GENETIC DIVERSITY AMONG POPULATIONS OF THE TAKE-ALL FUNGUS *Gaeumannomyces graminis*

by

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Errata

The following amendments to this thesis should be noted

Chapter 2, pp. 10 and 21: for 'Hartyl' read 'Hartl'

Chapter 3, p. 25: for 'asses' read 'assess'

Chapter 3, p. 30: for 'hierachical' read 'hierarchical'

Chapter 3, p. 36: for 'G. graminis' read '*G. graminis*'

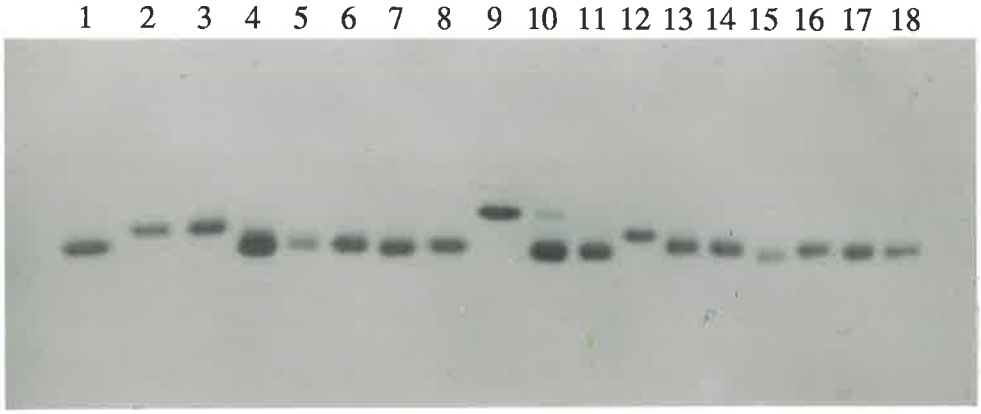
Chapter 5, p. 73: for 'reproduceability' read 'reproducibility'

Throughout the text:

For	Read	For	Read
'BamH I'	'*Bam*H I'	'Hpa II'	'*Hpa* II'
'Dra I'	'*Dra* I'	'Pst I'	'*Pst* I'
'Hae III'	'*Hae* III'	'Pvu II'	'*Pvu* II'
'Hind III'	'*Hind* III'		

The following is a typical example of an isozyme gel (Chapters 3 and 6).

Figure Allozyme phenotypes of 18 *G. graminis* isolates at the glucose-6-phosphate isomerase (GPI) locus. Relative mobility (R_f) values of each allele are given in parenthesis. Lanes: 1-18; *Ggt*- 500 (0.94), *Gga*- 192M (1.00), *Ggg*- 51652 (1.00), CV16 (0.94), NN17 (0.94), WN14 (0.94), BB26 (0.94), DN12 (0.94), *Ggg*- H4 (1.06), MD27 (0.88), WI14 (0.88), HM28 (1.00), BT22 (0.94), HS13 (0.94), TW15 (0.88), WP30 (0.94), GM30 (0.94), TM24 (0.94). See tables 3.1 and 6.1 for identities of isolates.



Declaration

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

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

Paul Harvey

To Joanne

(050168 - 110792)

Anar kaluva tielyanna!

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Summary

This project sought to assess the extent and distribution of genetic diversity within and between populations and varieties of the take-all pathogen *Gaeumannomyces graminis*, throughout the cereal belt of southern Australia. A prerequisite for this study was the establishment of a range of genetic markers to differentiate between the three taxonomic varieties of the pathogen (*G. graminis* var. *tritici* - *Ggt*, var. *avenae* - *Gga* and var. *graminis* - *Ggg*). Isozyme, RFLP and PCR techniques were used to assess levels of genetic diversity within a representative collection of twenty isolates. Each method provided a number of variety-specific markers. High levels of genetic diversity were detected within *Ggg* (Shannon's index: H_0 0.978 and 1.506 for isozymes and RFLPs, respectively) and *Ggt* (H_0 0.760 and 1.450) in comparison to *Gga* (H_0 0.305 and 0.466). Cluster analyses of genetic similarities revealed that *Gga* and *Ggt* isolates formed discrete groups, whereas *Ggg* was divided into two subgroups, one showing a closer relationship with *Ggt* than *Gga* and the other dissimilar to both varieties. These results justify the designation of the three pathogenic and morphologic variants as separate taxa.

