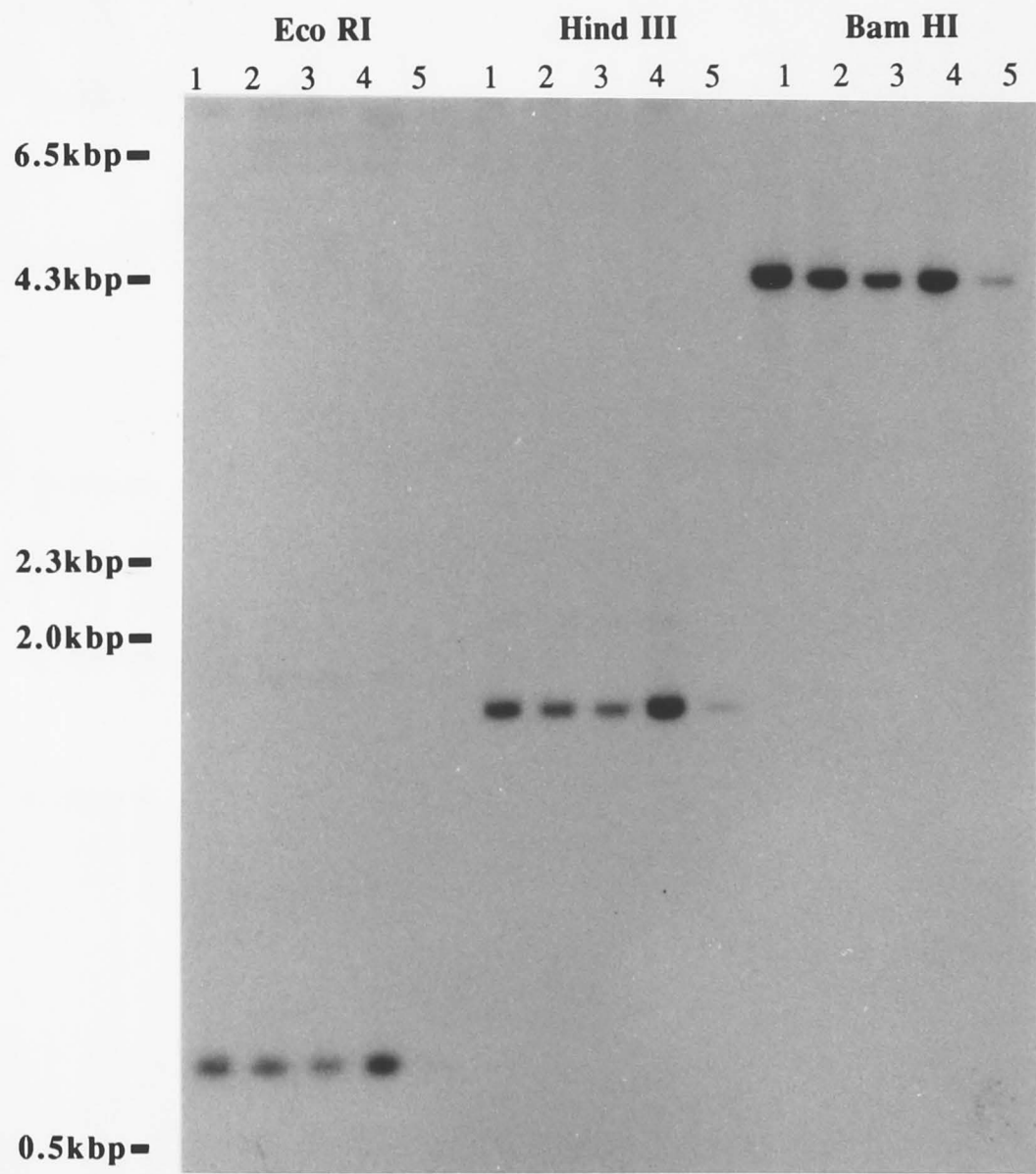
1b
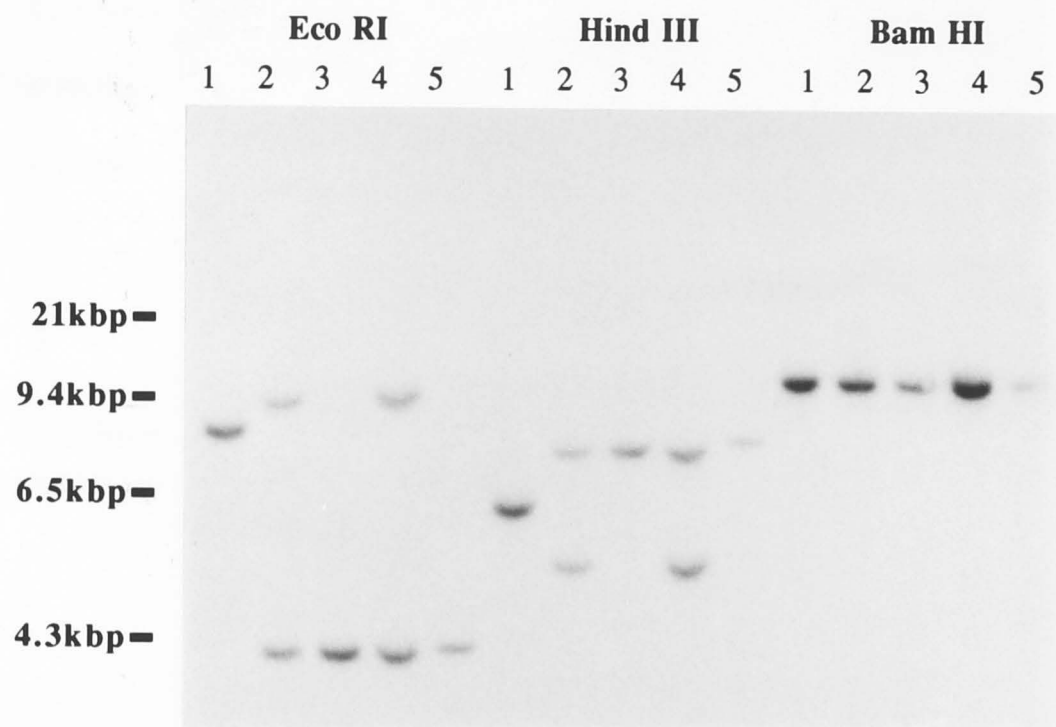
1c

Restriction enzyme/DNA combination															
Enzyme		*Eco RI*					*Hind III*					*Bam HI*			
DNA		LMS	CH5	271	228	P1C	LMS	CH5	271	228	P1C	LMS	CH5	271	228	P1C
pMLc	insert size (bp)															
3	1400	1	1	1	1	1	1	1	1†	1§	1¶	1	2	2†	2§*	2¶#
4	800	1	1	1	1	1	1	1	1†	1§	1¶	1	2	2†	2§	2¶#≡
8	1800	1	2	2†	2§*	2¶#≡	1	2	2†	2§	1#≡	1	1	1	2§*	1≡
15	200	1	2	1†	2§*	1¶≡	1	2	1†	2*§	1¶≡	1	1	1	1	1
16A	1300	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
16B	400	2	2	2†	2§	2¶	1	1	1	1	1	1	1	1	1	1
16C	200	2	2	2	2	2	2	2	2	2	2	1	1	1†	1§	1¶
17	600	1	1	1	1	1	1	1	2†	1*	1#	1	1	1	1	1
18	800	1	2	2†	2§	2¶	1	1	1	1	1	1	1	1	1	1
22	700	2	4	2	2	2¶#≡	2	3	2	2	2¶#≡	2	2	2†	2§*	2¶#≡
23	900	1	1	1†	1§	1¶#≡	1	2	1†	1§	1≡	1	1	1†	1§	1¶
28	500	2	3	3†	3§	3¶	2	2	2	2	2	2	2	2	2	2
30	400	2	3	3†	4§*	3¶≡	2	3	2	2	3¶#≡	1	2	2†	2§*	1#≡
31	600	1	2	2†	2§	1¶#≡	1	2	2†	2§*	1¶#≡	1	1	1†	2§*	1¶#≡
33	400	1	2	2†	2§	1#≡	1	1	1†	1§	1¶	1	1	1†	1§*	1¶#
35A	900	3	3	3	4§*	3≡	3	4	3†	4§*	3#≡	2	2	2†	3§*	2¶≡
35B	800	2	2	2	3§*	2	3	4	3†	4§*	3#	2	2	2†	2§	2¶
36	1100	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
37	600	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
38	1100	2	2	2†	2§	2¶	2	2	2	2	2	1	1	1	1	1
39	1150	1	1	1†#	2	1≡	2	3	2†	3§*	2¶≡	2	2	2†	3§*	2¶≡
40	350	1	1	1	1	1	2	2	2†	2§	3¶#≡	1	2	3†	2§*	3¶#≡

Table 5.1. Results of the RFLP analysis of the races of *M. lini*, LMS, CH5, 271, 228 P1C. Numbers indicate the number of genomic bands hybridizing to the cDNA probes. The data which is underlined is shown in Figure 5.1 a,b,c.

† : RFLP between LMS and 271.
§ : RFLP between LMS and 228.
¶ : RFLP between LMS and P1C.
* : RFLP between 271 and 228.
# : RFLP between 271 and P1C.
≡ : RFLP between 228 and P1C.
A, B, and C: *Eco RI* inserts contained within the one pMLc clone.

Figure 5.2. Autoradiographs of Southern blots hybridized to pMLc8 (2a), pMLc22 (2b) and pMLc33 (2c). The first two lanes contain genomic digests of the parent races P1C and 228 respectively. The next three lanes contain genomic digests of three hybrids from the cross, CH4, CH6 and CH5 respectively. The remaining lanes (numbered 1-19) contain genomic digests of the F_2 progeny from the self fertilization of CH5. The restriction enzyme *Eco RI* was used in this analysis and the same blot was used for each hybridization. Genomic DNA from progeny 9 digested poorly and was not included in the analysis. Arrow heads in Figure 5.2a indicate the position of bands in the P1C parent.

2a
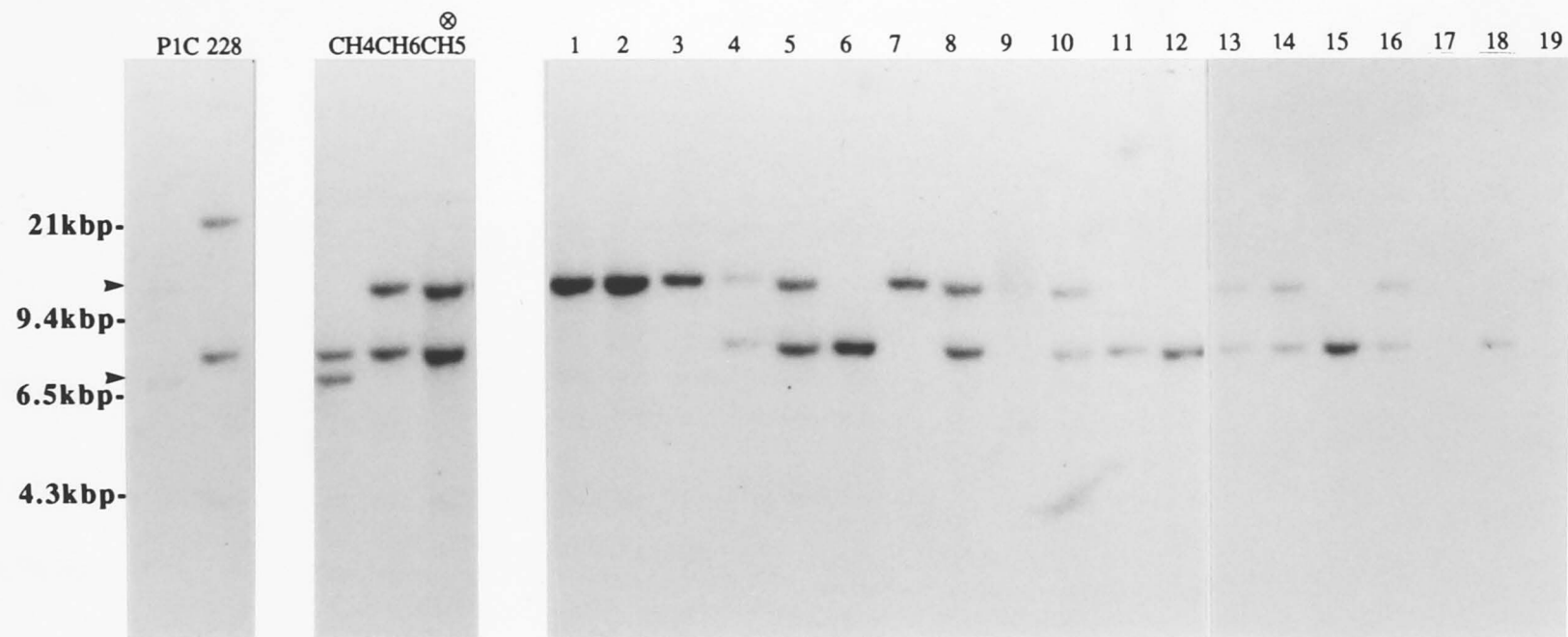
2b
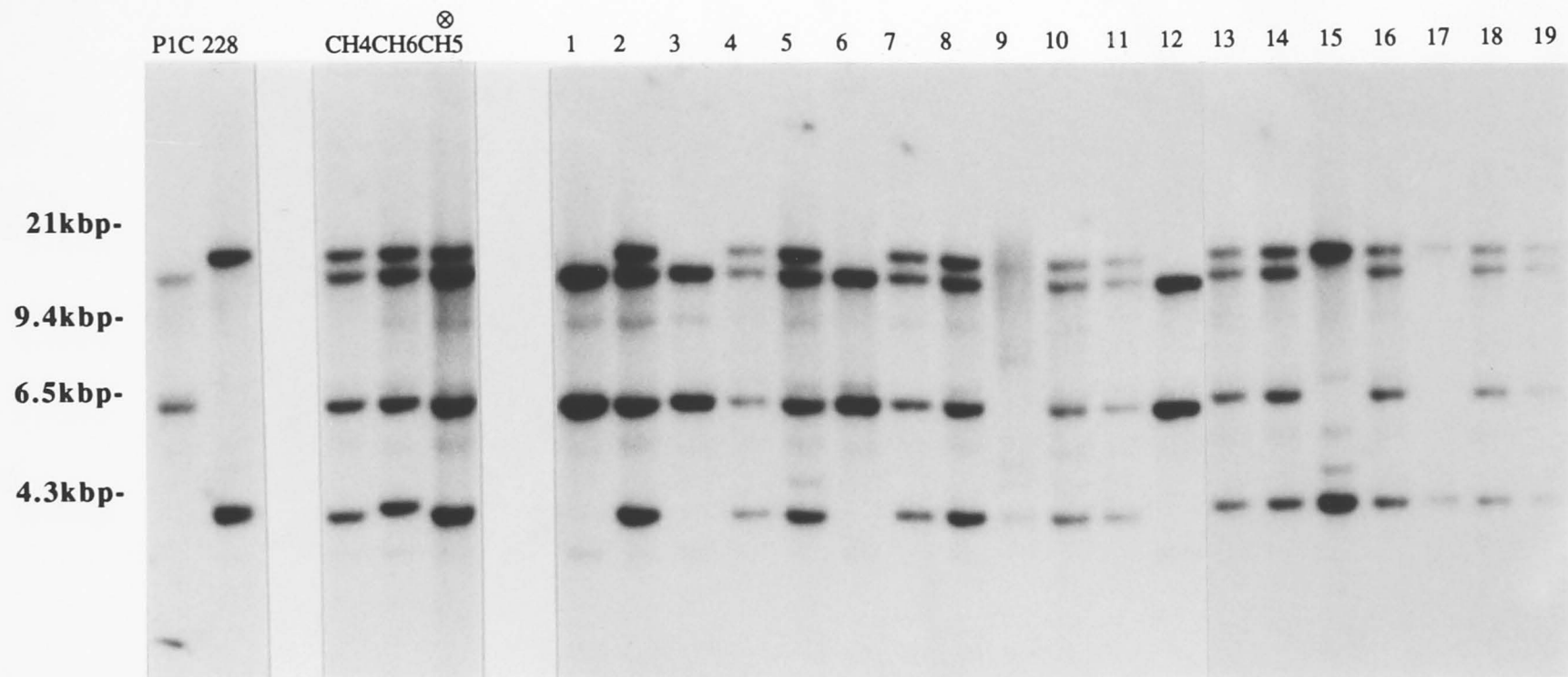
2c
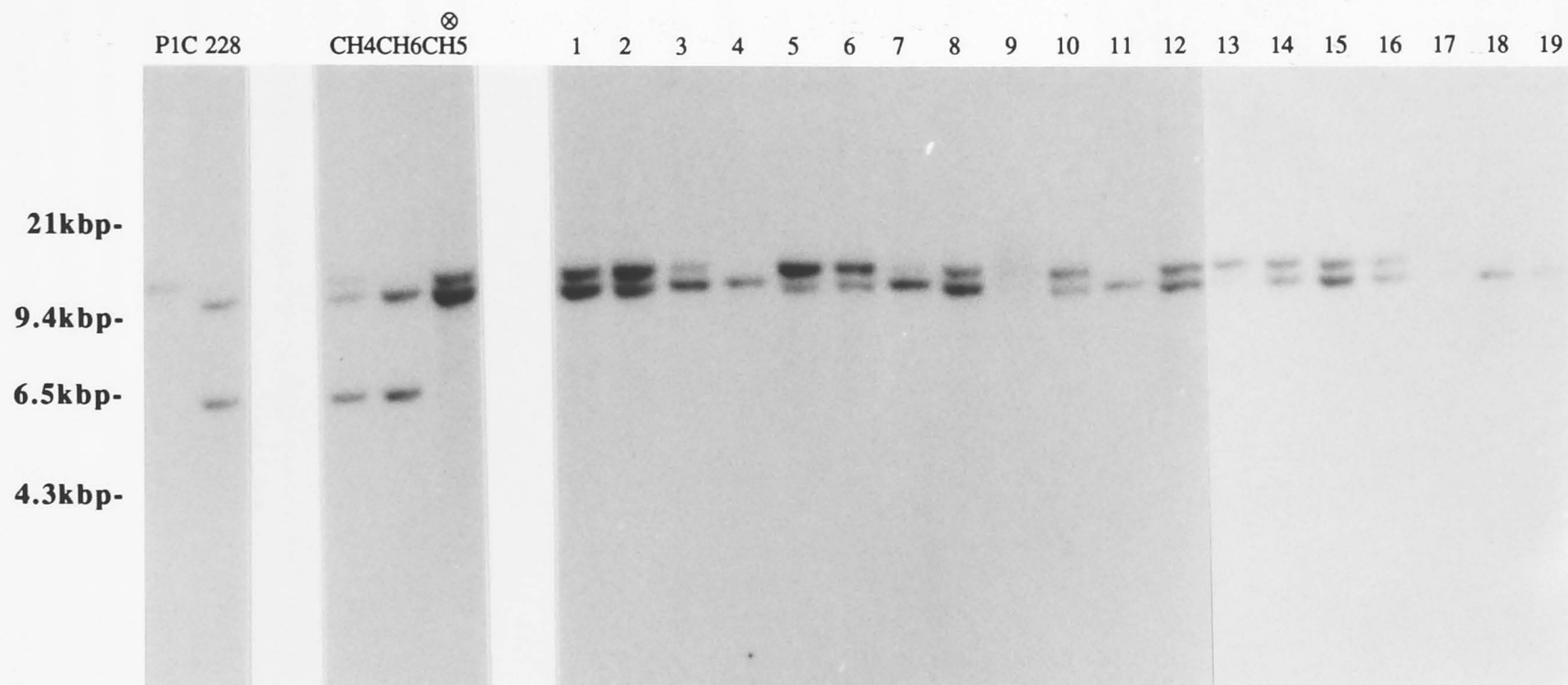
the P1C parental type, the heterozygote, and the 228 parental type ($\chi^2=1.0$, 0.5<p<0.7).

No. of bands per digest	% of digests	
	P. sorghi	M. lini
1	14	51
2	59	38
3	15	9
> 3	12	2

Table 5.2: Summary of the hybridization patterns of random cDNA probes from M. lini and P. sorghi. The number of hybridizing bands in each digest from all probe/DNA/enzyme combinations were grouped into 1, 2, 3, and > 3 bands per digest, and expressed as a percentage of the total number of probe/enzyme combinations. Data from M. lini was collected from 22 probes, 5 DNA samples cut with 3 restriction enzymes. P. sorghi DNA extracted from two races was analysed with 24 probes and in most cases with 6 restriction enzymes (refer to chapter 3).

	LMS	271	228	P1C
LMS	-	86	82	82
271		-	55	55
228			-	50
P1C				-

Table 5.3: The number of random cDNA sequences which detected RFLPs between races of M. lini, expressed as a percentage of the total number of probes tested.

The inheritance of bands in the other two RFLPs could not be explained in this manner. The pMLc22 probe hybridized to two bands which were different in both parental races (Figure 5.2b). Both bands were inherited from both parents in all three hybrids and in the F_2 progeny each parental type segregated together. The observed frequencies of the P1C parental type, the heterozygote, and the 228 parental type did not differ significantly from a 1:2:1 ratio ($\chi^2=2.4$, 0.2<p<0.3).

The inheritance of the RFLP bands detected by the last probe tested, pMLc33, revealed a further complication (Figure 5.2c). The pMLc33 probe hybridized to one band in P1C and two bands in 228. In the CH4 hybrid all three parental bands were found and yet in CH6 only the 228 bands were transmitted. In the CH5 hybrid only one of the 228 bands was inherited with the P1C band, both of which segregated in a 1:2:1 ratio in the F_2 progeny ($\chi^2=3.8$, 0.2<p<0.3).

The data collected from the transmission of all the parental bands that hybridized to the 7 probes that were tested, indicated that the level of heterozygosity at RFLP loci in *M. lini* is 0.55. That is, 55% of RFLP loci are heterozygous.

The segregation data of 9 unlinked avirulence genes was combined with the segregation data of the RFLP loci and entered into the Mapmaker program (Lander *et al*, 1987). The results indicated that there was no detectable linkage between any of the markers with the possible exception of pMLc30 and Avr_{P2}.

5.5 : Discussion

The high frequency with which clones selected from a cDNA library of *M. lini* detected RFLPs between races which infect cultivated and Australian native flax, indicated their potential usefulness in genetic studies of *M. lini*.

The level of multiple restriction bands observed in *M. lini* was not as high as had previously been reported in *P. sorghi*. Whereas 86% of all restriction/probe combinations gave multiple bands in *P. sorghi*, only 49% of the *M. lini* patterns contained multiple bands. In chapter 3 it was argued that the multiple bands in *P. sorghi* were the consequence of some form of genetic heterogeneity. While heterozygosity of alleles present in the different nuclei of the dikaryon could explain some of the complexity, some form of gene or sequence duplication was required to explain more complex patterns. Although the level of RFLP complexity was lower in *M. lini*, observation of the transmission of multiple bands in a sexual cross could provide some idea of the relative contribution of heterozygosity versus gene or sequence duplication in generating multiple banding patterns. Using 22 random cDNA clones as probes, 11 RFLPs were detected between parents of a genetic cross previously performed by Lawrence *et al* (1981b). The inheritance and segregation of 7 of these RFLP loci was observed in 3 hybrids and 19 F_2 progeny from one of these hybrids.