The genetic structure of 16 pathogen populations (totalling 480 isolates) from three cereal species was examined by analysis of variation with isozyme, RFLP and PCR markers. The taxonomic composition of pathogen populations was determined by low-copy RFLP phenotypes and PCR directed amplification of sequences derived from intron-exon splice junctions. Isozyme and DNA repeat-sequence phenotypes were highly variable within and between populations and were of limited value in determining the taxonomic identity of pathogen isolates.

Population analyses have indicated the widespread distribution of *Ggt* and *Gga* throughout the cereal producing regions of southern Australia and also their coexistence within many populations. The ability to discriminate between varieties existing within the one location implies that inter-variety sexual hybridisation must be a rare event. RFLP phenotypes of *Gga* isolates from turf grass (*Agrostis* spp.) were not detected in

populations isolated from cereals and implied that *Gga* may be differentiated into pathotypes which are adapted to parasitising cereals and turf grasses. RFLP and PCR analyses indicated that *Ggg* was absent from areas of intensive cereal production.

High levels of genetic diversity were detected within *G. graminis* in comparison to foliar plant pathogenic fungi. The RFLP and PCR analysis revealed approximately twice as much diversity (Hsp 2.212 and 2.539, respectively) as that resolved with isozymes (Hsp 1.001). In general, populations which were dominated by *Ggt* exhibited greater variation, whereas those populations which contained high frequencies of *Gga* contained lower levels of genetic diversity.

Each analysis indicated that most of the diversity was apportioned between populations of *G. graminis*. This distribution of variation implied that self-fertilisation, coupled with asexual propagation, may be the primary modes of reproduction and suggests that populations are evolving in relative isolation in the absence of high levels of inter-population gene flow. Consequently, most of the differentiation between populations may be attributed to genetic drift.

Cluster analyses of the genetic identities among populations showed no correlation between their genetic and geographical identities. This implied that geographically restricted pathotypes have not evolved within Australia. Populations were grouped according to their taxonomic composition (*Ggt* or *Gga*) and revealed two distinct evolutionary lineages within *G. graminis*, with average overall genetic identities of 0.60 and 0.15 for isozymes and RFLPs, respectively. By the criterion of genetic differentiation applied in defining species limits, these values were low enough to indicate that *Ggt* and *Gga* may represent different species. Analyses of genetic similarities estimated from DNA repeat-sequences indicated a random distribution of phenotypes between populations, which did not correlate with the genetic identities of populations estimated from isozyme or low-copy RFLP loci.

It is concluded that *G. graminis* is a highly variable species. This may be a reflection of the broad host range of the pathogen and the apparent lack of resistance in many of its host species. The relative intensity of host-mediated selection appears to be

an important factor affecting levels of genetic diversity within populations. Cultivation of oats applies strong selection which results in a predominance of genetically homogeneous *Gga* isolates within populations. In contrast, exposure to susceptible hosts, such as wheat and barley, appears to maintain variation within populations by not actively selecting out particular genotypes of the pathogen. Consequently, the genetic constitution of individual populations appears to be strongly influenced by genetic drift and fluctuations in size and taxonomic composition, resulting from host-mediated selection.

Chapter 1 Introduction

Gaeumannomyces graminis is a soil-borne, filamentous, ascomycete fungus known to parasitise a diverse range of Gramineous hosts (Scott, 1981; Nilsson and Drew Smith, 1981). The species has been taxonomically divided into three varieties on the basis of limited morphological characteristics and differences in pathogenicity upon cereal host species (Walker, 1972).

Take-all (caused by the fungus *Gaeumannomyces graminis* (Sacc.) von Arx & Olivier var. *tritici* Walker - henceforth referred to as *Ggt*) is the most serious root disease of wheat and barley occurring throughout the cereal producing regions of southern Australia (Cotterill and Sivasithamparam, 1989). *G. graminis* var. *avenae* (Turner) Dennis (*Gga*) also causes take-all in wheat and barley and is distinguished from *Ggt* by its pathogenicity upon oats. *G. graminis* var. *graminis* (*Ggg*) colonises the roots of wheat and barley, but is only mildly pathogenic and does not cause severe take-all symptoms (Walker, 1972).

G. graminis is known to cause substantial and chronic yield losses in cereal crops and has resulted in the cessation of wheat production in some areas of high disease incidence in the southern portion of the Western Australian cereal belt. Yield losses attributed to take-all in the cereal belt of southern Australia average 5-10% (Rovira, 1980; Murray and Brown, 1987) and can reach 40% (Cotterill and Sivasithamparam, 1989).

Wheat cultivars exhibiting resistance to *G. graminis* have not been identified, despite the screening of thousands of genotypes (Scott, 1981). Currently, the only effective method for short term disease control involves attempts to reduce inoculum levels by the use of non-susceptible hosts in regular rotation with cereals (Cotterill and Sivasithamparam, 1988).

The widespread and uniform susceptibility of wheat contrasts with the levels of resistance shown by the other temperate cereals, e.g. barley generally shows an increased tolerance to take-all, rye is considered to be moderately resistant and oats are

highly resistant to *Ggt* but susceptible to *Gga* (Scott, 1981). Within crops of these hosts the effects of take-all are not uniform, indicating that the pathogen populations exhibit considerable variation in virulence (Asher, 1981; Hollins and Scott, 1990). The combination of pathogen variability and contrasting levels of host susceptibility raises several important questions concerning the genetic composition of pathogen populations likely to be parasitising these crops:

(i) Do populations of *G. graminis* parasitising highly susceptible crops, exhibit comparable levels of genetic diversity with populations parasitising less susceptible crops?

(ii) Do strains capable of parasitising different host species coexist within the one pathogen population? If so, do these strains represent the three taxonomic varieties of *G. graminis* or are they different pathotypes (races) within the one variety?

(iii) What are the genetic relationships between the taxonomic varieties of *G. graminis*? Do they represent distinct gene pools which are reproductively isolated or are they arbitrary subdivisions of the one broad gene pool?

Answers to these questions require that pathogen populations be screened with molecular markers, capable of resolving various levels of genetic diversity. Intra-specific classification of *G. graminis* by morphological and pathogenic characteristics often proves to be inconclusive (Cunningham, 1981; Yeates, 1986). Therefore, biochemical markers, such as isozymes and direct genetic markers, such as DNA polymorphisms, are necessary. Markers useful in detecting intra-population variation need to resolve differences between closely related individuals, which may require targeting highly variable regions of the genome. Variety-specific markers, however, require the detection of conserved sequences within varieties but differentiation of these sequences between varieties (Michelmore and Hubert, 1987).

The ability to differentiate between the three varieties of *G. graminis* is of primary importance as it will provide information on take-all epidemiology and may indicate if

host-mediated selection is a factor responsible for changes in the genetic composition of pathogen populations (Leung and Williams, 1986; O'Dell *et al.*, 1989). Furthermore, the development of discrete, variety-specific markers may also determine if inter-variety hybridisation can be implicated in the evolution of pathotype diversity within *G. graminis*. Documentation of these events is crucial in developing an understanding of the evolutionary dynamics of pathogen populations (Burdon and Marshall 1981; Levy *et al.*, 1991; Priestley *et al.*, 1992).

The description of existing and potential variation within *G. graminis*, underlaid with knowledge of the agroecological and biological bases for variation, is of value in predicting changes within the genetic structure of pathogen populations and should provide information on the evolutionary capacity of the pathogen to respond to control methods. In the absence of broadly effective host resistance, long-term control of take-all will only be achieved through an enhanced knowledge of the genetic diversity within *G. graminis* and its genetic interactions with its cereal hosts.

In light of the above, this project was developed with the following aims:

1. To establish a suite of molecular markers which could be used to differentiate between the three taxonomic varieties of *G. graminis*. These markers could also be used to determine the evolutionary relationships between the varieties.
2. To establish the extent and distribution of genetic variation within and between populations and varieties of *G. graminis* using a broad range of genetic loci. The variety-specific markers would be used to determine the taxonomic composition of *G. graminis* populations and to identify inter-variety hybrids.
3. To determine the factors responsible for the generation and maintenance of genetic variation within and between populations. This will provide much needed information concerning take-all epidemiology and the evolutionary dynamics of interactions between the pathogen and its cereal hosts.

This investigation seeks to complement research concerned with the agroecology and biological control of *G. graminis*, with an integrated approach leading to the development of more effective control measures.

Chapter 2 Literature Review

2.1 Taxonomy and distribution of *G. graminis*

Take-all was first recognised as a disease of cereals in South Australia in 1852, although the causal agent of the disease was not identified until 1904. The first correct assignment of the take-all fungus with disease symptoms on wheat was in France, in 1890, where the pathogen was identified as *Ophiobolus graminis* Sacc. (Garrett, 1981). In 1952 von Arx and Olivier recognised that the wheat take-all fungus did not belong in the genus *Ophiobolus*, and established the new genus *Gaeumannomyces*, type species *G. graminis* (Walker, 1981).