At the locus defined by the clone pMLc8, RFLP bands segregated as alleles at a single locus. Each parent had 2 different bands one of which was transmitted to a hybrid and which then segregated in a 1:2:1 ratio in the F_2 progeny. Therefore, it could be concluded that each parent was heterozygous for different alleles at this RFLP locus.

At the pMLc22 locus both parents are presumably homozygous for alleles specifying a two banded pattern. Both bands from each parent were transmitted together to each hybrid and then segregated together in the F_2 progeny. If it were a single locus then an internal restriction site in either the coding region or in an intron would result in two bands hybridizing to the probe. The first of these possibilities can be eliminated as the pMLc22 cDNA clone did not contain a *Eco RI* restriction site (data not shown). It is not possible to distinguish between a restriction site within an intron or a tandem and tightly linked duplication of the pMLc22 sequence.

In all cases the inheritance of the RFLP loci in the F_2 progeny could be explained by the segregation of alleles at a single locus or closely linked loci. However, in the case of pMLc33 the complete lack of transmission of bands was observed in one of the 3 hybrids. One

explanation is the transmission of a null allele from the P1C parent. However, a further complication comes from the fact that the two bands from 228 are both transmitted to two of the hybrids, but only one of the bands is transmitted to the other hybrid. Therefore, the two 228 bands are likely to be derived from separate and unlinked or loosely linked loci. One 228 band was transmitted to all the hybrids and then segregated in a 1:2:1 ratio with the P1C band in the F_2 progeny. This suggested that the absence of the other 228 band in CH5 was due to the transmission of a null allele at this locus. It is unlikely that contamination of DNA samples could explain the aberrant nature of this inheritance as the filters used for this hybridization had been used for the other probes in which the hybrids behaved quite normally.

Null alleles are occasionally observed in genetic markers defined by isozymes and can be explained simply by loss of gene function. However, a null allele for a DNA sequence suggests some form of small or large scale deletion. Hartley and Williams (1971) argue that non-disjunction of chromosomes at mitosis may generate recombinant dikaryons in flax rust. If a homologue of the chromosome containing the pMLc33 gene was missing in P1C this could explain the aberrant inheritance of this marker. As the other RFLP bands were inherited normally it would have to be assumed that pMLc33 is the only gene out of the seven located on this missing chromosome or chromosome region. It should be pointed out that the segregation of null alleles in the form of chromosomes or chromosome regions is only one explanation of the observed aberrant inheritance. It is possible that these results are indicative of another aspect of the rust genetic system that has not been discovered or considered as yet.

The results of the linkage study indicated that the 15 loci segregating in the F_2 progeny appear to be unlinked with the possible exception of the pMLc30 and Avr_{P2}. The length of the genetic maps of *Aspergillus nidulans* and *Neurospora crassa* are 1800 and 620cM respectively (Caten, 1987). As the genome size of *M. lini* is likely to be similar or even larger than that of *A. nidulans* or *N. crassa*, and assuming the markers are evenly spread throughout the *M. lini* genome, then the average distance between the loci would be 80cM or greater. Thus the chance of detecting linkage is somewhat unlikely, and this is reduced even further by the small F_2 population size tested in this study.

The sexual transmission of multiple bands in *M. lini* indicates that 55% of RFLP loci are heterozygous. This level of heterozygosity is similar to that measured at avirulence loci (Johnson, 1954; Wilcoxson and Paharia, 1958; Luig and Watson, 1961; Samborski and Dyck, 1968,1976; Haggag *et al*, 1973), however, only 15 to 30% of loci were found to be heterozygous using isozyme markers (Burdon *et al*, 1986). If genetic heterozygosity can explain the incidence of multiple bands in other rust fungi such as *P. sorghi* where the hybridization patterns contain on average a greater number of bands than in *M. lini*, then this suggests that the genomes of rust fungi may be highly heterozygous. The origin of high levels of heterozygosity is unknown, however it is possible that current isolates of rust fungi may be highly successful hybrids between genetically different strains. In the event of limited sexual crossing within the hybrid, or some form of fixed heterozygosity, the genetically dissimilar and highly heterozygous state would remain constant in the population.

56

    This report provides further demonstration of the usefulness of RFLP markers in rust genetics. The results of this chapter provide evidence that the flax rust genome appears to be highly heterozygous, and other types of genetic changes, including deletion of sequences, are occurring in the flax rust genome. These results, in relation to the previous chapters, indicate that high levels of sequence heterogeneity appear to exist between the two nuclei of the dikaryon in rust fungi. Future work will be directed at determining the cause and consequences of this heterogeneity to natural variation in rust fungi, particularly by means of somatic recombination. Furthermore, RFLP markers may be used to construct a genetic map of a rust genome. Such a map would provide a basis from which the cloning of specific genes could be achieved by chromosome walking from closely linked markers (Young, 1990).

Chapter 6 : A transposon-like element in the maize rust fungus, *Puccinia sorghi*.

6.1 : Abstract

A 496bp sequence is responsible for an RFLP between two races of the maize rust fungus, *Puccinia sorghi*. The sequence, called the *P. sorghi* repeat element or 'PSR', is highly repeated in the genome of both races, but is absent from wheat stem rust (*P. graminis tritici*) and flax rust (*Melampsora lini*). The PSR element contains two 64bp direct repeats and hybridizes to a 1.4kb RNA. Longer sequences homologous to PSR have been observed in *P. sorghi*. One of these has been cloned and sequenced and found to have terminal inverted repeats. While these PSR related sequences have many features common to eukaryotic transposable elements, their transposition has not been demonstrated to occur in the *P. sorghi* genome.

6.2 : Introduction

In chapter 3 the use of randomly selected cDNA probes to detect RFLPs between two races of the maize rust, *Puccinia sorghi* Schw., was reported. One of the races was isolated from Australia and the other from the USA, and they both differed at 8 avirulence loci (refer to Table 3.1, chapter 3). A number of RFLPs were detected between the two races and in most cases the differences could be explained by the modification of restriction sites causing a change in fragment length. However one RFLP, detected by the cDNA clone pPSOc24, could not be explained in this way. In all of the 5 restriction digests of genomic DNA from race 1 and race 2 that were tested, this probe hybridized to a race 2 band that was 500bp longer than a band in race 1. This RFLP could be explained by the deletion of a 500bp sequence from race 1, or the insertion of a 500bp sequence in race 2. Events such as deletion and insertion are indicative of the movement of transposable elements.

Transposable elements have been described in a variety of organisms ranging from prokaryotes to yeasts and higher eukaryotes (reviewed by Berg and Howe, 1989). In filamentous fungi a number of sequences that resemble transposable elements have been described, however as yet, none have been shown to move. These include the Tad element in *Neurospora crassa* (Kinsey and Helber, 1989; Kinsey, 1990), the *repa* sequence in *Podospora anserina* (Deleu *et al*, 1990) and the MGR sequence in the rice blast fungus *Magnaporthe grisea* (Hamer *et al*, 1989). In *Ascobolus*, elements have been found that behave as classical transposons in genetic tests, none of which have been characterized at the molecular level (Nicolas *et al*, 1987).

This chapter provides a molecular explanation for an RFLP observed between two races of *P. sorghi*. This RFLP was found to be caused by the presence of a 496bp sequence in the race 2 genome. This sequence, designated the PSR element (*P. sorghi*

58

repeat), is repeated many times in the genome of both races of *P. sorghi*, and has a number
of characteristics similar to transposons described in other eukaryotes.

6.3 : Materials and Methods
<u>Rust races</u>

Race 1 and race 2 were maintained as pure cultures on maize plants with resistance
genes capable of differentiating between the two races. Inoculation procedures,
urediospore collection and germination, and total DNA and RNA extraction methods have
been described in chapter 2 and by Pryor and Boelen (1987).
<u>Genomic cloning</u>

To determine the molecular basis of the RFLP (Figure 1), two *Bam HI* fragments
showing the size difference were cloned (marked with an asterisk in Figure 1). Attempts to
clone these fragments directly from genomic digests were unsuccessful, so genomic
libraries from both races containing the large *Eco RI* fragment (shown by an arrow in
Figure 6.1) were constructed. Genomic DNA (20μg) from both races was cut to
completion with *Eco RI* (Pharmacia) and size fractionated on a 5-20% sucrose gradient.
Fractions containing fragments 10kbp or greater in size were pooled and ligated to *Eco RI*
arms of λ EMBL 4 (Promega), and transfected into the *E. coli* strain NW2 (Loenen *et al*,
1987). Positive plaques were detected by hybridization of phage lifts to the *P. sorghi*
cDNA clone, pPSOc24. Phage from these plaques were amplified and the DNA extracted.
The phage DNAs were digested with *Bam HI* and the two indicated RFLP fragments were
isolated and sub-cloned into the pGEM 3Zf(+) bacterial plasmid vector (Promega). All the
methods described were conducted according to Sambrook *et al* (1989).
<u>Analysis of the *Bam HI* fragments from race 1 and race 2</u>

To identify the exact region of the RFLP between race 1 and race 2, the two *Bam HI*
sub-clones pBR1 and pBR2 were mapped for restriction sites. The restriction maps
revealed a unique *Xba I* site in race 2, and the two fragments which flanked this site were
sub-cloned and sequenced. The corresponding region from the race 1 genome was also
sub-cloned and sequenced. The fragments from the *Sph I* site to the unique *Xba I* site in
race 2 and the corresponding *Sph I/Xba I* fragment from race 1 were used as probes to
Southern and northern blots using the methods described in chapter 3. Nucleotide
sequencing was done from single stranded DNA templates using fluorescent primers and an
auto-sequencing machine (Applied Biosystems Incorp.).
<u>Polymerase chain reaction</u> (PCR)

Oligonucleotides were made using a Applied Biosystems DNA synthesizer and
purified on a 12% acrylamide gel according to Sambrook *et al* (1989). The sequences of
the primers used were: primer 1 (21bp), 5' TCACTTTTCTAGTCTTCTCTG 3' and primer
2 (21bp), 5' GCTGAAGAATCAAGAGCACAG 3'. Reactions were performed using a
GeneAmp kit (Perkin-Elmer Cetus), with 0.5U of *Taq I* polymerase and 5ng of control
template (pBR2) or 100ng of total genomic *P. sorghi* DNA. The PCR products were

treated briefly with S1 nuclease (Sigma, 0.01U for 10 minutes at 37°C), phosphorylated using T4 polynucleotide kinase (Pharmacia), ligated into the *Sma I* site of pGEM 3Zf(+) (Promega) and transformed into *E. coli* cells (strain *JPA101*).

6.4 : Results

The RFLP between race 1 and race 2 detected by the pPSOc24 cDNA clone is due to the presence of a 496bp sequence in race 2 An autoradiograph of the pPSOc24 clone hybridized to blots of race 1 and race 2 genomic DNA is shown in Figure 6.1. For each restriction enzyme used, an RFLP equivalent to a difference in length of approximately 500bp was detected between race 1 and race 2. The *Bam HI* fragments from both races which differed by 500bp (shown by an asterisk) were selected for cloning according to the strategy described in Materials and Methods. The restriction digestion patterns and the maps of these clones (pBR1 and pBR2 from race 1 and race 2 respectively) are shown in Figures 6.2 and 6.3. The locations of the restriction sites were identical in the two clones, except for a unique *Xba I* site in race 2 (circled in Figure 6.3). The *Sph I/Xba I* and *Xba I/Xba I* fragments which flanked the unique *Xba I* site, and the corresponding region from race 1 (*Sph I/Xba I* fragment) were cloned and sequenced according to the strategy shown in Figure 6.4. The sequence data revealed that the RFLP between race 1 and race 2 was due to the presence of a 496bp sequence in race 2. The region flanking this sequence was virtually identical between the two races except for 4 nucleotide changes out of 595bp. The 496bp sequence was flanked by a 5bp duplication which was also found in race 1 at the point at which the 496bp sequence in race 2 commenced. Two 64bp direct repeats were found within the 496bp sequence, one of which was terminal and the other located in the middle. The complete nucleotide sequence and a summary of the structural features of the 496bp sequence are shown in Figures 6.5 and 6.6.

The 496bp sequence is highly repeated and specific to the *P. sorghi* genome Probes from the 496bp sequence and the corresponding region in race 1 were hybridized to Southern blots of race 1 and race 2 genomic DNA. The location of the probes and the resulting autoradiograph are shown in Figure 6.3 and 6.7. The race 1 probe hybridized to the same genomic fragments as the pPSOc24 cDNA sequence which was located upstream and adjacent to the *Bam HI* site. In contrast, the race 2 probe hybridized to many other genomic fragments from race 1 and race 2, indicating that the 496bp sequence is highly repeated in the *P. sorghi* genome. Furthermore, probes specific for two regions of the 496bp sequence, one outside the direct repeats and the other between the direct repeats, gave identical highly repeated patterns when hybridized to genomic DNA from *P. sorghi*. This indicates that the whole of the 496bp sequence is repeated in the genome (data not shown).

The repeated sequence defined by the *Sph I/Xba I* fragment from race 2 was hybridized to a Southern blot of genomic DNA from *P. graminis tritici* (wheat stem rust), *Melampsora lini* (flax rust) and race 1 of *P. sorghi*. No detectable homology was observed between this probe and genomic DNA from *P. graminis* and *M. lini* (Figure 6.8). On the

Figure 6.1. Autoradiograph of a Southern blot containing genomic DNA from race 1 and race 2 of *P. sorghi*, hybridized to the cDNA clone pPSOc24. DNA was cut with 5 different restriction enzymes and race 1 and race 2 DNA of each digest were run in adjacent lanes, with the race 1 digest run first. The order of the restriction digests is as follows; lanes 1 and 2, *Eco RI*; lanes 3 and 4, *Hind III*; lanes 5 and 6, *Bam HI*; lanes 7 and 8, *Bgl II*; lanes 9 and 10, *Pst I*; lanes 11 and 12, *Sac I*. The arrow indicates the *Eco RI* bands from race 1 and 2 which were cloned into the λ EMBL 4 vector (Promega). The race 2 DNA cut with *Eco RI* was a partial digest, and subsequent experiments have confirmed that a single band of similar length to that in race 1 hybridizes to the pPSOc24 probe. The two *Bam HI* fragments (indicated by an asterisk) were sub-cloned into the pGEM 3Zf(+) plasmid.
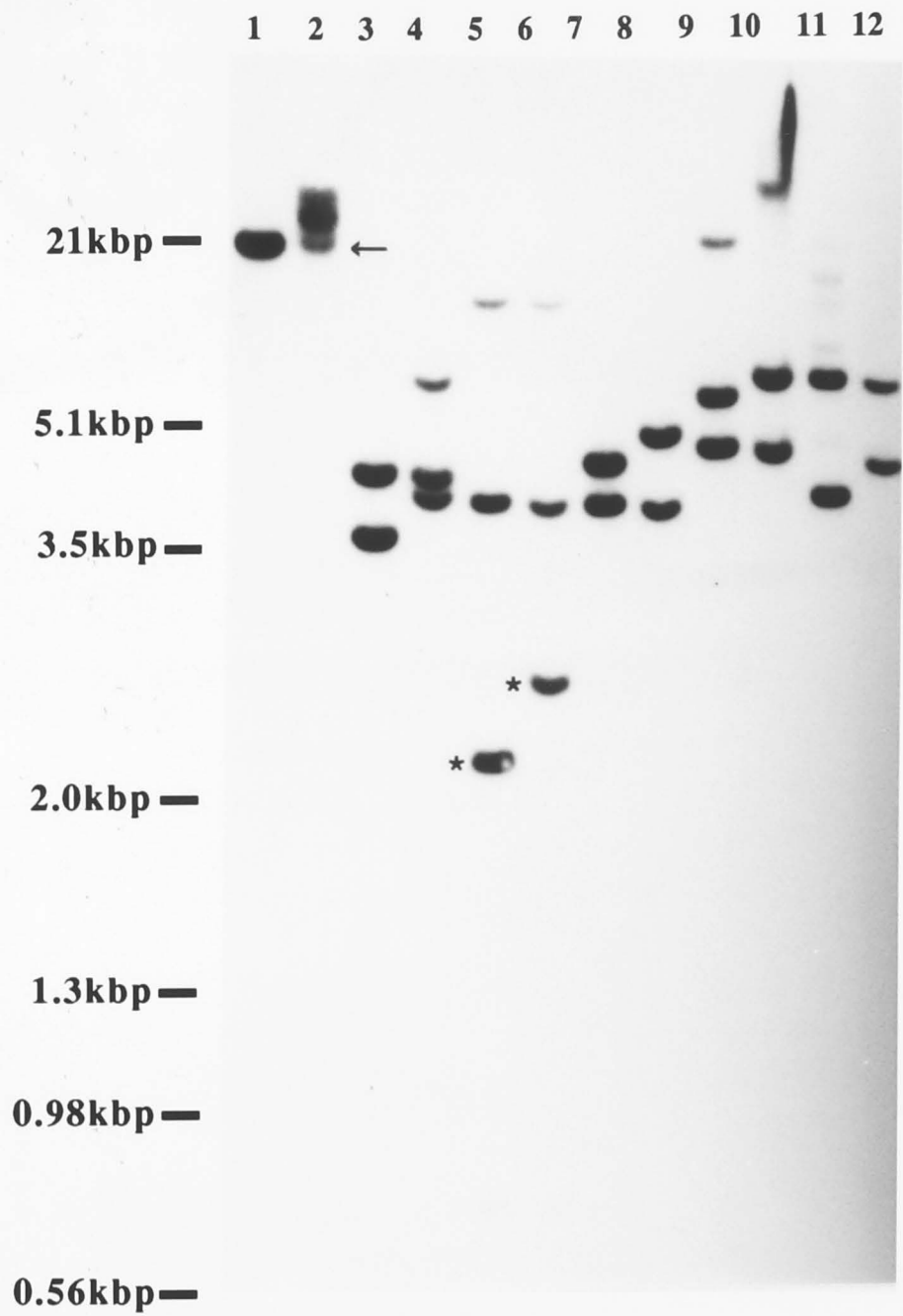
Figure 6.2. An ethidium bromide stained agarose gel of plasmid DNA from pBR1 and pBR2 cut with a number of restriction enzymes. Lane 1 contains *Eco RI* digested DNA of SPP 1 used as a size marker. Lane 2 is a *Bam HI* digest which cuts out the 2.3kbp pBR1 fragment and the 2.8kbp pBR2 fragment. Lanes 3-8 are digests of *Hinc II*, *Sph I*, *Xba I*, *Hinc II+Sph I*, *Hinc II+Xba I* and *Sph I+Xba I* respectively.

pBR1

1 2 3 4 5 6 7 8

8.51kbp—
4.84kbp—
2.81kbp—
1.86kbp—
1.16kbp—
0.72kbp—
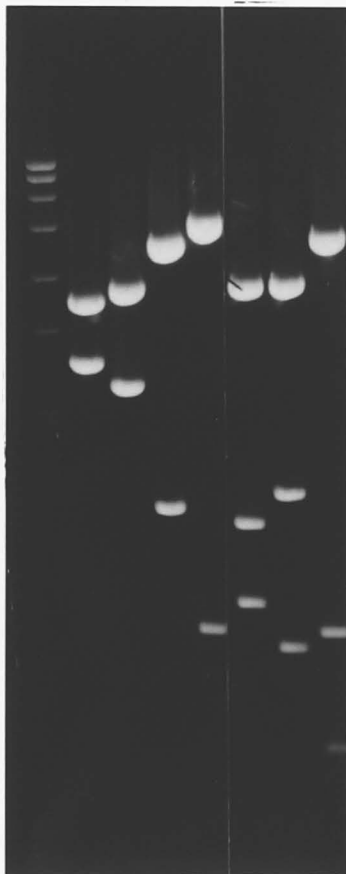
pBR2

1 2 3 4 5 6 7 8

8.51kbp—
4.84kbp—
2.81kbp—
1.86kbp—
1.16kbp—
0.72kbp—

Figure 6.3. A restriction map of the two *Bam HI* fragments from race 1 (pBR1) and race 2 (pBR2). The circled *Xba I* site in the race 2 clone is a site unique to race 2. The shaded bars, labelled B and C, represent the two fragments from either race which were used as probes to Southern and northern blots of race 1 and race 2 *P. sorghi*. The results of these hybridization are shown in Figures 6.7 and 6.9.

pBR1 (Race 1, 2.3kbp *Bam HI* genomic fragment)

Bam HI *Hinc II* *Sph I* *Xba I* *Bam HI*

B
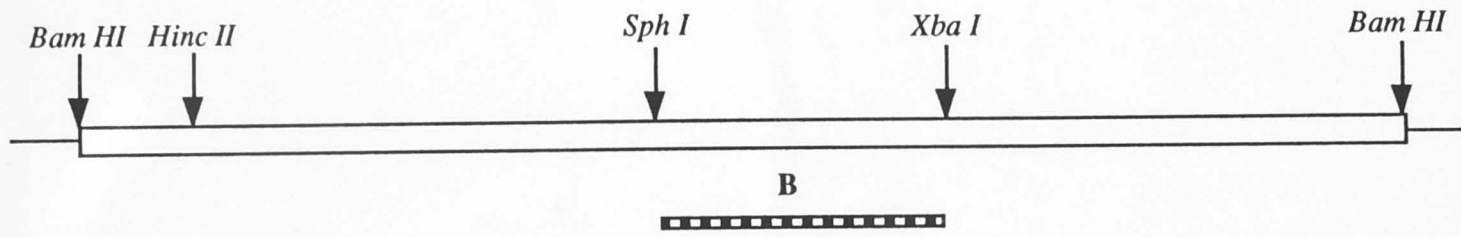
pBR2 (Race 2, 2.8kbp *Bam HI* genomic fragment)
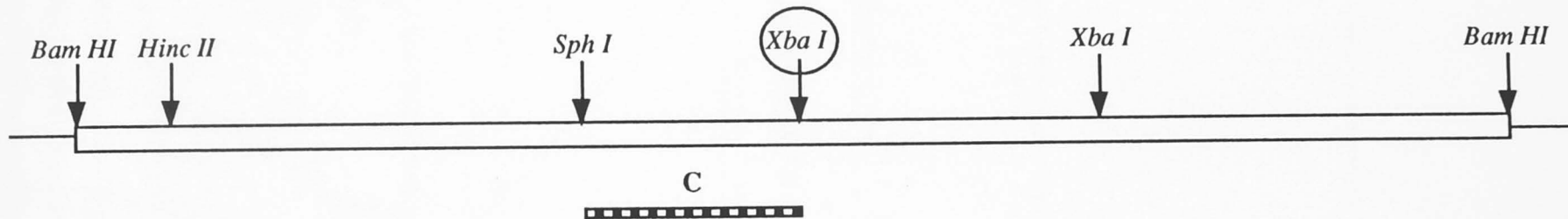
Bam HI *Hinc II* *Sph I* *Xba I* *Xba I* *Bam HI*

C

0 0.5kbp 1.0kbp

Figure 6.4. Sequencing strategy of the region flanking the *Xba I* site unique to race 2. The arrows indicate the direction and region of DNA that was sequenced. The shaded regions indicate homologous sequences between race 1 and race 2 genomic DNA, except for 4 base substitutions. The unshaded region is a 496bp sequence present in the race 2 genome that was absent from race 1.

Race 1

Sph I   Xba I

5'   3'

Race 2

5'   3'

Sph I   Pvu II   Xba I   Pvu II   Xba I

0   0.1   0.2   0.3   0.4   0.5   0.6   0.7   0.8   0.9   1.0 k b p
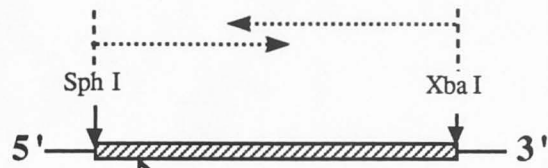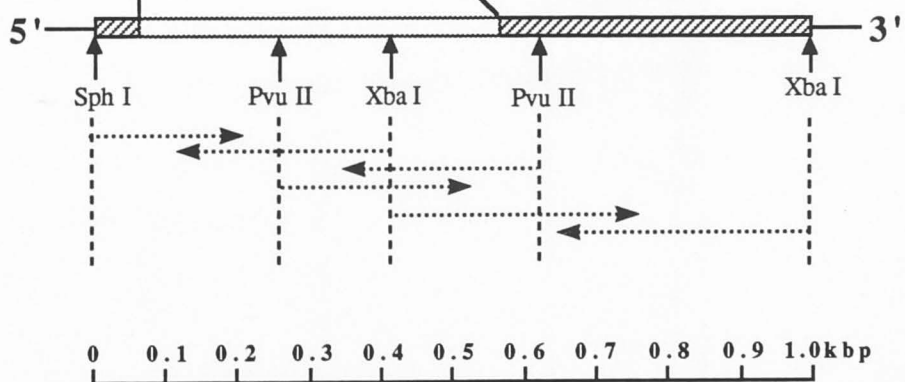
Figure 6.5. Summary of the structure of the PSR element showing two 64bp exact direct repeats (sequence I and II), and a terminal 5bp duplication (sequence III). The 5bp sequence was also found at the point of insertion in the race 1 genome.

PSR *Puccinia sorghi* repeat sequence
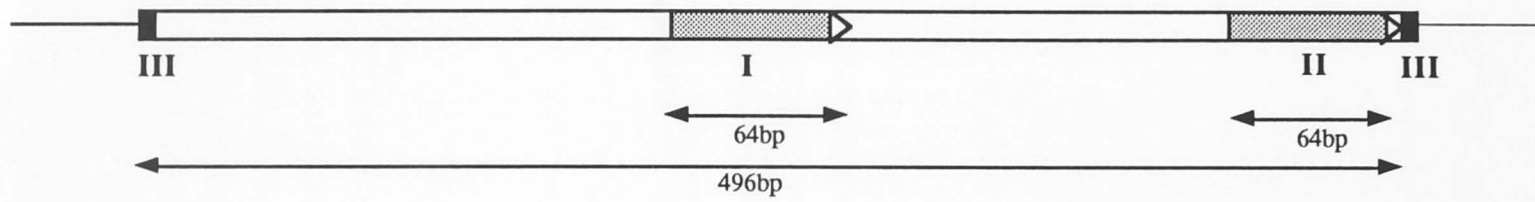
sequence I and II : CTGTGCTCTTGATTCTTCAGCTCACTTTTCTAGTCTTCTCTGGCCTTAGGCCATTATTTCTTCA

sequence III : CTTGA

Figure 6.6. The complete nucleotide sequence of the *Sph I/Xba I* fragment from race 1 and the *Sph I/Xba I* and *Xba I/Xba I* fragments from race 2. The correct arrangement of the two race 2 sequence was obtained from the sequence of a *Pvu II/Pvu II* fragment which bridged the two sequences. The long stretches of sequence which are highlighted are the two 64bp direct repeats and the smaller boxed sequence is a 5bp terminal duplication.

Race 1

Sph I

GCATGCAAGC AGACATTAGC CCACTTCACA TTGGAACCTT TAACACAGGT

TTTCACACCT TGAAACATGA AATTCCCAAC AAGTTTTTTG GTCATCAGAT

TCAGCCGTCA TCATGCAAAA ATCCTCACAA CAGTGCCCGC CACGCTAATA

GTAGCATTAG TATAAAGTAA ACCCCGCTGA CTGCTAACAA CTATTGTCAC

AGCTGTTAAT GGTTTTGACT AACGCTAAGG AACAAGCCAT AAAAGGCCTT

GTTACGCGAT GCTACACTTC TTTGTTATGA AAAATAGCTG TTACAAGAGT

TGTAGCATAG CATACACACG TAAACCGCCT GTTGAGGCCC TGTAGCGCCG

CTGATACTAC AGCATTTGTG CAAAAAAACC CGCTACACTA CACTTTTTGG

CCCTGTAGCG TAGGCCCACC ACTCCTTCTC AGTTGTTTAA AAAAGGAAAA

TTTGAGCTGG TAAAATGTCT TCTGACTTGA ATAAAATTCT CTAGA

Xba I

Race 2

Sph I

GCATGCAAGC AGACATTAGC CCACTTCACA TTGGAACCTT TAACACAGGT

TTTCACACCT TGATGTTGGA ATTGTGACTG CCCCCAAAGA GGGCTTCTTC

CTTTCTTTTT CCATTCCTT CTTTTCTTTT CTTCTGCACC TTAGGCCCTC

AACTCAGGCC CCTTTTCCAC GTTGTATTTC TTCTTGTCCC CATCAGAAAT

ACAACCTCAC TTAGTTCTGA GCCCGTAGCA CTGAGACATG CTCCTCTGGA

GTCCCTCTCA GCTGTTGTCC TAAGTCCCTG AGCATCCTGT GCTCTTGATT

CTTCAGCTCA CTTTTCTAGT CTTCTCTGGC CTTAGGCCAT TATTTCTTCA

Xba I

TGGTTAAAAA CCTTGCTCAA CCTCCTTCGC TAACCACTGA AATCTAGATT

CTCAACCTTC TTCAGTAACT ACAATCTAAA TCCTCAATCT TTAAACCTTT

CTCATCTACC CTCCCTTCGA TTCGGCCACA CCATTCAAAT CCTCACTGTG

CTCTTGATTC TTCAGCTCAC TTTTCTAGTC TTCTCTGGCC TTAGGCCATT

ATTTCTTCAC TTGAAACATG AAAATTCCCAA CAGGTTTTTT GGTCATCAGA

TTCAGCCGTC ATCATGCAAA AATCCTCACA ACAGTGCCGC TACGCTAATA

GTAGCATTAG TATAAGGTAA ACCGCTGACT GCTAACAACT ATTGTCACAG

CTGTTAATGG TTTTGAGCTA ACGCTAAGGA ACAAGCCATA AAAGGCCTTG

TTACGCGATG CTACACTTCT TTGTTATGAA AAATAGCTGT TACAAGAGTT

GTAGCATAGC ATAACACACG TAAACGCCTG GTTAGGCCCT GTACGCCGCT

AATACTACAG CATTTGTGCA AAAAAACCCG CTACACTACA CTTTTTGGCC

CTGTAGCGTA GGCCCACCAC TCCTTCTCAG TTGTTTAAAA AAGGAAAATT

TGAGCTGGTA AAATGTCTTC TGACTTGAAT AAAATTCTCT AGA

Xba I

Figure 6.7. Autoradiographs of a Southern blot of genomic DNA from race 1 and race 2 of *P. sorghi*, hybridized to probe B and then re-hybridized to probe C (see Figure 6.3). The lanes contain the following DNA digests; lane 1, race 1/*Bam HI*; lane 2, race 2/*Bam HI*; lane 3, race 1/*Hind III*; lane 4, race 2/*Hind III*. Several RFLPs can be seen between race 1 and race 2 detected by probe C.
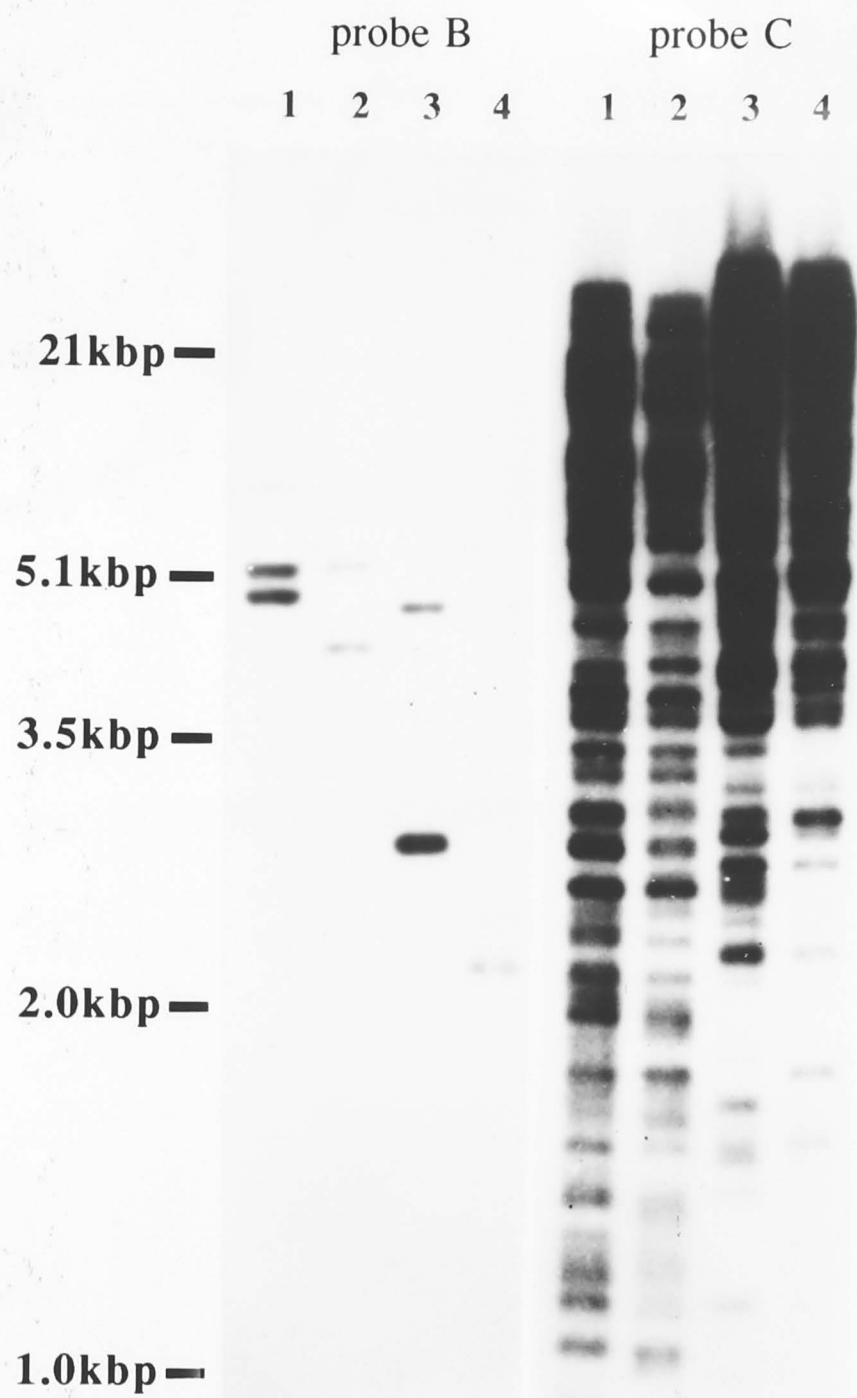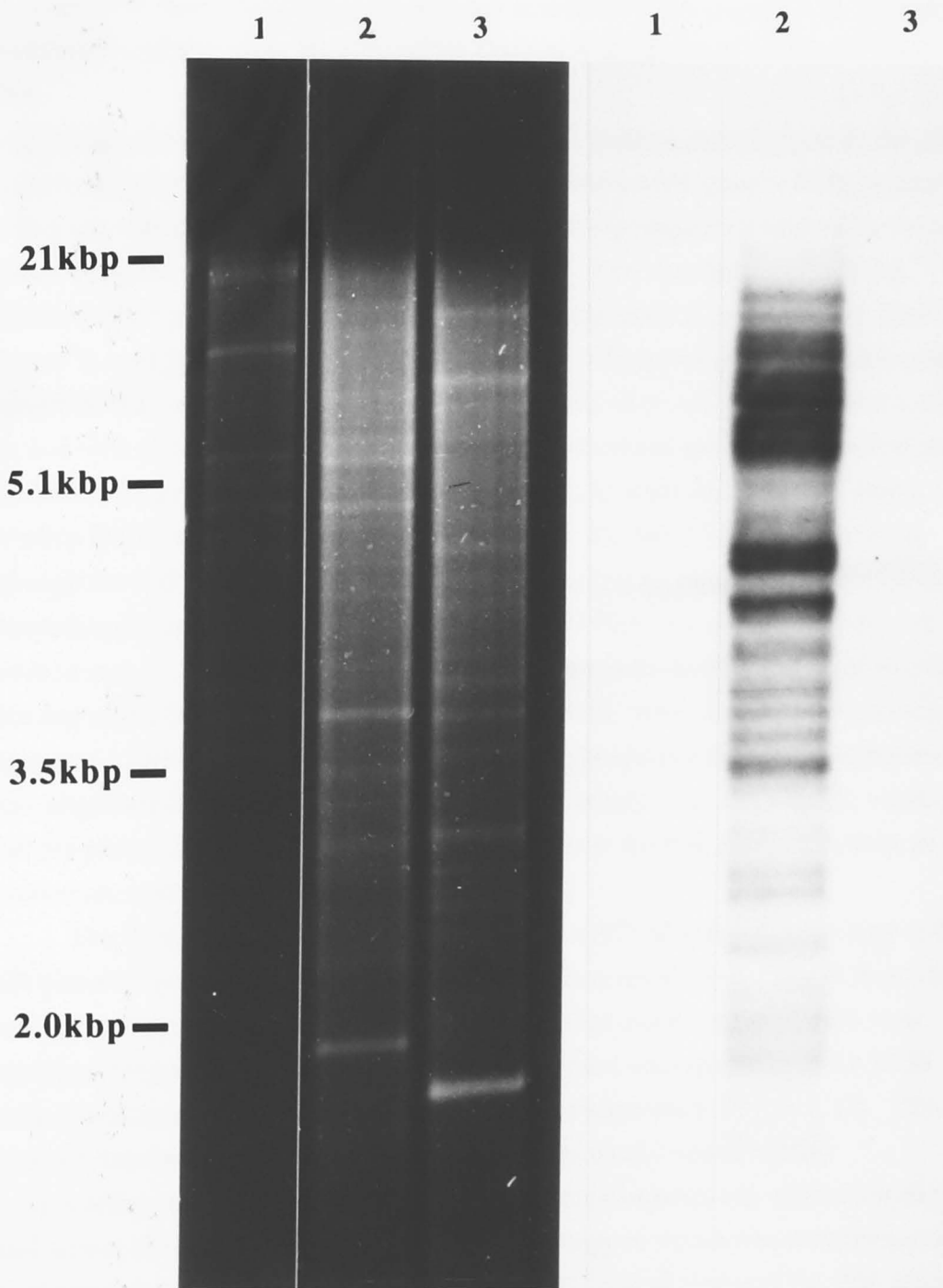
Figure 6.8. An ethidium bromide stained agarose gel and the autoradiograph of the corresponding Southern blot, containing DNA from *P. graminis tritici* (lane 1), *P. sorghi* (lane 2) and *Melampsora lini* (lane 3), all cut with *Eco RI*, and hybridized to probe C (see Figure 6.3).

60

basis of these results the 496bp sequence was termed the *Puccinia sorghi* repeat element
(PSR).

<u>The PSR element hybridizes to a 1.4kb RNA</u> The PSR element defined by the *Sph I/Xba I*
fragment from race 2 hybridized to a 1.4kb RNA on a northern blot, whereas the equivalent
fragment from race 1 did not hybridize to any detectable RNA (Figure 6.9). Clearly, from a
consideration of size alone the 496bp PSR element is incapable of coding for the 1.4kb
RNA.

<u>Longer sequences with PSR homology and terminal inverted repeats occur in the race 2</u>
<u>genome</u> In order to recover larger genomic sequences homologous to PSR, primers
made from within the 64bp repeats were used to amplify sequences flanked by the direct
repeats using the polymerase chain reaction (PCR). Two non-overlapping 21bp
oligonucleotide primers were synthesized which corresponded to nucleotides 22→42
(primer 1), and 21→0 (primer 2) of the 64bp repeat. The position and orientation of the
primers is shown in Figure 6.10. When both primers were used in a polymerase chain
reaction with pBR2 plasmid DNA (as a positive control) and genomic DNA from race 1
and race 2, the expected 209bp fragment was amplified from the pBR2 and race 2
template DNA, but not from race 1 DNA (Figure 6.11, lanes 3, 6, 9). Therefore,
although the PSR sequences are present in race 1 (Southern blot), it is likely that the
orientation and arrangement of the repeats within the PSR element in race 2 are not
found in race 1. When primer 1 alone was used there were no PCR products amplified
with any of the DNAs. When primer 2 was used alone there were no PCR products
using race 1 DNA, however using race 2 DNA as a template a number of sequences
were amplified (Figure 6.11, lane 8). The fact that primer 1 did not amplify sequences
whereas primer 2 did indicates that the direct repeats in the PSR element exist in an
inverted orientation in the genome.

The PCR products shown in lane 8 (Figure 6.11) all showed homology to the
PSR element by Southern blot and hybridization (data not shown). The PCR products
were cloned into the *Sma I* site of pGEM 3Zf(+). One clone, called pPSRB, was
sequenced (Figure 6.12). It contained a 1.05kbp insert corresponding to the PCR
product indicated by an asterisk in Figure 6.11 was sequenced (Figure 6.12). This
sequence data confirmed that the insert contained terminal inverted repeats
corresponding to the primer 2 sequence. The sequence immediately adjacent to the left
hand primer of pPSRB showed almost exact homology to the reverse complement of
the sequence immediately adjacent to primer 2 in the internal repeat of the PSR element
(shown in Figure 6.13). This indicates that the internal direct repeat of the PSR element
could have originated from an inversion event.

The remainder of pPSRB showed no homology to the PSR element. A
fragment from the internal *Bgl II* site to the *Bam HI* site in the polylinker of the pGEM
3Zf(+) plasmid was sub-cloned. This fragment was used as a probe to genomic blots
of race 1 and race 2 DNA. The hybridization pattern of this probe (shown in Figure

Figure 6.9. Autoradiographs of two northern blots of total RNA from race 1 (lane 1) and race 2 (lane 2) of *P. sorghi*, hybridized to probes B and C (see Figure 6.3).

probe B   probe C
1   2   1   2

9.5kbp —
7.5kbp —
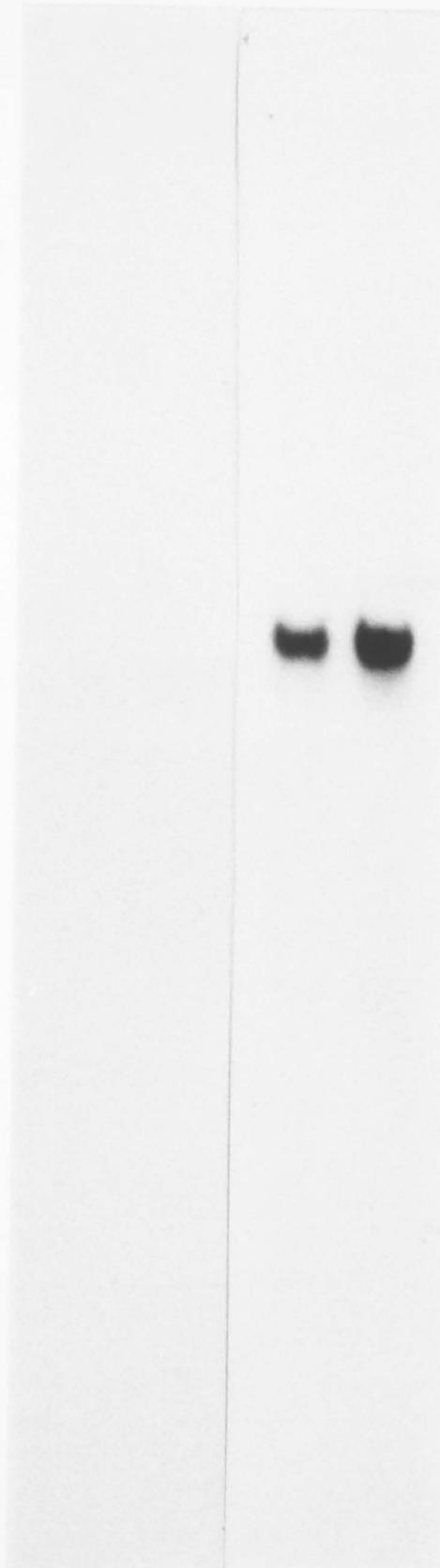
4.4kbp —

2.4kbp —

1.35kbp —

0.24kbp —

Figure 6.10. Map of the PSR element showing the orientation and location of two oligonucleotide primers (21bp) homologous to the direct repeats of the PSR element. Using both primers and the genomic clone (pBR2) as a template DNA, a 209bp fragment would be amplified by the polymerase chain reaction.

PSR *Puccinia sorghi* repeat sequence
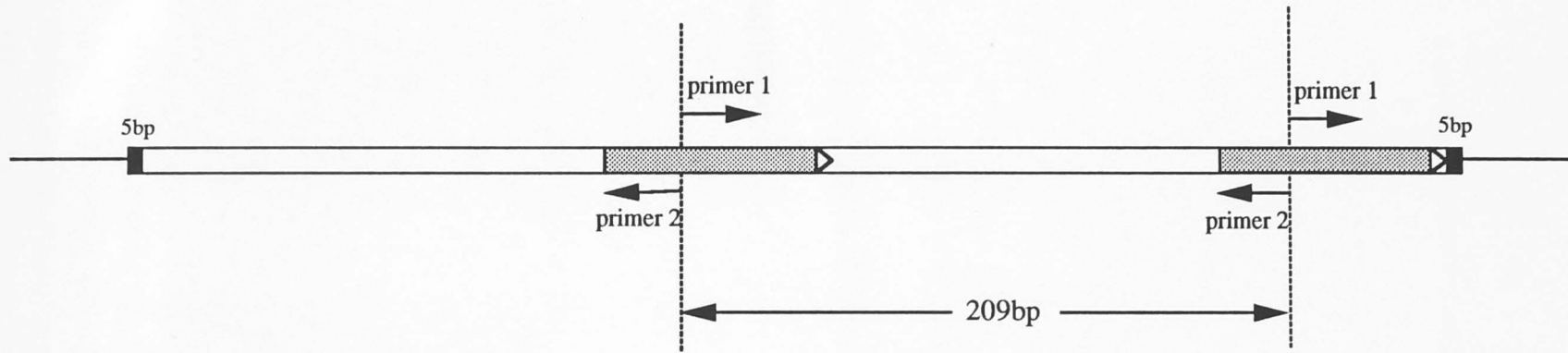
Figure 6.11. An ethidium bromide stained agarose gel showing the size of PCR products made using primers 1 and 2. Lane 1, SPP 1 cut with *Eco RI* (Bresatec); lane 2, reaction containing 100μM of each primer with no template DNA added; lane 3, both primers with 5ng of pBR2 DNA (linearised with *Eco RI*); lane 4, primer 1 with 100ng of race 1 genomic DNA; lane 5, primer 2 with 100ng of race 1 genomic DNA; lane 6, both primers with 100ng of race 1 genomic DNA; lane 7, primer 1 with 100ng of race 2 genomic DNA; lane 8, primer 2 with 100ng of race 2 genomic DNA; lane 9, both primers with 100ng of race 2 genomic DNA. The smear of staining material at the bottom of the gel which is particularly evident in lane 2 (indicated by the arrow), is unincorporated primers. The asterisk indicates the 1.05kbp PCR product from lane 8 that was cloned and sequenced (pPSRB).
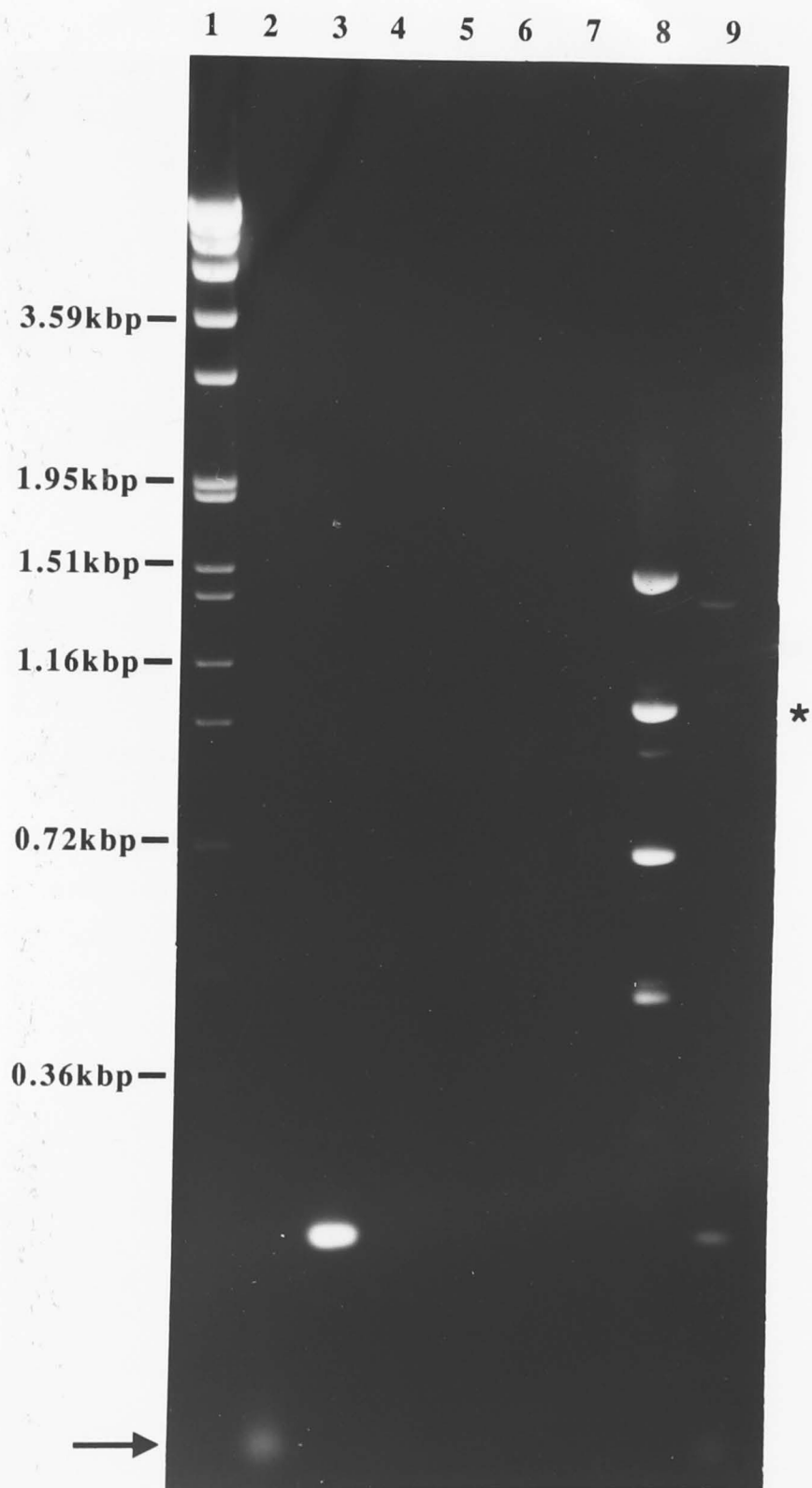
Figure 6.12. The complete nucleotide sequence of the pPSRB clone. The boxed sequence at either end is primer 2, with the right hand end being the reverse complement of the primer 2 sequence. The total length of the clone is 1063bp. The *Bgl II/Bam HI* fragment that is underlined was sub-cloned and used as a probe to a blot of race 1 and race 2 genomic DNA. The *Bam HI* site was located adjacent to the insert in the polylinker region of the plasmid DNA. An autoradiograph of this hybridization is shown in Figure 6.14.

pPSR B

GCTGAAGAAT CAAGAGCACA GGATGCTCAG GGACTTAGGA
CAACAGCTGA GAGGGACTCT ATAGGAGCAT GTCTCAGTGC
TACGGGATCA GAACTAAGTG GGGTTGTATT TCTGATGGGG
ACAGAAACAA ATACAACATG CAAAAGGGGG CCTGAGTTGA
GGGCCTAAGA TGAAACAGAA CAAAAGAGAA AAGAAATGAA
GAAAGACCAT TTGAGCCATG GTCAGAATTC TAACAACGAT
AAGAAAGCAG AGCGACCTAA AGGAAAAAGA AGAAAAGAAG
AGACACATGA GAAAGGGAAG AGAGTGAGAG AAAGAACAAG
GGAAACCAGA CCTGGAGGCG ACACAGAGGA GATGAAGTTG
ACGAGTGAGA CACACGGATC AGAAAGAAGG AGAAAATCAG
                                            Bgl II
ATGAAGAAAA AGAGAAACAA CAAGGTGTGT AGATCTTGAA
CTATATACAT CTGATTATCC CAATTAGGTT TCCAACCATG
TTACTCTCAC TACCCCGCTC CTCAGCTGTG CCGCAGCGGT
TTGGAAATGC TTCCCCGGAC TGGAAGTTCA ACATAAGCTA
GCCCGCCACC AAAAGCTGAG CTAAGCCACG CTAGCCCAAT
CAAATATGCT CAACTGCACC GGGAACAAAA AAGTAATAAA
CAACGCTGGC TCCTCAAGGA AATGTTTGAA GAAGATGACA
AACAAACCAA TGAAGAAGAT GATGAATTTG ATTTTCTCGA
AGAACTTGAG ACTGACGAGG AGGAAGGATC ACAAAGTCGA
GCCTCCCGCC AGCCAAACAA GGAACAAAAT CATGCCAAAG
GACACATCAA ATTGCTTTGG GACTACTTGA ATGCAGGATT
GAAGTGATTT TCAGCTCCGC TTCAGACTTC AAAAGGAGCT
GTTCTTGAAG ATGGTCTCTG ATGTTGAACT TTATTGTATT
TTGTGCAAAA TCCTGGAAGA AGTCTCTGTC CTATTTTTTA
TGAAAAATAT ATAACTAATT TGGTAATTAT TGCAATAGGA
TTGTACTGCA AACATGGGTT TGTCAAGCCT GCAGAAGATT
GCCTGTGCTC TTGATTCTTC AGC

Figure 6.13. A comparison of the structure of pPSRB and the PSR element. The sequence adjacent to the internal repeat of the PSR element was homologous to the reverse complement sequence of the right-hand end of the pPSRB clone. This indicated that the direct repeats in the PSR element were most likely to have once been in an inverted orientation.
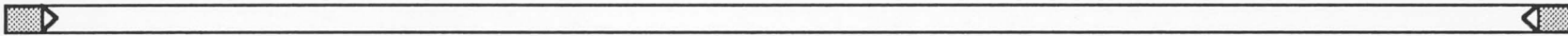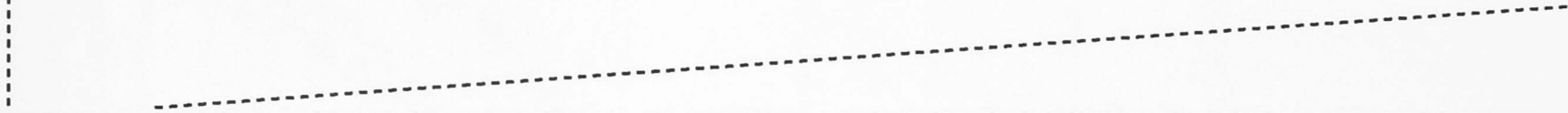
PSR element
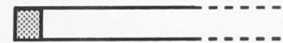
- strand: primer 2

GCTGAAGAATCAAGAGCACAG GATGCTCAGGGACTTAGGACAACAGCTGAGAGGGACTCCAGAGGAGCATGTCTCAGTGCTACGGGCTCAGAACTAAGTGAGGTTGTATTT →

GCTGAAGAATCAAGAGCACAG GATGCTCAGGGACTTAGGACAACAGCTGAGAGGGACTCTATAGGAGCATGTCTCAGTGCTACGGGATCAGAACTAAGTGGGGTTGTATTT →

+ strand:

pPSR B

Figure 6.14. Autoradiograph of a Southern blot containing genomic DNA from race 1 and race 2 of *P. sorghi*, hybridized to the *Bgl II*/*Bam HI* fragment from pPSRB. Lanes 1 and 2 contain genomic DNA from race 1 and race 2 respectively, cut with *Bam HI*. Lanes 3 and 4 contain genomic DNA from race 1 and race 2 respectively, cut with *Hind III*.

61
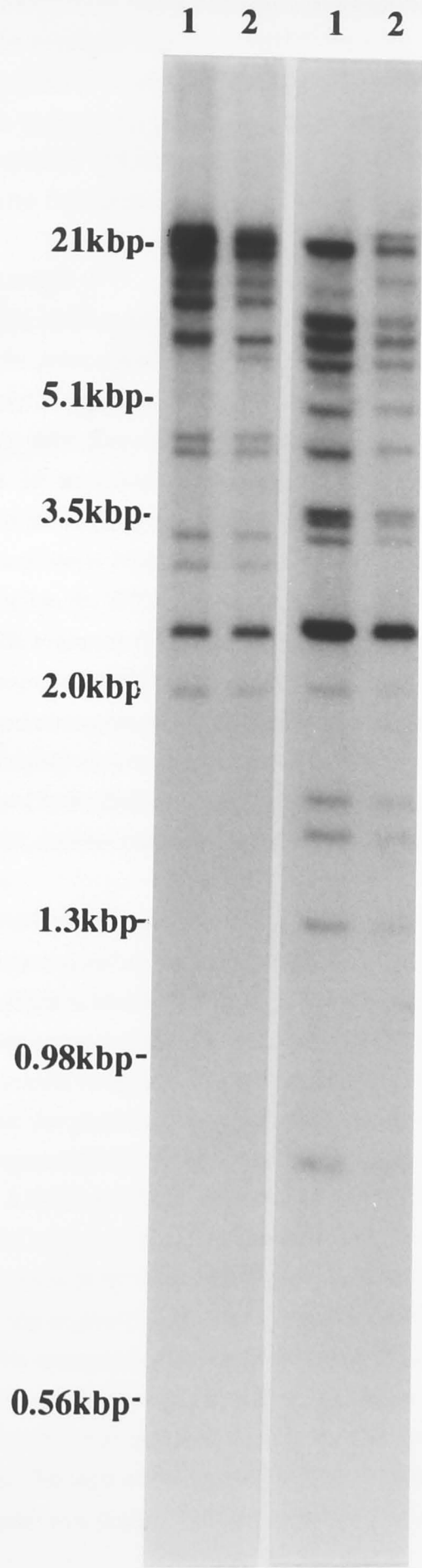
6.14) is substantially simpler to that of the PSR element. No clearly visible RFLPs were observed between race 1 and race 2 using the probe from pPSRB.

All the sequence data from the PSR element and pPSRB clone was analysed for open reading frames. In addition these sequences and the flanking regions of the PSR element were analysed for homology to sequences in a number of sequence data bases using the Wisconsin DNA analysis package. No extended open reading frames were detected and no significant homology to other sequences in the data bases were found.

6.5 : Discussion

An RFLP between two races of the maize rust fungus, *Puccinia sorghi*, was found to be due to the presence of a 496bp sequence in the race 2 genome. This sequence is highly repeated in the genome of both races, but no homologous sequences were detected in genomic DNA from flax rust (*Melampsora lini*) or wheat stem rust (*P. graminis tritici*). For these reasons the sequence has been termed the *P. sorghi* repeat, or the PSR element.

A number of RFLPs were detected between race 1 and race 2 when the PSR element was used as a probe to blots of genomic DNA from the two races. Thus the PSR element should be suitable for 'DNA fingerprinting' of rust isolates in much the same way as the repeated MGR sequence from the rice blast fungus has been used to measure pathotype diversity (Levy *et al*, 1991). Identification of a sequence analogous to PSR in more destructive and economically significant rust pathogens such as *P. graminis* would be very useful for studying the population structure of the species. As pointed out, under the conditions used here, there is no detectable homology between the PSR element and *P. graminis* DNA, however related sequences may be identified by lowering the stringency of hybridization.

The PSR element is flanked by a 5bp sequence present also in the race 1 genome at the location corresponding to the PSR element, and contains two 64bp exact direct repeats, only one of which is located terminally. The 496bp PSR element hybridizes to a 1.4kb RNA but contains no substantial open reading frames. This suggests that the PSR element may be a truncated version of a longer sequence. Using the PCR technique it was possible to demonstrate the presence of other longer sequences within the *P. sorghi* genome that were homologous to PSR. Furthermore, these sequences contained repeats in an inverted orientation. A 1063bp PCR product was cloned (pPSRB) and sequence data showed that the 64bp direct repeats of the PSR element may once have been inverted repeats, but became direct repeats by inversion. If these inverted repeats had been terminal and still flanked by a 5bp duplication then this sequence would exhibit many of the features associated with transposable elements described in other eukaryotic organisms including maize and *Drosophila* (reviewed by Berg and Howe, 1989).

Transposition or mobility of the PSR or its related sequences has not been demonstrated, although a number of RFLPs were detected between race 1 and race 2 using the PSR element as a probe. This indicates that PSR sequences may have moved during the

separation of the two races. The right hand end of the pPSRB clone, which had no homology to the PSR element, hybridized to substantially fewer restriction bands than PSR. This is similar to transposable elements in other eukaryotes where, for example, in maize there are many copies of defective *Ds* elements and one or few copies of an intact *Ac* element. In *Ac* the internal sequence is responsible for coding for the transposase function. The reduced hybridization pattern of the pPSRB clone showed no clear RFLPs between race 1 and race 2, indicating that if the pPSRB clone is related to a longer functional transposable element, then this sequence is stable.

Demonstration of transposition of the PSR or related elements might be difficult unless the particular conditions necessary to initiate excision can be met. The mobility of the *P* element of *Drosophila* and the *Mu* element of maize is stimulated in some crosses and not in others, a phenomenon known as hybrid dysgenesis in *Drosophila* (Kidwell *et al*, 1977; Engels, 1983). In *Neurospora crassa* mobility of the Tad element of has been demonstrated only during meiotic division (Kinsey and Helber, 1989). It is possible that such requirements are needed for the activation of the transposable elements in rust fungi. However, it is not known how often *P. sorghi* completes its sexual cycle, and it remains to be tested as a means of mobilizing the PSR element. Alternatively, somatic events involving nuclear exchange and parasexual recombination are believed to occur in rust fungi (Flor, 1964; Ellingboe, 1961; Watson and Luig, 1958). Such a mixing of nuclei and gene recombination may provide the stimulus for the mobility of the PSR element and related sequences.

A notable characteristic of rust pathogens is the frequent appearance of new virulent strains in nature. Previous studies by Flor (1960) and Schwinghamer (1959) suggest that induced mutation to virulence is accompanied by a loss of avirulence gene function (reviewed by Day, 1974). In relation to studies in other organisms, the insertion of transposable elements is thought to be a major contributor to the occurrence of spontaneous mutations (Harada *et al*, 1990; Green, 1980; Spradling and Rubin, 1981). In evolutionary terms this mechanism has the advantage of inactivating rather than deleting genetic information, and such an argument has obvious implications for the appearance of virulence mutations in rust fungi. Therefore, it is tempting to link the occurrence of mutations to virulence in *P. sorghi* to the insertion of transposable elements related to PSR. However, before such an hypothesis can be tested in *P. sorghi*, a clear demonstration of the mobility of the PSR element is required.

Chapter 7 : General Discussion

The general introduction to this thesis emphasized that almost all that is known about the genetics of rust fungi can be attributed primarily to the use of avirulence gene markers (*Avr*), and in several cases to the use of isozyme markers. These studies have documented the Mendelian inheritance of genes as well as the likelihood of somatic recombination events occurring in the asexual cycle of rust fungi. However, a detailed description of the nature of these genetic mechanisms has been limited by the lack of available genetic markers. Furthermore, while there is some knowledge of rust genetics, there is almost nothing known about the organization and structure of the rust genome. The work described in this thesis has provided more information about the organization and structure of the rust genome, and related this information to what is known about the genetics of these organisms.

7.1: Genetic heterogeneity

The available data about fungal heterogeneity and genome organization stem primarily from the study of laboratory strains of several saprophytic fungi, namely *Neurospora*, *Aspergillus* and several yeasts (Van Etten *et al*, 1981). Measurements of the size and complexity of the genome of phytopathogenic fungi, including the downy mildew fungus of lettuce, *Bremia lactucae*, and the maize rust fungus, *Puccinia sorghi*, have been reported by Francis *et al* (1990) and in chapter 2 of this thesis. In each case, the genomes were similar in size to most other fungi that have been analysed, however the levels of repeated DNA appeared to be atypical of fungi characterized previously. Francis *et al* argue it is quite possible that changes have occurred in the genomes of saprophytic fungi when they are maintained in controlled laboratory conditions on a defined nutrient source. Whether the levels of repeated DNA have any bearing on the stability of genome structure and the generation of variability in fungal pathogens awaits the characterization of genomic DNA from more pathogenic fungi, and also wild isolates of species of saprophytic laboratory maintained fungi.

The results of the RFLP analysis described in chapters 3, 4, and 5, indicated that molecular probes will provide a useful source of markers to complement existing characters used in genetic and taxonomic studies. cDNA probes detected a number of RFLPs between two races of *P. sorghi* that were analysed, and showed little or no homology to genomic DNA from other related rusts. Hybridization patterns of the probes to genomic digests of *P. sorghi* using a methyl sensitive enzyme indicated that genomic DNA extracted from germinated urediospores of maize rust may be methylated. This preliminary observation has not been followed up, however studies of methylation of genomic DNA of other eukaryotic organisms suggest an involvement in controlling gene expression (Bird, 1986). A developmental study of rust DNA

methylation, particularly in relation to gene expression, would be a feasible and informative experiment.

The hybridization of 24 randomly selected cDNA probes to Southern blots of *P. sorghi* DNA revealed a consistent complexity in the hybridization patterns. Almost all the cDNA probes hybridized to two or more genomic fragments in all the restriction digests that were tested. This complexity was not caused by internal restriction sites within the genomic sequences homologous to the probe. Other results also eliminated cloning artifacts, or the presence of large introns within rust genes, as causing the observed complexity. It was concluded that the complex hybridization patterns were the result of some form of genetic heterogeneity, most likely to be a combination of gene heterozygosity and sequence duplication.

A test of this hypothesis was not possible in *P. sorghi* because of biological constraints. However, it was possible to observe the transmission of multiple banded RFLPs in the progeny of a sexual cross in the flax rust, *Melampsora lini*. The results of this analysis indicated that simple genetic heterozygosity could explain much of the complexity. However, even this system provided only a partial test since the level of complexity observed in RFLP patterns of *M. lini* were substantially less than in *P. sorghi*. Furthermore, the segregation of one RFLP marker could only be explained by invoking the transmission of a null allele. While this phenomenon is not fully understood, it clearly indicates that in *M. lini* some, as yet, unexplained genetic mechanisms are operating during sexual reproduction. It is not known if similar events occur in *P. sorghi*.

Given these limitations, the inheritance study indicated that most multiple banding patterns were the result of gene heterozygosity. The level of heterozygosity at RFLP loci in *M. lini* was measured to be 0.55, which is similar to that reported at avirulence loci in other rust fungi. Both of these results are much higher than levels recorded using isozyme markers. If gene heterozygosity is the major cause of the multiple banding patterns in *P. sorghi*, then as the level of multiple banding is higher in *P. sorghi* than in *M. lini*, it appears that maize rust may have a higher level of heterozygosity than *M. lini*. This implies that if the multiple bands in *P. sorghi* represent allelic differences between the two nuclei of the dikaryon, then the two nuclei are likely to be genetically dissimilar at the sequence level.

Simple heterozygosity can explain a hybridization pattern containing two bands, however an explanation of more complex patterns requires some form of sequence duplication. Whatever this event, it must have occurred prior to the separation of race 1 and race 2 since the majority (88%) of bands from the two races co-migrate. Sequence duplication may range from small regions up to whole nuclear duplication. Several observations discussed in the next section indicate that nuclear duplication or polyploidy may have occurred in some rust fungi.

7.2: The origin of genetic heterogeneity in rust fungi

The phylogeny of rust fungi is not well understood (reviewed by Wahl *et al*, 1984). This can be attributed, in part, to the lack of reliable taxonomic markers that are available, and to the complete absence of any fossil record of rusts and most other fungi. However, it can be safely assumed that because of the specialized and biotrophic interaction that rust fungi have with a limited range of host plants, rust evolution is closely associated with the evolution of the host plant.

There is good evidence that polyploidy has occurred frequently during the evolution of plants, particularly the grasses (reviewed by Simmonds, 1976). For example, the evolution of the Triticae has involved the hybridization of related species followed by chromosome doubling to establish a stable and fertile allopolyploid. The evolution of hexaploid wheat (2n=42) is a good example of this. Two diploid wheats (AA and BB) crossed to produce a fertile allotetraploid (AABB), which in turn crossed with a wild diploid relative (DD) to produce the allohexaploid bread wheat, *Triticum aestivum* (AABBDD). Similarly, *Zea mays* (family Maydeae) is thought to be an ancient and stable allotetraploid.

Exactly how the evolution of the rusts that infect the grasses has been influenced by the evolution of the host plant is unknown, however there are several observations, including the results of this thesis, that suggest hybridization and/or polyploidy may have also been involved in rust evolution.

McGinnis (1953, 1954, and 1956) reported that the chromosome number of *P. graminis tritici*, *P. recondita* and *P. sorghi* was n=6, whereas several other rusts of grasses had a chromosome number of n=3. Closer observation of mitotic chromosomes of *P. graminis* revealed the terminal attraction of chromosomes on the metaphase plate. McGinnis postulated that rust species with a chromosome number of 6 may be allopolyploids. This result has not been substantiated, most probably due to the difficulty of preparing chromosomes from rust fungi. However, it seems possible that the grass rusts may adapt to evolutionary jumps in the host by a similar mechanism of wide crossing and polyploidy. This has not been suggested in the literature, however there are observations that offer some support for this suggestion.

Watson and Luig (1959, 1972) argue that hybridization events can occur between different physiological races of rust fungi during the asexual stage of the life cycle. They postulated that a rust which grows on the rough wheat grass *Agropyron scabrum*, but not on wheat or rye, originated from a somatic hybridization event between two *formae speciales* of *P. graminis*, one of which grows on rye and the other on wheat. Burdon *et al* (1981) used isozyme variants to support this argument and suggested that the hybrid resulted from the exchange of whole nuclei between the parental rusts. Such nuclear exchange could generate a dikaryon containing two very different nuclei.

Somatic hybridization between genetically dissimilar rust fungi provides an explanation for the high level of heterogeneity detected in the RFLP studies of *P. sorghi* and *P. graminis*. The conservation of a dikaryon comprising of two dissimilar nuclei might be maintained as a system of fixed heterozygosity due to genetic systems such as balanced lethals, or chromosome translocations, mating type specificities, or other mechanisms (Cleland, 1972). Alternatively, if *P. sorghi* is a stable allopolyploid then this would explain why the multiple bands were observed for all the cDNA probes that were analysed, and why the multiple patterns were conserved between race 1 and race 2.

These hypotheses will require experimental testing. Undoubtedly, the use of RFLP markers will provide the means to test the origin of genetic heterogeneity in rust fungi. It would be of interest to follow the hybridization patterns of specific probes in the rust fungi that infect the putative diploid ancestors of wheat and maize.

7.3: The PSR element - future research.

In chapter 6 the isolation of a small transposon-like sequence from *P. sorghi* was reported. This species specific sequence, designated the *P. sorghi* repeat (PSR), was highly repeated in the *P. sorghi* genome and did not hybridize to DNA from *P. graminis* or *M. lini*. The structure of the PSR element was similar to transposable elements that have been described in other organisms, however mobility of the PSR or related sequences has not been demonstrated.

Future work on the PSR element will involve attempts to trigger the transposition of the sequence by sexual and somatic crossing. Out crossing has been shown to stimulate the movement of the *P* element of *Drosophila melanogaster* and *Mu* in *Zea mays* (reviewed by Berg and Howe, 1989). Attempts will also be made to isolate the DNA sequences which code for the 1.4kb RNA that has homology to the PSR element. This could be acheived by isolating the cDNA sequence complementary to the 1.4kb RNA, or using the PCR technique to amplify sequences long enough to encode the transcript. The characterization of the 1.4kb transcript may indicate the function, if any, of the PSR element and related sequences.

If the PSR element is related to a functional mobile element, and conditions are found to stimulate its movement, then the possibility exists to clone genes of interest by insertional mutagenesis, or gene tagging. It may be possible to relate the insertion of a transposable element to the inactivation of an avirulence gene, generating a new virulence specificity. Such possibilities present exciting developments in research into rust fungi.

7.4: Concluding remarks

The results of this thesis provide a clear demonstration of the application of molecular techniques to study the genome organization and genetics of rust fungi. In

particular, the RFLP study indicates that the rust genomes are heterogeneous, especially those of *Puccinia* species. The origin and nature of this heterogeneity will only be determined by a thorough survey of more rust isolates and analysis of more genetic progeny, preferably from *Puccinia* species. On a broader scale, there are a number of other important uses for RFLPs in rust research.

(i) RFLP markers used in conjunction with conventional virulence markers will provide a powerful tool to track the movement of specific virulent strains. In Australia, it is thought that three major introductions of *P. graminis tritici* have occurred, and subsequent variation in virulence has been attributed to single step-wise mutations. Using RFLPs it will be possible to determine the origin of introduced strains and how they spread through agricultural districts. Such information will assist greatly in predictive plant breeding.

(ii) RFLPs can be used to construct a genetic or physical map of a rust fungal genome, from which specific genes could be cloned by chromosome walking (Young, 1990). The cloning and characterization of mating type and pathogenesis genes, in particular avirulence genes, will not only provide basic scientific knowledge about plant-microbe interactions, but may possibly lead to novel mechanisms to control disease.

(iii) RFLP markers will be useful in establishing a phylogenetic tree of rust fungi. The difficulties at present lie in the unreliability of spore morphology as a taxonomic character. Molecular probes may be used to quantify levels of sequence similarity between rust species, and when used as RFLP markers could provide an unlimited number of taxonomic characters to add to the existing morphological traits, so that the phylogeny of the uredinales can be confidently established.

This thesis has opened up a number of new research directions, and demonstrated the effectiveness of molecular techniques in expanding the knowledge of genome organization and the genetic systems of obligate fungal pathogens of plants.

68

Appendices

Appendix A: Comparison of total and invitro labelled protein profiles from race 1 and race 2 of *P. sorghi*.

Race 1 and race 2 of *P. sorghi* have been the biological material that has been used for much of this thesis. These races were selected because of their different geographic origin and spectrum of avirulence genes. The results of chapter 3 indicated that molecular probes could successfully detect RFLPs between genomic DNA from race 1 and race 2. Another comparison of total and invitro labelled proteins was less successful at detecting polymorphisms between the two races. The results of this analysis are discussed in the next section.

Total protein

Total protein was extracted from ungerminated and germinated urediospores of race 1 and race 2, as described by Howes *et al* (1982). Ungerminated urediospores were broken open with glass beads according to the method described by Dickinson and Pryor (1989), whereas germinated urediospores were ground to a powder in liquid nitrogen. Total protein was precipitated in nine volumes of acetone, and separated under denaturing conditions (0.1% sodium dodecyl sulphate) on a 15% polyacrylamide gel and stained with coomassie blue (Sambrook *et al*, 1989). The electrophoretic profile of total protein from ungerminated and germinated urediospores of race 1 and race 2 is shown in Figure A1. Approximately 50-60 resolvable bands were detected and one difference in the profiles of race 1 and race 2 is indicated by an arrow.

Invitro translated proteins

Poly A$^+$ RNA was prepared according to the methods described in section 3.3 (chapter 3). Proteins were translated from this RNA using a rabbit reticulocyte lysate system (Amersham) to which the ^{35}S labelled amino acid methionine had been added. Reactions were done according to Sambrook *et al* (1989). The efficiency with which ^{35}S was incorporated into protein was measured by the radioactivity (dpm) precipitated in 10% trichloroacetic acid (TCA). The labelled proteins were separated by SDS-polyacrylamide gel electrophoresis.

The optimization of reaction time and RNA concentration in the invitro translation reactions were measured and the results are shown in Figures A2 and A3. The maximum level of incorporation using germinated and ungerminated urediospore RNA was reached between 30 and 60 minutes. All subsequent reactions were incubated for 45 minutes. The effects of increasing the concentration of RNA from germinated urediospores caused the amount of incorporated ^{35}S RNA to rise and then plateau, whereas increasing the amount of RNA from ungerminated urediospores caused the incorporation of label to increase and then drop almost to background levels.

Figure A1: Electrophoretic profile of SDS soluble proteins extracted from germinated and ungerminated urediospores of race 1 and race 2 of *P. sorghi*. Lane 1, race 1 ungerminated urediospore proteins; lane 2, race 2 ungerminated urediospore proteins; lane 3, race 1 germinated urediospore proteins; lane 4, race 2 germinated urediospore proteins. The arrow indicates an electrophoretic variant between race 1 and race 2. Size markers are in kilodaltons.
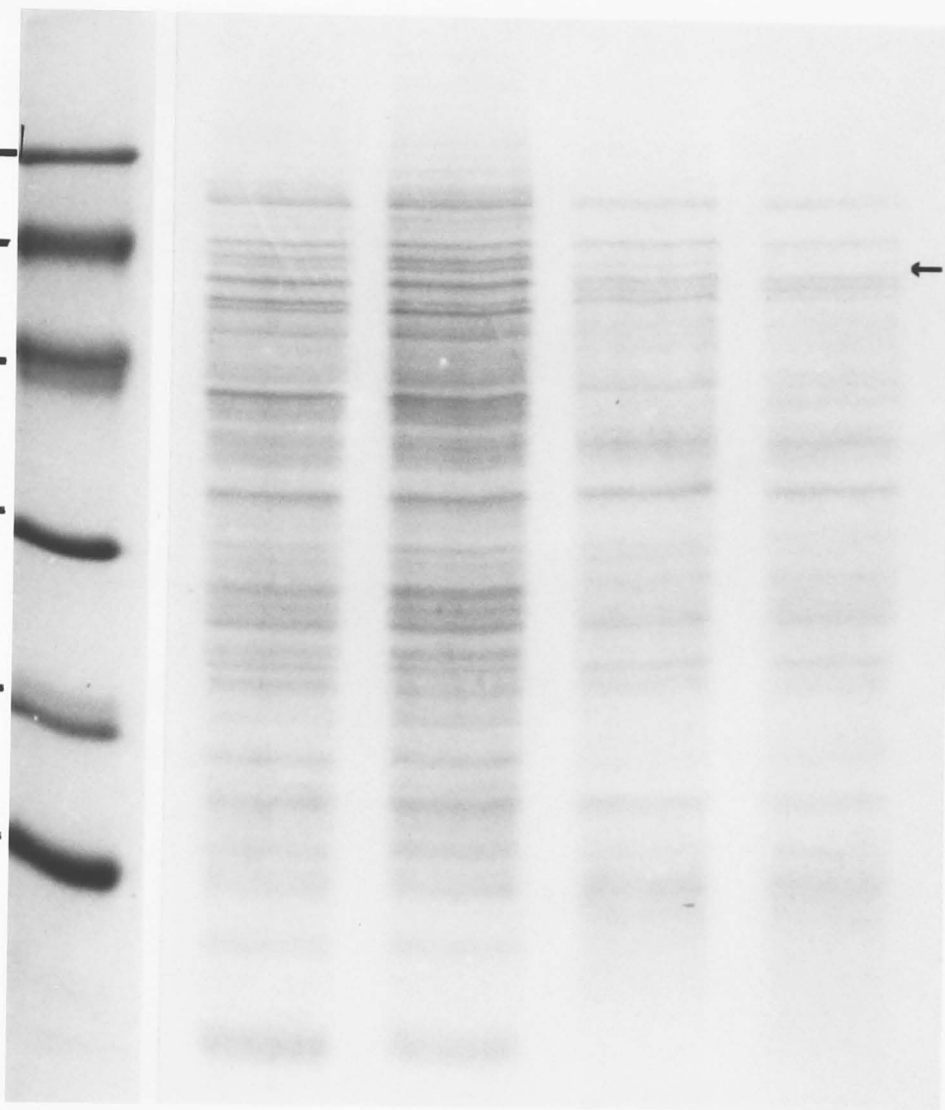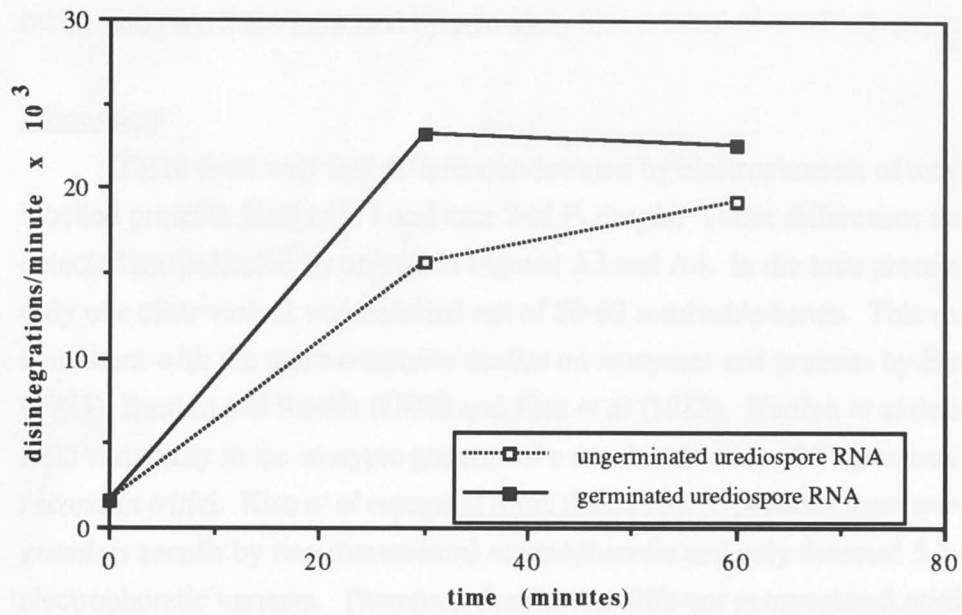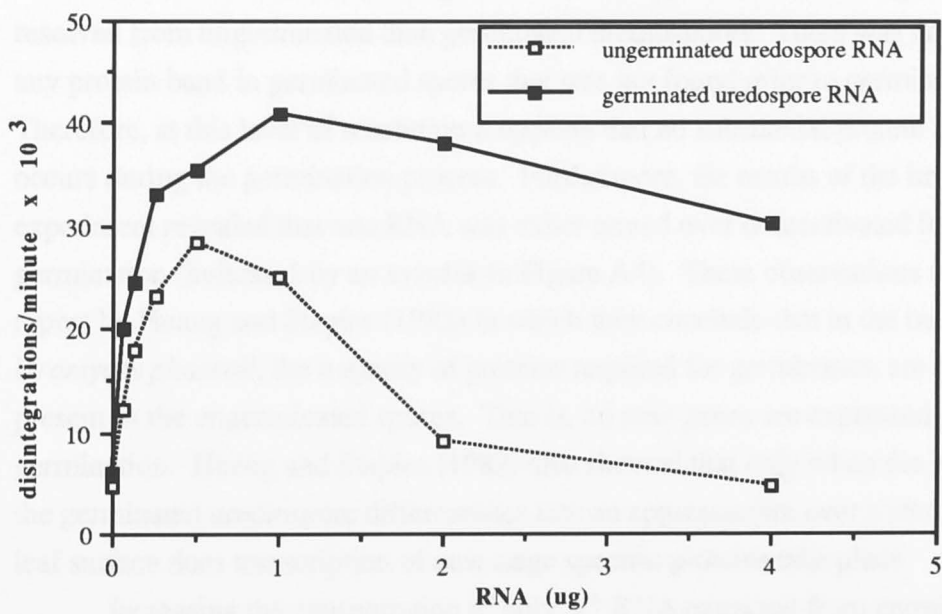
Figure A2: Graph showing the relationship between the reaction time and the incorporation of TCA precipitable ^{35}S counts into invitro translated protein, using poly A+ RNA extracted from germinated and ungerminated urediospores of *P. sorghi*. Each point represents the mean of two measurements.

Figure A3: Graph showing the relationship between the amount of germinated and ungerminated urediospore RNA (μg) used in a standard reaction volume of 20μl, and the incorporation of ^{35}S into invitro labelled protein. Each point represents the mean of two measurements.

A2

disintegrations/minute x 10³ (y-axis, 0–30)

time (minutes) (x-axis, 0–80)

Legend:
- ungerminated urediospore RNA
- germinated urediospore RNA

A3

disintegrations/minute x 10³ (y-axis, 0–50)

RNA (ug) (x-axis, 0–5)

Legend:
- ungerminated uredospore RNA
- germinated uredospore RNA

69

The invitro labelled proteins from germinated and ungerminated urediospores of race 1 and race 2 were separated on a 10-20% gradient SDS-polyacrylamide gel. The electrophoretic conditions, infiltration of scintilint into the gel and autoradiography are all described by Sambrook *et al* (1989). The autoradiograph of the electrophoretic profile of the invitro labelled proteins is shown in Figure A4, and differences between race 1 and race 2 are indicated by arrows.

Discussion

There were very few differences detected by electrophoresis of total and invitro labelled proteins from race 1 and race 2 of *P. sorghi*. Those differences that were detected are indicated by arrows in Figures A3 and A4. In the total protein profiles, only one clear variant was detected out of 50-60 resolvable bands. This result is consistent with the more extensive studies on isozymes and proteins by Burdon *et al* (1983), Burdon and Roelfs (1986) and Kim *et al* (1985). Burdon *et al* detected very little variability in the isozyme patterns of a number of races of *P. graminis tritici* and *P. recondita tritici*. Kim *et al* separated more than 280 polypeptides from two races of *P. graminis secalis* by two-dimensional electrophoresis and only detected 5-7 different electrophoretic variants. Therefore, despite the different geographical origin, virulence specificity and RFLP patterns of race 1 and race 2, variation at the protein level between the two races is quite low. However, it should be noted that only variation in size and/or protein charge can be detected by these methods.

Within the limits of this experiment, there appeared to be more protein bands resolved from ungerminated than germinated urediospores. There was no evidence for any protein band in germinated spores that was not found prior to germination. Therefore, at this level of resolution it appears that no substantial protein synthesis occurs during the germination process. Furthermore, the results of the invitro labelling experiment revealed that one RNA was either turned over or inactivated following germination (indicated by an asterisk in Figure A4). These observations agree with a report by Huang and Staples (1982) in which they conclude that in the bean rust, *Uromyces phaseoli*, the majority of proteins required for germination are already present in the ungerminated spores. That is, no new genes are expressed during germination. Huang and Staples (1982) also showed that only when the germ tube of the germinated urediospore differentiates into an appressorium over a stomate on the leaf surface does transcription of new stage specific proteins take place.

Increasing the concentration of poly A^+ RNA extracted from germinated urediospores (up to 1µg/20µl reaction) stimulated ^{35}S incorporation, but increasing the concentration of RNA further inhibited invitro translation. Such a response was not observed using poly A^+ RNA from germinated urediospores. This result was repeatable and specific to RNA extracted from ungerminated urediospores. There are a number of explanations for this result, including either the presence of a low

Figure A4: Autoradiograph of the electrophoretic profile of invitro ^{35}S labelled proteins separated on a 10-20% gradient SDS polyacrylamide gel. Lane 1 shows the proteins translated invitro from germinated urediospore polyA+ RNA from race 1 of *P. sorghi*. Lane 2 is the same reaction using polyA+ RNA from germinated urediospores of race 2, and lane 3 is the reaction using polyA+ RNA from ungerminated urediospores of race 1. Arrows indicate electrophoretic variants between race 1 and race 2, and the asterisk shows a protein translated from an RNA in ungerminated urediospores that is not present or not translated in germinated urediospores. Size markers are in kilodaltons.
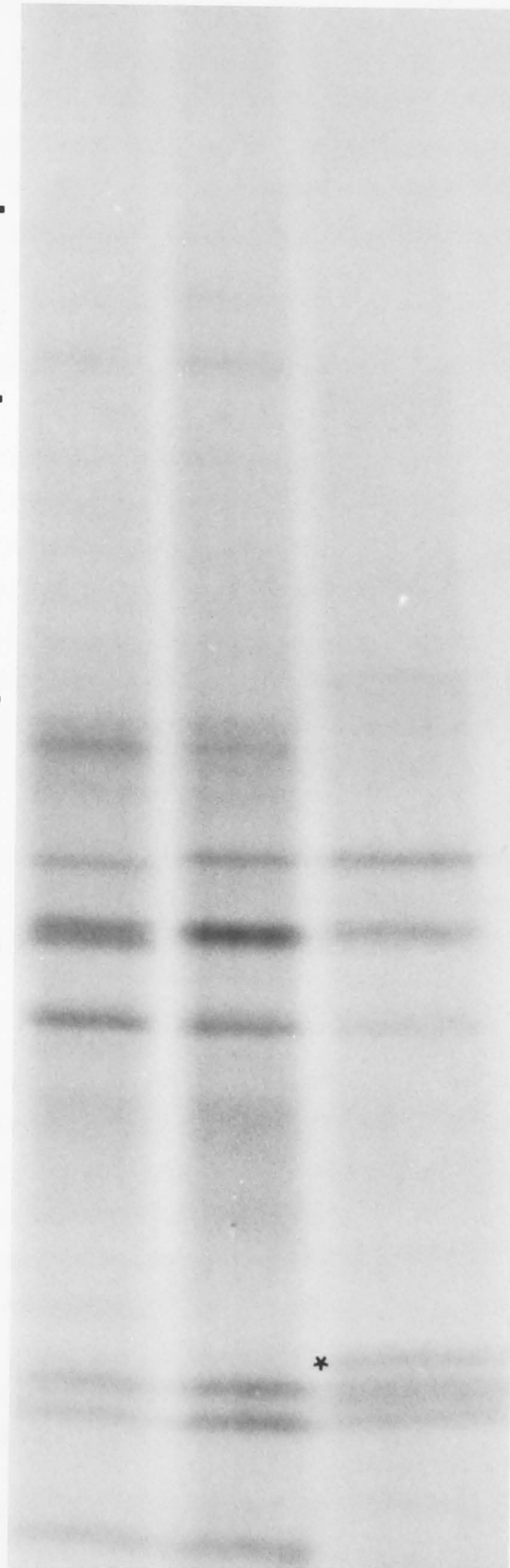
concentration of a ribonuclease in ungerminated spores, or a specific molecule inhibiting translation. It seems understandable that a population of stored mRNAs would be equipped with some mechanism to inhibit translation until the correct environmental conditions are present. An inhibitor of germination has been reported in a number of rust fungi (Macko, 1981), and it is possible that this inhibitor may act by preventing translation of mRNA transcripts required for germination. If this inhibitor copurifies with RNA extracted from ungerminated urediospores, then this would explain the data presented here. This speculative model shows some analogy to other systems. A population of stored mRNAs is present in pollen of several plant species, and inhibition of invitro translation of this mRNA population has been observed (Mascarenhas, 1975). Upon pollen recognition on the stigma, translation is initiated and the pollen tube emerges and grows down the style (Mascarenhas, 1989).

Appendix B: RFLP analysis of race 2 and race 2-1 of *P. sorghi*.

The *Rp1* locus confers resistance in *Zea mays* to infection by the maize rust fungus *Puccinia sorghi*. Inoculation of maize plants containing the *Rp1g* resistance allele with race 2 urediospores of *P. sorghi*, gives rise to necrotic flecks on the leaf which are indicative of the hypersensitive or resistance response. However, the occasional fully developed pustule can be seen on the leaf surface. Urediospores collected from these pustules are found not only to be virulent against the *Rp1g* allele, but also virulent against the *Rp5* resistance gene. Both *Rp1* and *Rp5* are located on the tip of the short arm of chromosome 10 of maize, with *Rp5* being 1.1 map units distal to *Rp1* (Saxena and Hooker, 1968). The spontaneous and simultaneous acquisition of virulence against *Rp1g* and *Rp5* has been observed in a number of independent inoculations (Pryor, personal communication). This new rust has been named race 2-1 and the virulence specificity of this race and race 1 and 2 is shown in Table B1.

The origin of race 2-1

As the change from race 2 to race 2-1 is repeatable and occurs frequently, this suggests that if this is caused by mutation it is most likely to be a single event. A single mutation causing the simultaneous acquisition of virulence to two different resistance genes contradicts the 'gene for gene' model proposed by Flor (1942) to explain the interaction between flax and the flax rust, *Melampsora lini*. Despite many years of genetic research in host pathogen interaction, the 'gene for gene' model has proved durable. Assuming the validity of the 'gene for gene' model, there are a number of genetic events that could cause the simultaneous loss of two avirulence genes. These events leading to the formation of race 2-1 are described below.

		Rp5 and the alleles of Rp1								
	Origin	b	j	c	m	d	f	td	a	g	Rp5
race 1	Australia	+	+	+	+	+	-	-	-	-	-
race 2	USA	-	-	-	-	+	+	+	+	-	-
race 2-1		-	-	-	-	+	+	+	+	+	+

Table B1: Reaction types of race 1, 2 and 2-1 of *P. sorghi* on isogenic maize plants containing the different alleles of *Rp1* and *Rp5* (Hooker and Russell, 1962). +: compatible reaction, -: incompatible reaction.

Firstly, it is possible that *Rp1g* and *Rp5* are the same gene, in which case a single mutation would give rise to virulence against a single resistance gene. This would be within the bounds of the 'gene for gene' model. Pryor (1987) argues that the *Rp1* locus in maize may be an extremely large and complex gene. Although *Rp1g* has been mapped at the *Rp1* locus, which is 1.1cM proximal to *Rp5*, it has been suggested that the *Rp1* locus could extend this distance and the *Rp1g* and *Rp5* alleles could be identical (Bennetzen, unpublished data). The relationship between physical distance and genetic distance in *Z. mays* has been estimated to be approximately 2Mb per cM (Caten, 1987). This could make *Rp1* and *Rp5* several million basepairs apart. However, Pryor (personal communication) suggests that the tip of chromosome 10 in *Z. mays* may be a hot spot for recombination, in which case the relationship between physical and genetic distance in the region of *Rp1g* and *Rp5* may be greatly skewed. Therefore, the possibility still remains that these two alleles are identical.

A second explanation could be that virulence against *Rp1g* and *Rp5* are different genes and that a somatic recombination event within the race 2 genome has generated a new combination of virulence genes. Heterokaryosis and parasexual recombination have been suggested to contribute to changes observed in the virulence specificity of rust fungi (section 1.2, chapter 1). If the genes controlling virulence against *Rp1g* and *Rp5* were both heterozygous (Avr_{Rp1g}/avr_{Rp1g} and Avr_{Rp5}/avr_{Rp5}), then the fusion of hyphal cells followed by nuclear reassortment could bring together into the one cytoplasm the two recessive virulent alleles. The hybrid mycelium would be virulent against *Rp1g* and *Rp5* and would result in the formation of a pustule.

One problem with this model is that nuclear reassortment generating a virulent dikaryon which is homozygous at both loci would require the association of nuclei of the same mating type. The function of mating type genes is to ensure that self fertilization does not occur in the sexual stage of the rust life cycle (Lawrence, 1980; reviewed in section 1.2). However, Buller (1941) and Ellingboe and Raper (1962) reported that the association of nuclei during somatic nuclear reassortment in *Schizophyllum commune* is controlled by mating type genes. Therefore, if mating type genes are active during the somatic stage of the rust life cycle, nuclear fusion and somatic recombination would be required for the formation of a new combination of

virulence genes in a compatible dikaryon. Watson and Luig (1958) and Ellingboe (1961) have reported somatic recombination and parasexuality in the wheat stem rust, *Puccinia graminis tritici*. It is not known if such events occur in *P. sorghi*.

A further explanation of the origin of race 2-1 is by deletion or whole chromosome loss (aneuploidy). If race 2 was heterozygous for avirulence against *Rp1g* and *Rp5* (as described above), then either deletion or whole chromosome loss could lead to the uncovering of both recessive virulence genes. The resulting genotype would be virulent against the *Rp1g* and *Rp5* resistance genes. For this to happen both avirulence loci would have to be closely linked or on the same chromosome.

In order to assess the possibility of large scale somatic rearrangements as opposed to single gene or point mutations, several RFLP probes from *P. sorghi* (chapter 3) were employed. Figure B1 shows an autoradiograph of the maize rust cDNA, pPSOc24, hybridized to a Southern blot containing digests of race 1, race 2 and race 2-1 genomic DNA. In each digest the hybridization pattern between race 2 and race 2-1 was identical. This was the case for 9 other cDNA probes that were tested.

Discussion

It appears from the cDNA hybridization experiments that large scale somatic events such as whole nuclei exchange have not occurred in the formation of race 2-1. If this were the case then at least one band in each of the race 2 digests would be expected to segregate in race 2-1. However, these preliminary results cannot rule out smaller chromosomal rearrangements. Assuming the chromosome number of *P. sorghi* is 6 (McGinnis, 1956), and that the cDNA probes are evenly distributed in the genome, then about 30 different probes would need to be analysed to be 95% confident that one of the six chromosomes had not been lost. Such an extensive analysis was not carried out, but on the basis of the available data it seems likely that whole nuclear reassortment is not responsible for the formation of race 2-1. The data is insufficient to decide on the likelihood of other possibilities.

Appendix C: Protoplast preparation and separation of chromosomes from *Puccinia sorghi*.

One of the prominent differences in the cellular organization of plants, fungi and animals is the presence or absence of a cell wall. In animals, the cytoplasm of the cell is enclosed in a plasma membrane whereas in plants and fungi this membrane is further enclosed by a cell wall. Plant cell walls are composed mainly of cellulose whereas chitin is the major cell wall constituent of fungi. Chitin is a linear β-1,4-linked homopolymer of N-acetyl-D-glucosamine and thus very similar to cellulose (Farkas, 1985). Removal of the cell wall from fungal or plant cells exposes the plasma membrane and the resulting body is known as a protoplast.

Figure B1: Autoradiograph of a Southern blot containing genomic DNA from race 1, race 2 and race 2-1 of *P. sorghi* hybridized to the cDNA clone, pPSOc24. Lanes 1, 2, and 3 contain *Eco RI* digested genomic DNA from race 1, race 2 and race 2-1, respectively. The same order of DNAs were present in the remaining lanes, with lanes 4, 5, and 6 containing *Hind III* digests; 7, 8, and 9 containing *Bam HI* digests; 9, 10, and 11 containing *Bgl II* digests and 13, 14, and 15 containing *Sac I* digests. In each case the race 2 and race 2-1 patterns were identical (except for loading differences). Note the presence of the PSR element in both race 2 and race 2-1 (chapter 6).

73

Protoplasts have been an important part of plant and mycological research for over twenty years. However, recent interest has developed in the use of protoplasts for DNA mediated transformation (Ullrich *et al*, 1985; Specht *et al*, 1988). Even more recently, the development of the pulsed field electrophoretic technique has enabled the separation of intact chromosomes from simple eukaryotes such as fungi (Schwartz and Cantor, 1984). Successful separation of chromosomes from a number of fungal species, including some phytopathogens, has been achieved (reviewed in section 1.4.1, chapter 1). In several cases protoplasts have served as the starting material for the extraction and electrophoresis of intact chromosomal DNA (Kinscherf and Leong, 1988).

Young (1990) has introduced the concept of map based cloning of specific genes from plant pathogenic micro-organisms. The basis of this technique is to use pulsed field electrophoresis in conjunction with a conventional linkage map, defined by RFLP markers, to develop a physical map of extended regions of genomic DNA. Specific genes could be located on large DNA molecules, and cloned by chromosomal walking from closely linked RFLP loci. There can be no doubt about the potential application of such research to plant pathology, and in particular, to biotrophic pathogens such as rust fungi where conventional genetics is difficult. For this reason attempts have been made to develop methods for protoplast isolation and pulsed field electrophoresis of intact chromosomal DNA from *Puccinia sorghi*. It was hoped that single copy cDNA sequences, used in the RFLP analysis (chapter 3), could be used to generate a large scale physical map of the *P. sorghi* genome.
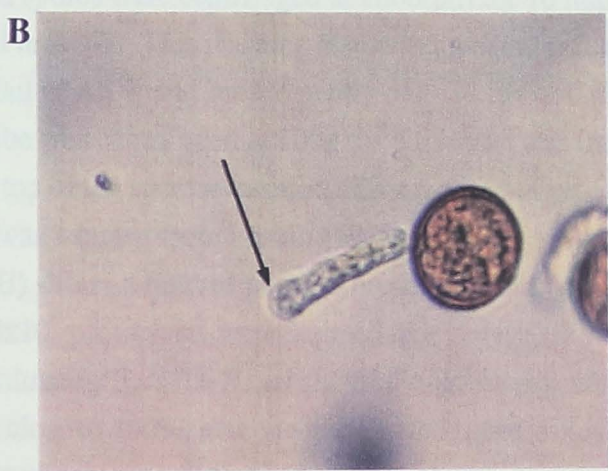
Protoplast isolation

(i) Fungal strains: Urediospores of *P. sorghi* were harvested and germinated as described in section 2.2 (chapter 2). Spores were germinated for 4 hours or until most spores had produced a germ tube at least three times the diameter of the spore.

(ii) Osmotic stabilizers: In order to find the optimal osmotic conditions for protoplast formation several sugars were tested. Huang *et al* (1990) have recently reported the preparation and regeneration of protoplasts from the wheat stem rust, *Puccinia graminis tritici*, using 0.8M MgSO$_4$ as an osmotic stabilizer. The experiments reported here predate this report and salts such as MgSO$_4$ have not been tested. Mannitol was found to be the best osmotic stabilizer and Figure C1 shows germinated urediospores of *P. sorghi* exposed to different concentrations of mannitol. Plasmolysis, or the separation of the plasma membrane from the cell wall, was observed in conditions of 0.8M mannitol or greater concentration (indicated by arrows in Figure C1(B) and C1(C)). As plasmolysis had not occurred in 0.6M mannitol it was assumed that an optimal concentration for incipient plasmolysis and protoplast formation would be in a concentration range of 0.6-0.8M.

Figure C1: Urediospores of *P. sorghi* that have been germinated on an air/water interface for 1 hour and then incubated for a further hour in mannitol at concentrations of A: 0.6M, B: 0.8M, and C: 1.0M. Arrows indicate plasmolysis of the plasma membrane. Photographs were taken under 400x magnification.

A

B

C

Several other experiments were performed to optimize other parameters for protoplast isolation and purification. These included the cellulytic enzyme used, the enzyme concentration and the protoplast purification steps. The method found to be the best compromise for protoplast yield and purity is described below.

(iii) Isolation and purification of protoplasts: Urediospores were germinated, collected and weighed to measure the amount of water trapped in the mesh of germinated spores. Mannitol and $MgSO_4$ were added to a final concentration of 0.6M and 7mM respectively. Phosphate buffer (Na_2HPO_4, pH 5.5) was added to a final concentration of 5mM. Approximately 50mls of the osmotic solution were used for every 500mgs of ungerminated urediospores, and the germinated urediospores were equilibrated in this solution for 1 hour at room temperature with gentle shaking. Novozyme (Novo Industries) was added to a final concentration of 3mg/ml and incubated for a further 3-4 hours. The formation of protoplasts was monitored by light microscopy (Figure C2(A)). An equal volume of artificial sea water (ASW) of the same osmotic potential (600mos) was added and mixed for 10 minutes. The protoplasts and spores were centrifuged at 1000rpm for 10 minutes, and the pellet gently resuspended in ASW. This washing step was repeated and the pellet resuspended in 500µl of ASW and loaded gently onto 10mls of 0.6M sucrose in a 15ml corex tube. The tube was centrifuged at 100g for 5 minutes and the protoplasts collected from the top of the sucrose cushion. This method of protoplast purification on a sucrose cushion was adapted from Larkin (1976).

Figure C2(B) shows a field of purified protoplasts extracted from *P. sorghi*. Approximately $1.3x10^7$ protoplasts were isolated from every 100mg of urediospores. As there are approximately $3.0x10^8$ *P. sorghi* urediospores per gram, and assuming the germination rate is close to 100%, and one germinated spore would form one protoplast, then this represents a 50% yield of protoplast.

(iv) Viability and nuclear content of protoplasts: To test the viability of the protoplasts, fluorescein diacetate (FDA) was added (0.1 mg/ml final concentration) and the protoplasts observed under ultraviolet light using a fluorescent microscope. The basis of the viability test is that leakage of FDA from dead membranes is much greater than from intact membranes (Larkin, 1976). Figure C2(C) shows FDA stained protoplasts illuminated with U.V. light. Approximately 60-70% of the protoplasts were fluorescing strongly and, therefore, are likely to be viable.

The fluorochromatic stain DAPI (4,6-diamidino-2-phenyl-indole) is specific for nucleic acids and fluoresces under U.V. light. DAPI was added to a protoplast suspension (100µg/ml final concentration) and illuminated with U.V. light. Figure C2(D) shows a field of protoplasts under U.V. light. Approximately 50% of protoplasts contained intact nuclei and those not containing nuclei are most probably the result of the plasma membrane budding off before the nuclei are enclosed within the protoplast.

Figure C2: **A**; Protoplast formation from a germinated urediospore of *P. sorghi* after treatment for 30 minutes with 3mg/ml Novozyme in 0.6M mannitol, 7mM $MgSO_4$ and 5mM Na_2HPO_4, pH 5.5. **B**; A field of purified *P. sorghi* protoplasts. **C**; A field of protoplasts stained with FDA (Fluorescein diacetate) viewed under U.V. light. **D**; DAPI stained protoplasts viewed under U. V. light showing one protoplast containing two nuclei.
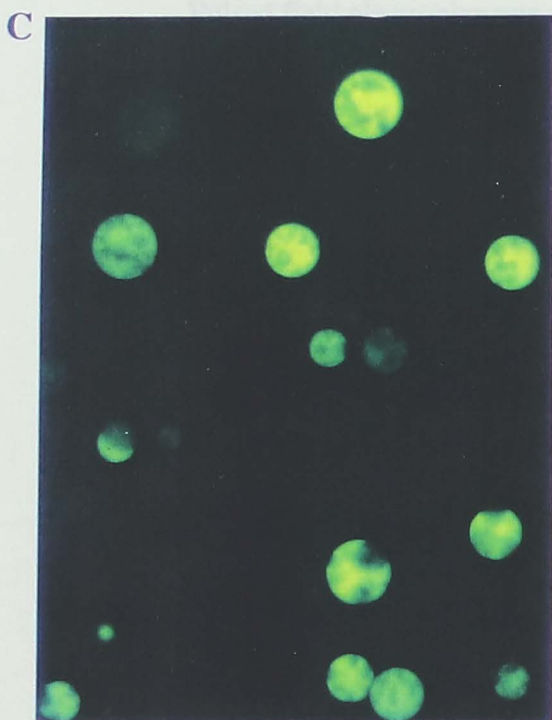
These results are important in estimating the number of urediospores required to make enough protoplasts to visualize chromosomal DNA by pulsed field electrophoresis. Kinscherf and Leong (1988) have estimated that approximately 2.0×10^9 protoplasts/ml are required to see ethidium bromide stained chromosomal DNA from the maize smut fungus *Ustilago maydis*. If this estimation is applied to protoplast isolation from *P. sorghi*, then 28 grams of urediospores would be required for every millilitre of protoplasts made. This is not feasible. However the 10^4 fold greater sensitivity of Southern hybridization to ethidium bromide staining of DNA, lowers this requirement to 2.8mg of urediospores for every millilitre of protoplasts. For this reason, attempts to use protoplasts as a source of DNA for separation of *P. sorghi* chromosomes by pulsed field electrophoresis were continued.

Pulsed field electrophoresis

Protoplasts were suspended in ASW to a concentration of 5.0×10^7/ml and heated to 37°C. An equal volume of 2.25% low melting point agarose (Seaplaque) in ASW and 50mM EDTA at 50°C was added and mixed quickly. The mixture was immediately drawn into a 1ml glass pipette (2.5mm diameter) and placed at 4°C for 15 minutes. The protoplast agarose mixture was then expelled from the pipette and cut into plugs (6mm long) and incubated at 50°C overnight in 0.1% sarcosyl, 0.5M EDTA and 2mg/ml proteinase K (Sigma). The plugs were washed twice for 16 hours in 0.5M EDTA at 50°C and stored for up to 6 months at 4°C.

Pulsed field electrophoresis was performed using a CHEF apparatus. The CHEF system and the conditions of electrophoresis are described by Chu *et al* (1987). Chromosomes from the yeast *Saccharomyces cerevisiae* were used as size markers and to standardize the relationship between the size of molecules resolved and the length of the pulse time. Chromosomal DNA from *S. cerevisiae* was made according to the method described by Carle and Olson (1985). Following electrophoresis, DNA was transferred to a nylon membrane by Southern blotting according to the method described by Orbach *et al* (1988), and hybridized to *P. sorghi* total genomic DNA that had been radioactively labelled by nick translation. The results of the electrophoresis and blotting of *P. sorghi* chromosomal DNA are summarised in Figure C3.

Discussion

The successful isolation of protoplasts from *P. sorghi* indicates that this system is likely to be applicable to a number of biological studies of rust fungi. Regeneration of protoplasts into a form capable of infecting the host plant would provide the basic requirement to develop a DNA mediated transformation system for rust fungi. Regeneration of protoplasts from *P. graminis tritici* has been reported (Huang *et al*, 1990), however successful transformation has not yet been achieved. The

Figure C3: Autoradiographs of chromosomal DNA from *S. cerevisae* (lane 1) and *P. sorghi* (lane 2) electrophoresed under different pulsed field conditions (A-D), blotted and hybridized to radioactively labelled total genomic DNA from *P. sorghi*. **A**; 70 second pulse time at 5 V/cm for 22 hours. **B**; 5 minute pulse time at 3.3 V/cm for 45 hours. **C**; 7 minute pulse time at 3.3 V/cm for 51 hours. **D**; 30 minute pulse time at 1.67 V/cm for 120 hours. Blots from electrophoretic conditions A and D are accompanied by photographs of the ethidium bromide stained agarose gel before blotting showing the separation of *S. cerevisae* chromosomes.
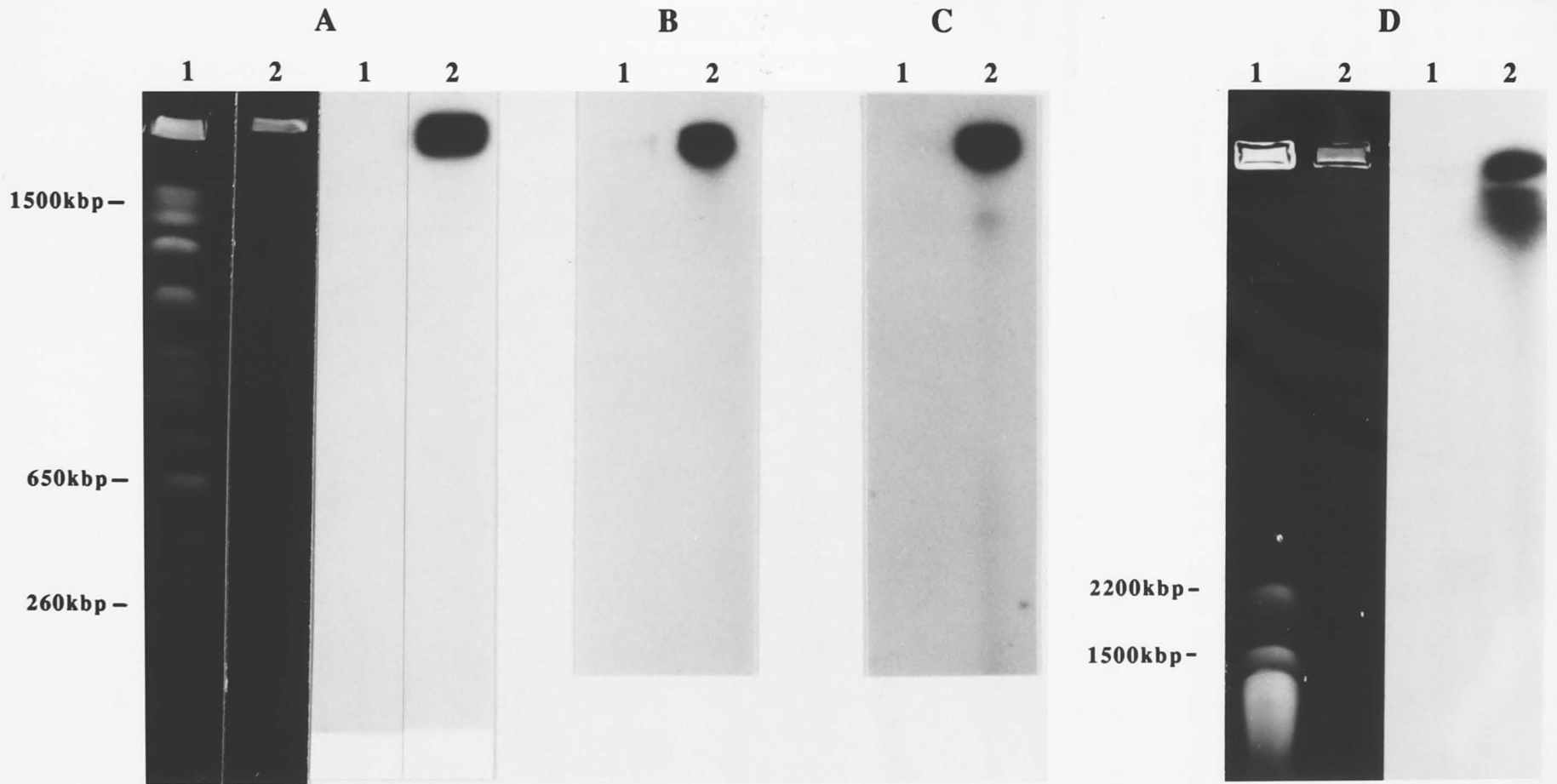
transformation of rust fungi is a vital step towards cloning and characterizing specific genes such as avirulence.

The application of protoplasts in providing a source of *P. sorghi* chromosomal DNA for electrophoretic karyotyping was not successful. It appears that some chromosomal DNA from *P. sorghi* entered the gel matrix under conditions of a 30 minute pulse time at 1.67 V/cm. Increasing the pulse time to 60 minutes or greater may lead to the successful resolution of chromosomal DNA, however this experiment has not yet been done. An important consideration is that the size of the *P. sorghi* genome has been estimated to be 4.5×10^7 bp (chapter 2), and the chromosome number has been estimated to be n=6 (McGinnis, 1956). On the basis of this data the average length of a *P. sorghi* chromosome would be 7Mb. This is at the upper limit of resolution of pulsed field electrophoresis (Smith *et al*, 1987).

With the improvement in electrophoretic techniques, successful separation of chromosomes from *P. sorghi* will undoubtedly be achieved. However, in the mean-time, infrequently cutting restriction endonucleases such as *Not I* and *Sfi I* could be used to construct long range restriction maps of chromosomal DNA of rust fungi (Young, 1990).

Bibliography

Akins, R.A., and Lambowitz, A.M. 1984. The [poky] mutant of Neurospora contains a 4-base-pair deletion at the 5' end of the mitochondrial small rRNA. PNAS (USA) **81**:3791-3795.

Allen, R.F. 1934. A cytological study of heterothallism in *Puccinia sorghi*. J. Agric. (Washington, D.C.) Res. **49**:1047-1068.

Arthur, J.C. 1904. The aecidium of maize rust. Bot. Gaz. (Chicago) **38**:64-67.

Arthur, R., Herr, F., Straus, N., Anderson, J., and Horgen, P. 1982. Characterization of the genome of the cultivated mushroom, *Agaricus brunnescens*. Exp. Mycol. **7**:127-132.

Balazs. I., Purrello, M., Alhadeff, B., Grzeschik, K.-H., and Szabo, P. 1984. Isolation and subregional mapping of a human cDNA clone detecting a common RFLP on chromosome 12. Hum. Genet. **68**:57-61.

Bartos, P., Fleischmann, G., Samborski, D.J., and Shipton, W.A. 1969. Studies on asexual variation in the virulence of oat rust, *Puccinia coronata* f. sp. *avenae* and wheat leaf rust, *Puccinia recondita*. Can. J. Bot. **47**:1383-1387.

Baum, B.R., and Savile, D.B.O. 1985. Rusts (Uredinales) of Triticeae: evolution and extent of coevolution, a cladistic analysis. Bot. J. Linn. Soc. **91**:367-394.

Berg, D.E., and Howe, M.M. (eds) 1989 Mobile DNA. American Society for Microbiology, Washington, D.C.

Bird, A.P. 1986 CpG-rich islands and the function of DNA methylation Nature **321**:209-211.

Birren, B.W., Lai, E., Clark, S.M., Hood, L., and Simon, M.I. 1988. Optimized conditions for pulsed field gel electrophoretic separations of DNA. Nuc. Acids Res. **16**(15):7563-7582.

Böckelmann, B., Osiewacz, H.D., Schmidt, F.R., and Schulte, E. 1985. Extrachromosomal DNA in fungi-organisation and function. In 'Fungal virology' (Buck, K.W. ed.), pp. 237-268. CRC Press, Florida.

Boeke, J.D. 1989 Transposable elements in *Saccharomyces cerevisiae* In 'Mobile DNA' (Berg, D.E. and Howe, M.M. eds) American Society for Microbiology, Washington, D.C.

Botstein, D., White, R.L., Skolnick, M., and Davis, R.W. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am. J. Human Genet. **32**:314-331.

Braithwaite, K., Irwin, J.A.G., and Manners, J. 1990. Ribosomal DNA as a molecular taxonomic marker for the group species *Colletotrichum gloeosporioides*. Mycol. Res. (in press).

Brambl, R., Plesofsky-Vig, N., Hammett, J.R., and Russell, P.J. 1987. Preserved polyadenylated ribonucleic acid in dormant conidia of *Neurospora crassa* and new RNA synthesis during spore germination. Exp. Mycol. **11**:317-330.

Britten, R.J., Graham, D.E., and Neufeld, B.R. 1974. Analysis of repeating DNA sequences by reassociation. In 'Methods in Enzymology. Vol 29: Nucleic acids and protein synthesis. Part E'. (Grossman, L., and Moldave, K. eds.), pp. 363-418. Academic Press, New York.

Broach, J.R. 1982. The yeast plasmid 2μ circle. Cell **28**:203-204.

Buckner, B., Novotny, C.P., and Ullrich, R.C. 1988 Developmental regulation of the methylation of the ribosomal DNA in the basidiomycete fungus *Schizophyllum commune*. Curr. Genet. **14**:105-111.

Buller, A.H.R. 1941. The diploid cell and the diploidization process in plants and animals, with special reference to the higher fungi. Bot. Rev. **7**:335-431.

Burnett, J.H. 1976. Fundamentals of Mycology. 2nd ed. Edward Arnold, London.

Burdon, J.J., and Roelfs, A.P. 1985a. Isozyme and virulence variation in asexually reproducing populations of *Puccinia graminis* and *P. recondita* on wheat. Phytopathology **75**:907-913.

Burdon, J.J., and Roelfs, A.P. 1985b. The effect of sexual and asexual reproduction on the isozyme structure of populations of *Puccinia graminis*. Phytopathology **75**:1068-1073.

Burdon, J.J., and Roelfs, A.P. 1986 Isozymic variation in *Puccinia graminis* f. sp. *tritici* detected by starch gel electrophoresis. Plant Dis. **70**:1139-1191.

Burdon, J.J., Luig, N.H., and Marshall, D.R. 1983. Isozyme uniformity and virulence variation in *Puccinia graminis* f. sp. *tritici* and *P. recondita* f. sp. *tritici* in Australia. Aust. J. Biol. Sc. **36**:403-410.

Burdon, J.J., Marshall, D.R., and Luig, N.H. 1981. Isozyme analysis indicates that a virulent cereal rust pathogen is a somatic hybrid. Nature (Lond.) **293**:565-566.

Burdon, J.J., Marshall, D.R., Luig, N.H., and Gow, D.J.S. 1982 Isozyme studies on the origin and evolution of *Puccinia graminis* f.sp. *tritici* in Australia. Aust. J. Biol. Sci. **35**:231-238.

Burdon, J.J., Roelfs, A.P., and Brown, A.H.D. 1986 The genetic basis of isozyme in the wheat stem rust fungus (*Puccinia graminis tritici*). Can. J. Genet. Cytol. **28**:171-175.

Callow, J.A. 1984. Cellular and molecular recognition between higher plants and fungal pathogens. In 'Cellular Interactions' (Linskens, H.F., and Heslop-Harrison, J. eds.). Encyclopedia of Plant Physiology. **17**:212-237.

Callow, J.A. 1987 Models for host-pathogen interaction In 'Genetics and Plant Pathogenesis' (Day, P.R., and Jellis, G.J., eds.), pp. 283-295. Blackwell Sc. Pub., Oxford.

Carle, G.F., and Olson, M.V. 1985. An electrophoretic karyotype for yeast. PNAS (USA) **82**:3756-3760.

Casselton, L.A., and Herce, A.F. 1989. Heterologous gene expression in the basidiomycete fungus *Coprinus cinereus*. Curr. Genet. **16**:35-40.

Castle, A.J., Horgen, P.A., and Anderson, J.B. 1987 Restriction fragment length polymorphisms in the mushrooms *Agaricus brunnescens* and *Agaricus bitorquis*. Appl. Environ. Micro. **53**:816-822.

Caten, C.E. 1987. The genetic integration of fungal life styles. In 'Evolutionary biology of the fungi' (Rayner, A.D.M., Brasier, C.M., and Moore,D. eds.), pp. 215-229. University Press, Cambridge.

Chang, C., Bowman, J., De John, A., Lander, E., and Meyerowitz, E. 1988. Restriction fragment length polymorphism linkage map for *Arabidopsis thaliana*. PNAS (USA) **85**:6856-6860.

Christiansen, S.K., and Giese, H. 1990. Genetic analysis of the obligate parasitic barley powdery mildew fungus based on RFLP and virulence loci. Theor. Appl. Genet. **79**:705-712.

Chu, G., Vollrath, D., and Davis, R.W. 1986. Separation of large DNA molecules by contour-clamped homogeneous electric fields. Science **234**:1582-1585.

Clark-Walker, G.D., and Sriprakash, K.S. 1981. Sequence rearrangements between mitochondrial DNAs of *Torulopsis glabrata* and *Kloeckera africana* identified by hybridization with six polypeptide encoding regions from *Saccharomyces cerevisiae* mitochondrial DNA. J. Mol. Biol. **151**:367-387.

Cleland, R.E. 1972. Oenothera: Cytogenetics and Evolution. Academic press, London.

Collins, R.A., Stohl, L.L., Cole, M.D., and Lambowitz, A.M. 1981. Characterization of a novel plasmid DNA found in mitochondria of *N. crassa*. Cell **24**:443-452.

Craigie, J.H. 1927. Discovery of the function of the pycnia of rust fungi. Nature **120**:76.

Cullen, D., and Leong, S. 1986. Recent advances in the molecular genetics of industrial filamentous fungi. Trends in Biotech. **4**:285-288.

Cummings, D.J., MacNeil, I.A., Domenico, J., and Matsura, E.T. 1985. Excision-amplification of mitochondrial DNA during senescence in *Podospora anserina*. DNA sequence analysis of three unique "plasmids". J. Mol. Biol. **185**(4):659-680.

Day, P.R. 1974. Genetics of Host-Parasite Interaction. W.H. Freeman, San Francisco.

Debets, A.J.M., Swart, K., Holub, E.F., Goosen, T., and Bos, C.J. 1990 Genetic analysis of *amds* transformants of *Aspergillus niger* and their use in chromosome mapping. Mol. Gen. Genet. **222**:284-290.

Deleu, C., Turcq, B., and Begeret, J. 1990 *Repa*, a repetitive and dispersed DNA sequence of the filamentous fungus *Podospora anserina* Nuc. Acids Res. **18**:4901-4903.

DeLey, J. 1968. Molecular biology and bacterial phylogeny. In 'Evolutionary Biology', Vol. 2 (Dobzhansky, T., Hecht, M.K., and Steere, W.C. eds.), pp. 103-156. North Holland Pub. Co., Amsterdam.

DeLey, J., Cattoir, H., and Reynaerts, A. 1970. The quantitative measurement of DNA hybridization from renaturation rates. Eur. J. Biochem. **12**:133-142.

De Vries, H., and De Jonge, J.C. 1984. Deletion of a protein gene on mitochondrial DNA causes the stopper phenotype in *Neurospora crassa*. In 'EMBO workshop, Gene expression in filamentous fungi', p. 51. Rhenen, Netherlands.

Dickinson, C.H., and Lucas, J.A. 1982. Plant Pathology and Plant Pathogens. 2nd ed. Blackwell Sc. Pub., Oxford.

Dickinson, M.J., and Pryor, A. 1989. Isometric virus-like particles encapsidate the double-stranded RNA found in *Puccinia striiformis*, *Puccinia recondita*, and *Puccinia sorghi*. Can. J. Bot. **67**:3420-3425.

Dons, J.J.M., De Vries, O.M.H., and Wessels, J.G.H. 1979. Characterization of the genome of the basidiomycete *Schizophyllum commune*. Biochem. Biophys. Acta **563**:100-112.

Dutta, S.K., Penn, S.R., Knight, A.R., and Ojha, M. 1972. Characterization of DNAs from *Coprinus lagopus* and *Mucor azygospora*. Experientia **28**:582-584.

Duvell, A., Hessberg-Stutzke, H., Oeser, B., Rogmann-Backwinkel, P., and Tudzynski, P. 1988. Structural and functional analysis of mitochondrial plasmids in *Claviceps purpurea*. Mol. Gen. Genet. **214**:128-134.

Ellingboe, A.H. 1964. Somatic recombination in dikaryon K of *Schizophyllum commune*. Genetics **49**:247-251.

Ellingboe, A.H. 1961. Somatic recombination in *Puccinia graminis* var. *tritici*. Phytopathology **51**:13-14.

Ellingboe, A.H. 1982. Genetical aspects of host defense. In 'Active Defence Mechanisms in Plants' (Wood, R.K.S. ed.), pp. 179-192. Plenum Press, New York.

Ellingboe, A.H., and Raper, J.R. 1962. Somatic recombination in *Schizophyllum commune*. Genetics **47**:85-98.

Engels, W.R. 1983 The P family of transposable elements in *Drosophila*. Ann. Rev. Genet. **17**:315-344.

Evans, I.H. 1983. Molecular genetic aspects of yeast mitochondria. In 'The Molecular Biology of the Yeast *Saccharomyces*, Life Cycle and Inheritance' (Strathern, J.N., Jones, E.W., and Broach, J.R. eds.), p505. Cold Spring Harbor, New York.

Fawcett, D.H., Lister, C.K., Kellett, E., and Finnegan, D.J. 1986. Transposable elements controlling 1-R hybrid dysgenesis in *D. melanogaster* are similar to mammalian LINEs. Cell **47**:1007-1015.

Farkas, V. 1985. The fungal cell wall. In 'Fungal protoplasts: applications in biochemistry and genetics' (Peberdy, J.F., and Ferenczy, L. eds.), pp. 3-29. Marcel Dekker, New York.

Fincham, J.R.S., Day, P.R., and Radford, A. 1979. Fungal Genetics. 4th ed. Blackwell Sc. Pub., Oxford.

Firtel, R.A., and Kindle, K. 1975. Structural organization of the genome of the cellular slime mold *Dictyostelium discoideum*: interspersion of repetitive and single-copy DNA sequences. Cell **5**:401-411.

Flangas, A.L., and Dickson, J.G. 1961. The genetic control of pathogenicity, serotypes and variability in *Puccinia sorghi*. Amer. J. Bot. **48**:275-285.

Flor, H.H. 1942. Inheritance of pathogenicity in *Melampsora lini*. Phytopathology **32**:653-669.

Flor, H.H. 1947. Inheritance of reaction to rust in flax. Ag. Res. **74**:241-262.

Flor, H.H. 1955. Host-parasite interaction in flax rust - its genetics and other implications. Phytopathology **45**:680-685.

Flor, H.H. 1960. The inheritance of X-ray induced mutations to virulence in a urediospore culture of race 1 of *Melampsora lini*. Phytopathology **50**:603-605.

Flor, H.H. 1964. Genetics of somatic variation for pathogenicity in *Melampsora lini*. Phytopathology **54**:823-826.

Flor, H.H. 1965. Inheritance of smooth-spore-wall and pathogenicity in *Melampsora lini*. Phytopathology **55**:724-727.

Flor, H.H. 1971. Current status of the gene-for-gene concept. Annu. Rev. Phytopathol. **9**:275-296.

Förster, H., Kinscherf, T.G., Leong, S.A., and Maxwell, D.P. 1988. Estimation of relatedness between *Phytophthora* species by analysis of mitochondrial DNA. Mycologia **80**(4):466-478.

Fouquet, H., Bierweiler, B., and Sauer, H.W. 1974. Reassociation kinetics of nuclear DNA from *Physarum polycephalum*. Eur. J. Biochem. **44**:407-410.

Francis, D.M., Hulbert, S.H., and Michelmore, R.W. 1990. Genome size and complexity of the obligate fungal pathogen, *Bremia lactucae*. Exp. Mycol. **14** (in press).

Froeliger, E.H., Munoz-Rivas, A.M., Specht, C.A., Ullrich, R.C., and Novotny, C.P. 1987. The isolation of specific genes from the basidiomycete *Schizophyllum commune*. Curr. Genet. **12**:547-554.

Gabriel, D.W., and Rolfe, B.G. 1990. Working models of specific recognition in plant-microbe interactions. Annu. Rev. Phytopathol. **28**:365-391.

Garber, R.C., Turgeon, B.G., and Yoder, O.C. 1984. A mitochondrial plasmid from the plant pathogenic fungus *Cochliobolus heterostrophus*. Mol. Gen. Genet. **196**:301-310.

Giasson, L., and Lalonde, M. 1987. Analysis of a linear plasmid isolated from the pathogenic fungus *Ceratocystis fimbriata* Ell. & Halst. Curr. Genet. **11**:331-334.

Giasson, L., Specht, C.A., Milgrim, C., Novotny, C.P., and Ullrich, R.C. 1989. Cloning and comparison of Aα mating-type alleles of the basidiomycete *Schizophyllum commune*. Mol. Gen. Genet. **218**: 72-77.

Goddard, M.V. 1976a. The production of a new race, 105E 137 of *Puccinia striiformis* in glasshouse experiments. Brit. Mycol. Soc. Trans. **67**:395-398.

Goddard, M.V. 1976b. Cytological studies of *Puccinia striiformis* (yellow rust of wheat). Brit. Mycol. Soc. Trans. **66**:433-437.

Green, M.M. 1980. Transposable elements in *Drosophila* and other Diptera. Annu. Rev. Genet. **14**:109-120.

Haggag, M.E.A., Samborski, D.J., and Dyck, P.L. 1973. Genetics of pathogenicity in three races of leaf rust on four wheat varieties. Can. J. Genet. Cytol. **15**:73-82.

Hamer, J.E., Farrall, L., Orbach, M.J., Valent, B., and Chumley, F.G. 1989. Host species-specific conservation of a family of repeated DNA sequences in the genome of a fungal plant pathogen. PNAS (USA) **86**:9981-9985.

Harada, K., Yukuhiro, K., and Mukai, T. 1990. Transposition rates of movable genetic elements in *Drosophila melanogaster*. PNAS (USA) **87**:3248-3252.

Harshey, R.M., Jayaram, M., and Chamberlin, M.E. 1979. DNA sequence organization in *Phycomyces blakesleeanus*. Chromosoma **73**: 143-151.

Hartley, M.J., and Williams, P.G. 1971. Genotypic variation within a phenotype as a possible basis for somatic hybridization in rust fungi. Can. J. Bot. **49**:1085-1087.

Hashiba, T., Homma, Y., Hyakumachi, M., and Matsuda, I. 1984. Isolation of a DNA plasmid in the fungus *Rhizoctonia solani*. J. Gen. Micro. **130**:2067-2070.

Helentjaris, T., Slocum, M., Wright, S., Schaefer, A., and Nienhuis, J. 1986. Construction of genetic linkage maps in maize and tomato using restriction fragment length polymorphisms. Theor. Appl. Genet. **72**:761-769.

Higgins, T.J.V. and Donald Spencer 1991. The expression of a chimeric cauliflower mosaic virus (CaMV 35S)-Pea Vicilin gene in Tobacco. Plant Science (in press)

Hintz, W.E., Mohan, M., Anderson, J.B., and Horgen, P.A. 1985. The mitochondrial DNAs of *Agaricus*: Heterogeneity in *A. bitorquis* and homogeneity in *A. brunnescens*. Curr. Genet. **9**:127-132.

Holliday, R. 1961. The genetics of *Ustilago maydis*. Genet. Res. **2**: 04-230.

Honeyman, A.L., and Currier, T.C. 1986. Isolation and characterization of linear DNA elements from the mitochondria of *Gaeumannomyces graminis*. Appl. Environ. Microbiol. **52**:924-929.

Hooker, A.L., and Russell, W.A. 1962. Inheritance of resistance to *Puccinia sorghi* in six corn inbred lines. Phytopathology **52**:122-128.

Howes, N.K., Kim, W.K., and Rohringer, R. 1982. Detergent-soluble polypeptides extracted from uredospores of four physiologic races of *Puccinia graminis* f. sp. *tritici*. Physiol. Plant Pathol. **21**(3):361-366.

Howlett, B.J. 1989. An electrophoretic karyotype for *Phytophthora megasperma*. Exp. Mycol. **13**:199-202.

Hulbert, S.H., and Michelmore, R.W. 1988. DNA restriction fragment length polymorphism and somatic variation in the lettuce downy mildew fungus, *Bremia lactucae*. Mol. Plant-Microbe Interact **1**:17-24.

Hudspeth, M.E.S., Timberlake, W.E., and Goldberg, R.B. 1977. DNA sequence organization in the water mold *Achlya*. PNAS (USA) **74**:4332-4336.

Huang, B.-F., and Staples, R.C. 1982. Synthesis of proteins during differentiation of the bean rust fungus. Exp. Mycol. **6**:7-14.

Huang, D., Staples, R.C., Bushnell, W.R., and Maclean, D.J. 1990. Preparation and regeneration of protoplasts from axenic mycelia derived from the wheat stem rust fungus. Phytopathology **80**:81-84.

Johnson, S.A., and Lovett, J.S. 1984. Base sequence complexity of the polyadenylated and non-polyadenylated stored messenger RNA in *Blastocladiella* zoospores. Exp. Mycol. **8**:117-131.

Johnson, T. 1954. Selfing studies with physiologic races of wheat stem rust *Puccinia graminis* var. *tritici*. Can. J. Bot. **32**:506-522.

Kapooria, R.G. 1973. Cytological Studies of basidiospores of *Melampsora lini*. J. Gen. Microbiol. **75**: 241-244.

Karsch, T., Kuck, U., and Esser, K. 1987. Mitochondrial group 1 introns from the filamentous fungus *Podospora anserina* code for polypeptides related to maturases. Nuc. Acids Res. **15**(16):6743-6744.

Kawano, S., Suzuki, T., and Kuriowa, T. 1982. Structural homogeneity of mitochondrial DNA in the mitochondrial nucleoid of *Physarium polycephalum*. Biochim. Biophys. Acta **696**:290-298.

Kidwell, M.G., Kidwell, J.F., Sved, J.A. 1977 Hybrid dysgenesis in *Drosophila melanogaster*: A syndrome of aberrant traits including mutation, sterility, and male recombination. Genetics **36**: 813-823.

Kim, W.K., Shang, H.S., and Samborski, D.J. 1985. Electrophoretic analysis of detergent-soluble polypeptides of *Puccinia recondita* f. sp. *tritici, P. recondita* f. sp. *secalis, P. hordei*, and *P. coronata*. Can. J. Plant Pathol. **7**:287-293.

Kinscherf, T.G., and Leong, S.A. 1988. Molecular analysis of the karyotype of *Ustilago maydis*. Chromosoma **96**:427-433.

Kinsey, J.A. 1990. Tad, a LINE-like transposable element of *Neurospora*, can transpose between nuclei and heterokaryons. Genetics **126**:317-323.

Kinsey, J.A., and Helber, J. 1989. Isolation of a transposable element from *Neurospora crassa*. PNAS(USA) **86**:1929-1933.

Kistler, H.C., and Leong, S.A. 1986. Linear plasmid-like DNA in the plant pathogenic fungus *Fusarium oxysporum* f. sp. *conglutinans*. J. Bacteriol. **167**:587-593.

Kistler, H.C., Bosland, P.W., Benny, U., Leong, S., and Williams, P.H. 1987. Relatedness of strains of *Fusarium oxysporum* from crucifers measured by examination of mitochondrial and ribosomal DNA. Phytopathology **77**:1289-1293.

Konstrad, J.W., and Leong, S.A. 1989. Isolation of two alleles of the b locus of *Ustilago maydis*. PNAS(USA) **86**:978-982.

Krumlauf, R., and Marzluf, G.A. 1979. Characterization of the sequence complexity and organisation of the *Neurospora crassa* genome. Biochem. **18**(17):3705-3713.

Kuck, U., Osiewacz, H.D., Schmidt, U., Kappelhoff, B., Schulte, E., Stahl, U., and Esser, K. 1985. The onset of senescence is affected by DNA rearrangements of a discontinuous mitochondrial gene in *Podospora anserina*. Curr. Genet. **9**:373-382.

Kuiper, M.T.R., and Lambowitz, A.M. 1988. A novel reverse transcriptase activity associated with mitochondrial plasmids of *Neurospora*. Cell **55**:693-704.

Lamb, C.J., Lawton, M.A., Dron, M., and Dixon, R.A. 1989. Signals and transduction mechanisms for activation of plant defenses against microbial attack. Cell **56**:215-224.

Lambowitz, A.M., Akins, R.A., Kelley, R.L., Pande, S., and Nargang, F.E. 1986. Mitochondrial plasmids of *Neurospora* and other filamentous fungi. In 'Extrachromosomal Elements in Lower Eukaryotes' (Wickner, R.B., Hinnebusch, A., Lambowitz, A.M., Gunsalus, I.C., and Hollaender, A. eds.), pp. 83-92. Plenum Press, New York.

Lander, E.S., Green, P., Abrahamson, J., Barlow, A., Daly, M.J., Lincoln, S.E., and Newburg, L. 1987 MAPMAKER: An interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics **1**:174-181.

Landry, B.S., Kesseli, R.V., Farrara, B., and Michelmore, R.W. 1987. A genetic map of lettuce (*Lactuca sativa* L.) with restriction fragment length polymorphism, isozyme, disease resistance and morphological markers. Genetics **116**:331-337.

Larkin, P.J. 1976. Purification and viability determinations of plant protoplasts. Planta (Berl.) **128**:213-216.

Lauer, G.D., Roberts, T.M., and Klotz, L.C. 1977. Determination of the nuclear DNA content of *Saccharomyces cerevisiae* and implications for the organization of DNA in yeast chromosomes. J. Mol. Biol. **114**:507-526.

Lawrence, G.J. 1980. Multiple mating-type specificities in the flax rust *Melampsora lini*. Science **209**:501-503.

Lawrence, G.J. 1988. *Melampsora lini*, rust of flax and linseed. In 'Advances in Plant Pathology' (Ingram, D.S., and Williams, P.H. eds), Vol. 6, pp. 313-331. Academic Press, London.

Lawrence, G.J., Boelen, M.B., and Pryor, A. 1988. Transmission of double-stranded RNAs in flax rust, *Melampsora lini*. Can. J. Bot. **66**:61-66.

Lawrence, G.J., Mayo, G.M.E., and Shepherd, K.W. 1981a. Interactions between genes controlling pathogenicity in the flax rust fungus. Phytopathology **71**:12-19.

Lawrence, G.J., Shepherd, K.W., and Mayo, G.M.E. 1981b. Fine structure of genes controlling pathogenicity in flax rust, *Melampsora lini*. Heredity **46**:297-313.

Levy, M., Romao, J., Marchetti, M.A., and Hamer, J.E. 1991. DNA fingerprinting with a dispersed repeated sequence resolves pathotype diversity in the rice blast fungus. The Plant Cell **3**:95-102.

Linde, D.C., Groth, J.V., and Roelfs, A.P. 1990. Comparison of isozyme and virulence diversity patterns in the bean rust fungus *Uromyces appendiculatus*. Phytopathology **80**:141-147.

Little, R., and Manners, J.G. 1969a. Somatic recombination in yellow rust of wheat (*Puccinia striiformis*). I. The production and possible origin of two new physiologic races. Brit. Mycol. Soc. Trans. **53**:251-258.

Little, R., and Manners, J.G. 1969b. Somatic recombination in yellow rust of wheat (*Puccinia striiformis*). II. Germ tube fusions, nuclear number and nuclear size. Brit. Mycol. Soc. Trans. **53**:259-267.

Loenen, W.A.M., Daniel, A.S., Braymer, H.D. and Murrays, N.E. 1987 Organization and Sequence of the *hsd* Genes of *Escherichia coli* K-12 J. Mol. Biol. **198**:159-170.

Luck, D.J.L., and Reich, E. 1964. DNA in mitochondria of *Neurospora crassa*. Nat. Acad. Sciences **52**:931-938.

Luig, N.H. 1979. Mutation studies in *Puccinia graminis tritici*. Wheat Genet. Symp. Proc. **5**: 533-539.

Luig, N.H., and Watson, I.A. 1961. A study of inheritance of pathogenicity in *Puccinia graminis* var. *tritici*. Proc. Linn. Soc. N.S.W. **86**:217-219.

Luig, N.H., and Watson, I.A. 1970. The effect of complex genetic resistance in wheat on the variability of *Puccinia graminis* f. sp. *tritici*. Proc. Linn. Soc. N.S.W. **95**:22-45.

Luig, N.H., and Watson, I.A. 1972. The role of wild and cultivated grasses in the hybridization of *formae speciales* of *Puccinia graminis*. Aust. J. Biol. Sci. **25**:335-342.

McDonald, B.A., and Martinez, J.P. 1990. Restriction fragment length polymorphisms in *Septoria tritici* occur at a high frequency. Curr. Genet. **17**:133-138.

McGinnis, R.C. 1954. Cytological studies of chromosomes of rust fungi. I. The mitotic chromosomes of *Puccinia graminis*. Can. J. Bot. **31**:522-526.

McGinnis, R.C. 1953. Cytological studies of chromosomes of rust fungi. II. The mitotic chromosomes of *Puccinia coronata*. Can. J. Bot. **32**:213-214.

McGinnis, R.C. 1956. Cytological studies of chromosomes of rust fungi. III. The relationship of chromosome number to sexuality in *Puccinia*. J. Heredity **47**:254-259.

Macko, V. 1981. Inhibitors and stimulants of spore germination and infection structure formation in fungi. In "The Fungal Spore:Morphogenetic Controls" (Turian, G., and Hohl, H.R. eds.), pp. 565-584. Academic Press, New York.

Maclean, D.J. 1982. Axenic culture and metabolism of rust fungi. In 'The Rust Fungi' (Scott, K.J., and Chakravorty, A.K. eds), pp. 37-120. Academic Press, London.

Manners, J.G. 1988 *Puccinia striiformis*, yellow rust (stripe rust) of cereals and grasses In 'Advances in Plant Pathology' (Ingram, D.S., and Williams, P.H. eds), Vol. 6, pp. 373-387. Academic Press, London.

Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. **3**:208-218.

Marmur, J., and Doty, P. 1962. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. J. Mol. Biol. **5**:109-118.

Masel, A., Braithwaite, K., Irwin, J., and Manners, J. 1990. Highly variable molecular karyotypes in the plant pathogen *Colletotrichum gloeosporioides*. Curr. Genet. **18**:81-86.

Mascarenhas, J.P. 1975. The biochemistry of angiosperm pollen development. Bot. Rev. **41**:259-314.

Mascarenhas, J.P. 1989. The male gametophyte of flowering plants. Plant Cell. **1**:657-664.

Meinhardt, F., and Esser, K. 1984. Linear extrachromosomal DNA in the morel *Morchella conica*. Curr. Genet. **8**:15-18

Meinhardt, F., Kempken, F., and Esser, K. 1986. Proteins are attached to the ends of a linear plasmid in the filamentous fungus *Ascobolus immersus*. Curr. Genet. **11**:243-246.

Metz, B.A., Ward, T.E., Welker, D.L., and Williams, K.L. 1983. Identification of an endogenous plasmid in *Dictyostelium discoideum*. EMBO J. **2**:515-519.

Michelmore, R.W., and Hulbert, S.H. 1987. Molecular markers for genetic analysis of phytopathogenic fungi. Annu. Rev. Phytopathol. **25**:383-404.

Murrin, F., Holtby, J., Nolan, R.A., and Davidson, W.S. 1986. The genome of *Entomophaga aulicae (Entomophthorales, Zygomycetes)*: Base composition and size. Exp. Mycol. **10**:67-75.

Mutasa, E.S., Tymon, A.M., Gottgens, B., Mellon, F.M., Little, P.F.R., and Casselton, L.A. 1990. Molecular organization of an α mating type factor of the basidiomycete fungus *Coprinus cinereus* Curr. Genet. **18**: 223-230.

Nargang, F.E. 1985. Fungal mitochondrial plasmids. Exp. Mycol. **9**:285-293.

Nargang, F.E., Bell, J.B., Stohl, L.L., and Lambowitz, A.M. 1984. The DNA sequence and genetic organization of a *Neurospora* mitochondrial plasmid suggest a relationship to introns and mobile elements. Cell **38**:441-453.

Natvig, D.O., May, G., and Taylor, J.W. 1984. Distribution and evolutionary significance of mitochondrial plasmids in *Neurospora* spp. J. Bacteriol. **159**:288-293.

Nelson, R.R., Wilcoxson, R.D., and Christensen, J.J. 1955. Heterocaryosis as a basis for variation in *Puccinia graminis* var. *tritici*. Phytopathology **45**:639-643.

Newton, A.C. 1987. Markers in pathogen populations. In 'Genetics and Plant Pathogenesis' (Day, P.R., and Jellis, G.J., eds.), pp. 187-194. Blackwell Sc. Pub., Oxford.

Newton, M., and Johnson, T. 1927. Colour mutations in *Puccinia graminis tritici* (Pers.) Erikss. and Henn. Phytopathology **17**:711-725.

Newton, A.C., Caten, C.E., and Johnson, R. 1985. Variation for isozymes and double-stranded RNA among isolates of *Puccinia striiformis* and two other cereal rusts. Plant Pathology **34**:235-247.

Newton, M., Johnson, T., and Brown, A.M. 1930. A study of the inheritance of spore colour and pathogenicity in crosses between physiologic forms of *Puccinia graminis tritici*. Sci. Agr. **10**:775-798.

Nicolas, A., Hamaza, H., Mekki-Berrada, A., Kalogeropoulos, A. and Rossignol, J.-L. (1987) Premeiotic and meiotic instability generates numerous *b2* mutation derivatives in *Ascobolus* Genetics **116**:33-43

O'Brien, S.J. 1990 (ed) Genetic maps: locus maps of complex genomes 5th Ed. Cold Spring Harbor N.Y.

O'Dell, M., Wolfe, M.S., Flavell, R.B., Simpson, C.G., and Summers, R.W. 1989. Molecular variation in populations of *Erysiphe graminis* on barley, oats and rye. Plant Pathol. **38**:340-351.

Ojha, M., Turler, H., and Turian, G. 1977. Characterization of the *Allomyces* genome. Biochim. Biophys. Acta. **478**:377-391.

Ono, B., and Ishino-Arao, Y. 1988. Inheritance of chromosome length polymorphisms in *Saccharomyces cerevisiae*. Curr. Genet. **14**:413-418.

Orbach, M.J., Vollrath, D., Davis, R., and Yanofsky, C. 1988. An electrophoretic karyotype of *Neurospora crassa*. Mol. Cell. Biol. **8**:1469-1473.

Person, C.O., and Mayo, G.M.E. 1974. Genetic limitations on models of specific interactions between a host and its parasite. Can. J. Bot. **52**:1339-1347.

Pontecorvo, G. 1956. The parasexual cycle in fungi. Annu. Rev. Microbiol. **10**:393-400.

Pryor, A. 1987. The origin and structure of fungal disease resistance genes in plants. Trends in Genetics 3(6):157-161.

Pryor, A.J., and Boelen, M.G. 1987. Virus-like particles containing dsRNA from the rust-fungus *Puccinia sorghi* Schw. Can. J. Bot. **65**:2380-2383.

Pryor, A., Boelen, M.G., Dickinson, M.J., and Lawrence, G.J. 1990. Widespread incidence of double-stranded RNAs of unknown function in rust fungi. Can. J. Bot. **68**:669-676.

Raeder, U., and Broda, P. 1984. Comparison of the lignin-degrading white rot fungi *Phanerochaete chrysosporium* and *Sporotrichum pulverulentum* at the DNA level. Curr. Genet. **8**:499-506.

Rambosek, J., and Leach, J. 1987. Recombinant DNA in filamentous fungi: progress and prospects. CRC Crit. Rev. in Biotech. 6(4):357-392.

Raper, C.A. 1983. Controls for development and differentiation of the dikaryon in basidiomycetes. In 'Secondary metabolism and differentiation in fungi' (Bennett, J.W., and Ciegler, A. eds.), pp. 195-238. Marcel Dekker, New York.

Raper, J.R., Krongelb, G.S., and Baxter, M.G. 1958. The number and distribution at the incompatibility factors in *Schizophyllum commune*. Am. Nat. **92**:221-232.

Roelfs, A.P., and Groth, J.V. 1988. *Puccinia graminis* f. sp. *tritici*, black stem rust of *Triticum* spp. In 'Advances in Plant Pathology' (Ingram, D.S., and Williams, P.H. eds.), Vol. 6, pp. 345-361. Academic Press, London.

Sahasrabudhe, N.A., and Ranjekar, P.K. 1985. Genome complexity of a powerful cellulolytic fungus *Penicillium funiculosum*. FEMS Micro. letters **30**:295-300.

Samac, D.A., and Leong, S.A. 1988. Two linear plasmids in mitochondria of *Fusarium solani* f. sp. *cucurbitae*. Plasmid **19**:57-67.

Samac, D.A., and Leong, S.A. 1989a. Lack of association of mitochondrial plasmids and pathogenicity in *Nectria haematococca (Fusarium solani* f. sp. *cucurbitae)*. Mol. Plant-Microbe Interact **2**:128-131.

Samac, D.A., and Leong, S.A. 1989b. Mitochondrial plasmids of filamentous fungi: characteristics and use in transformation vectors. Mol. Plant-Microbe Interact. Vol **2**(4):155-159.

Samborski, D.J., and Dyck, P.L. 1968. Inheritance of virulence in wheat leaf rust on the standard differential wheat varieties. Can. J. Genet. Cytol. **10**:24-32.

Samborski, D.J., and Dyck, P.L. 1976. Inheritance of virulence in *Puccinia recondita* on six back cross lines of wheat with single genes for resistance to leaf rust. Can. J. Bot. **54**:1666-1671.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor, New York.

Saxena, K.M., and Hooker, A.L. 1968. On the structure of a gene for disease resistance in maize. PNAS (USA) **61**:1300-1305.

Schafer, J.F., and Roelfs, A.P. 1985. Estimated relation between numbers of urediniospores of *Puccinia graminis* f. sp. *tritici* and rates of occurrence of virulence. Phytopathology **75**(7):749-750.

Schulz, B., Banuett, F., Dahl, M., Schlesinger, R., Schafer, W., Martin, T., Herskowitz, I., and Kahmann, R. 1990. The b alleles of *U. maydis*, whose combinations program pathogenic development, code for polypeptides containing a homeodomain-related motif. Cell **60**:295-306.

Schwartz, D.C., and Cantor, C.R. 1984. Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. Cell **37**:67-75.

Schwinghamer, E.A. 1959. The relation between radiation dose and the frequency of mutations for pathogenicity in *Melampsora lini*. Phytopathology **49**:260-269.

Scott, M.P., Tamkun, J.W., and Hartzell, G.W. III. 1989. The structure and function of the homeodomain. Biochim. Biophys. Acta **989**:25-48.

Shepherd, K.W. 1963 PhD thesis, University of Adelaide, South Australia.

Sidhu, G.S. (ed) 1988. Genetics of Plant Pathogenic Fungi. Vol. 6 of 'Advances in Plant Pathology' (Ingram, D.S., and Williams, P.H., eds.). Academic Press, London.

Simmonds, N.W. 1976 (ed) Evolution of crop plants. Longman, London.

Singh, S.J. and McIntosh, R.A. 1990 Linkage and expression of genes for resistance to leaf rust and stem rust in triticale. Genome **33**:115-118.

Smith, C.L., Matsumoto, T., Niwa, O., Kico, S., Fan, J.-B., Yanagida, M., and Cantor, C.R. 1987. An electrophoretic karyotype for *Schizosaccharomyces pombe* by pulsed field gel electrophoresis. Nucleic Acids Res. **15**:4481-4489.

Snell, R.G., and Wilkins, R.J. 1986. Separation of chromosomal DNA molecules from *C. albicans* by pulsed field gel electrophoresis. Nucleic Acids Res. **14**:4401-4406.

Sober, H.A. 19 (ed) Handbook of Biochemistry, Selected data for Molecular Biology Section H, p. 11. The Chemical Rubber Co. Cleveland, Ohio.

Sousa, C.N.A. de 1975 A comparison of strains of *Puccinia graminis* f.sp. *tritici* from Angola and Rhodesia with their counterparts in Australia. M.Ag. Thesis, University of Sydney.

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503-517.

Southern, E.M., Anand, R., Brown, W.R.A., and Fletcher, D.S. 1987. A model for the separation of large DNA molecules by crossed field gel electrophoresis. Nuc. Acids Res. **15**(15):5925-5943.

Specht, C.A., Munoz-Rivas, A., Novotny, C.P., and Ullrich, R.C. 1988. Transformation of *Schizophyllum commune*: an analysis of parameters for improving transformation frequencies. Exp. Mycol. **12**:357-366.

Spradling, A.C., and Rubin, G.M. 1981. *Drosophila* genome organization: conserved and dynamic aspects. Annu. Rev. Genet. **15**:219-264.

Steensma, H.Y., de Jongh, F.C.M., and Linnekamp, M. 1988. The use of electrophoretic karyotypes in the classification of yeasts: *Kluyveromyces marxianus* and *K. lactis*. Curr. Genet. **14**:311-317.

Stevens, B.J., and Moustacchi, E. 1971. ADN satellite α et molécules circulaires torsadées de petite taille chez la levure *Saccharomyces cerevisiae*. Exp. Cell. Res. **64**:259-266.

Storck, R., and Alexopoulos, C.J. 1970. Deoxyribonucleic acid of fungi. Bact. Reviews **34**(2):126-154.

Szecsi, A. 1981. Kinetic complexity and repetitiveness of *Fusarium graminearum* nuclear DNA. Exp. Mycol. **5**:323-329.

Szybalski, W. 1968. Use of cesium sulphate for equilibrium density gradient centrifugation. In 'Methods in Enzymology Vol. 12: Nucleic acids Part B. (Grossman and Moldave eds) Academic press, New York.

Tanksley, S.D., Bernatzky, R., Lapitan, N.L., and Prince, J.P. 1988. Conservation of gene repertoire but not gene order in pepper and tomato. PNAS (USA) **85**:6419-6423.

Taylor, E.C. 1983. PhD Thesis, University of Southampton, England.

Taylor, J.W. 1986. Fungal evolutionary biology and mitochondrial DNA. Exp. Mycol. **10**:259-269.

Timberlake, W.E. 1978. Low repetitive DNA content in *Aspergillus nidulans*. Science **202**:973-974.

Tudzynski, P., Duvell, A., and Esser, K. 1983. Extra-chromosomal genetics of *Claviceps purpurea*. I. Mitochondrial DNA and mitochondrial plasmids. Curr. Genet. **7**:145-150.

Turner, G., and Ballance, D.J. 1987. Structural variation and expression of fungal chromosomal genes. In 'Evolutionary biology of the fungi' (Rayner, A.D.M., Brasier, C.M., and Moore, D. eds.), pp. 17-32. University Press, Cambridge.

Turner, G., Earl, A.J., and Greaves, D.R. 1982. Interspecies variation and recombination of mitochondrial DNA in the *Aspergillus nidulans* species group and the selection of species-specific sequences by nuclear background. In 'Mitochondrial genes' (Slonimski, P., Borst, P., and Attardi, G. eds.), p441. Cold Spring Harbor, New York.

Tzeng, T., Chang, H., and Bronson, C.R. 1989 Electrophoretic confirmation of chromosome rearrangements in *Cochliobolus heterostrophus* translocation at *tox1*. Phytopathology **79**:1204.

Ullrich, R.C., Droms, K.A., Doyon, J.D., and Specht, C.A. 1980. Characterization of DNA from the basidiomycete *Schizophyllum commune*. Exp. Mycol. **4**:123-134.

Ullrich, R.C., Novotny, C.P., Specht, C.A., Froeliger, E.H., and Munoz-Rivas, A.M. 1985. Transforming basidiomycetes. In 'Molecular Genetics of Filamentous Fungi' (Timberlake, W.E. ed.), pp. 39-57. Alan R. Liss Inc., New York.

Valkoun, J., and Bartos, P. 1974. Somatic chromosome number in *Puccinia recondita*. Brit. Mycol. Soc. Trans. **63**:187-189.

Van Etten, H.D., Matthews, D E. and Matthews, P.S. 1989. Phytoalexin detoxification: importance for pathogenicity and practical implications Ann. Rev. Phytopathol. **27**:143-164.

Van Etten, J.L., Dahlberg, K.R., and Russo, G.M. 1981. Nucleic acids. In 'The Fungal Spore: Morphological Controls' (Turian, G., and Hohl, H.R. eds.), pp. 277-299. Academic Press, New York.

Vieira, J., and Messing, J. 1987. Production of single-stranded plasmid DNA. In 'Methods in Enzymology. Vol. 153: Recombinant DNA. Part D' (Wu, R., and Grossman, L. eds.), pp. 3-11. Academic Press, San Diego.

Wahl, I., Anikster, Y., Manisterski, J., and Segal, A. 1984 Evolution at the center of origin In 'The Cereal Rusts' Vol. 1 (Bushnell, W.R. and Roelfs, A.P. eds.) Academic press, Orlando, Florida.

Wallace, A.T., and Luke, H.H. 1961. Induced mutation rates with gamma rays at a specific locus in oats. Crop Sci. **1**:93-96.

Wallace, D.C. 1982. Structure and evolution of organelle genomes. Microbiol. Rev. **46**:208-240.

Wang, J., Holden, D.W., and Leong, S.A. 1988. Gene transfer system for the phytopathogenic fungus *Ustilago maydis*. PNAS (USA) **85**:865-869.

Ward, B.L., Anderson, R.S., and Bendich, A.J. 1981. The mitochondrial genome is large and variable in a family of plants (*Cucurbitaceae*). Cell **25**:793-803.

Watson, I.A. 1981. Wheat and its rust parasites in Australia. In 'Wheat science - today and tomorrow' (Evans, L.T., and Peacock, W.J. eds.), pp. 129-147. University Press, Cambridge.

Watson, I.A., and Luig, N.H. 1958. Somatic hybridization in *Puccinia graminis* var. *tritici*. Pro. Linn. Soc. N.S.W. **83**:190-195.

Watson, I.A., and Luig, N.H. 1959. Somatic hybridization between *Puccinia graminis* var. *tritici* and *Puccinia graminis* var. *secalis*. Proc. Linn. Soc. N.S.W. **84**:207-208.

Wesolowski, M., and Fukuhara, H. 1981. Linear mitochondrial deoxyribonucleic acid from the yeast *Hansenula mrakii*. Mol. Cell. Biol. **1**:387-393.

Wheeler, H.E., and Luke, H.H. 1955. Mass screening for disease-resistant mutants in oats. Science **122**:1229.

Whitehouse, H.L.K. 1949. Heterothallism and sex in the fungi. Biol. Rev. **24**:411-447.

Wright, R.G., and Lennard, J.H. 1980. Origin of a new race of *Puccinia striiformis*. Brit. Mycol. Soc. Trans. **74**:283-287.

Wilcoxson, R.D., and Paharia, K.D. 1958. A study of the progeny from the self-fertilization of race III of *Puccinia graminis* var. *tritici*. Phytopathology **48**:644-645.

Zolan, M.E., and Pukkila, P.J. 1985. DNA methylation in *Coprinus cinereus*. In 'Molecular Genetics of Filamentous Fungi' (Timberlake, W.E. ed.), pp. 333-344. Alan R. Liss Inc., New York.

Young, N.D. 1990. Potential applications of map-based cloning to plant pathology. Physiol. Mol. Plant Path. **37**:81-94.