Walker (1972) divided the species into three taxonomic varieties. *Ggt* is the causal agent of the take-all disease in wheat and barley and is ranked among the most damaging root disease of these crops world wide. *Gga* causes take-all disease in oats and turf grasses, in addition to wheat and barley, and generally has longer ascospores than *Ggt*. Both of these varieties also produce simple hyphopodia and distinction between them is based entirely upon ascospore length and pathogenicity toward oats (Walker, 1972). *Ggg* is only mildly pathogenic upon grasses and cultivated cereals, apparently due to its inability to invade the vascular tissues of the root (Skou, 1981). Nevertheless, it is known to colonise the outer cortical layers, and has been suggested as a possible biological control agent in preventing infection by *Ggt* and *Gga* (Wong, 1981). *Ggg* produces both lobed and simple hyphopodia and has ascospores in the same size range as the other two varieties (Walker, 1972). Consequently, *Ggg* isolates with simple hyphopodia may only be differentiated from *Ggt* and *Gga* by their inability to colonise stelar tissues and produce severe take-all symptoms.

Yeats *et al.* (1986) reported that isolates capable of infecting oats but morphologically characterised as *Ggt* were widely distributed throughout Western Australian cereal crops. Comparisons with *Gga* type specimens isolated from *Agrostis*

spp. (turf grass) showed that these oat-attacking isolates should be included as a specific race within *Ggt*, rather than reclassified as *Gga*. Chambers and Flentje (1967), however, considered that pathogenicity upon oats was a more reliable criterion for the separation of *Ggt* and *Gga* than ascospore length. Avenacin in oat roots, confers resistance to *Ggt* (Maizel *et al.*, 1964). The enzyme avenacinase, produced in high concentrations by only *Gga*, degrades this compound providing a mechanism to overcome the resistance (Osbourn *et al.*, 1991).

G. graminis has a cosmopolitan distribution in temperate climates and also occurs in cereal crops at high altitudes in subtropical and tropical regions (Garrett, 1981). It is known to parasitise a diverse range of plant species within the family Gramineae, most of which are highly susceptible to infection (Scott, 1981; Nilsson and Drew Smith, 1981). Severe take-all occurred in wheat crops in the first season of production after the land had been cleared of native vegetation (Garrett, 1981; Cotterill and Sivasithamparam, 1989), indicating that *G. graminis* was indigenous to Australia and was not introduced from Europe with the advent of cereal cropping. Consequently, *G. graminis* probably has a long history of coevolution with a diverse range of Gramineous hosts.

2.2 Ecology of *G. graminis*

Of critical importance to the evolutionary dynamics of plant pathogens is the environment in which they exist. This is composed of biotic factors (other organisms and especially their host plants) and abiotic factors (climatic and edaphic conditions).

Inoculum of *G. graminis* can be either soil-borne or wind-borne. Soil-borne inoculum is considered to be the primary source for repeated outbreaks of take-all within the cereal belt, whereas wind-borne inoculum, in the form of ascospores and infected plant materials, is thought to be of limited importance for the perpetuation of the disease (Hornby, 1981).

G. graminis parasitises many native and introduced annual grasses and the mycelium already present in the roots acts as the source of initial inoculum when

cereals are planted. Within the cereal-producing regions of southern Australia the absence of alternative host species (e.g. annual grasses) during the summer months is thought to restrict *G. graminis* to surviving saprophytically in host debris between cropping seasons (Cotterill and Sivasithamparam, 1989). Under more temperate conditions, *G. graminis* may be maintained between growing seasons in its parasitic form on the living roots of weed grasses and self-sown cereals, as well as saprophytically in host debris.

The density of inoculum changes due to reproduction, death and migration. The reservoir of inoculum in soil is increased or maintained by the parasitic potential of the fungus and its saprophytic activity. Death of inoculum results from antagonism and autolysis. Antagonism is a broad term that describes mechanisms that involve direct damage to the inoculum by biological agents other than the host and include competition and antibiosis. Autolysis, due to a shortage of either endogeneous or exogeneous nutrients, is considered to be the most important mechanism responsible for the reduction of soil-borne inoculum (Baker, 1968).

During the saprophytic phase, *G. graminis* may be subject to microbial antagonism associated with the decomposition of colonised host debris (Rovira and Wildermuth, 1981). In southern Australia a decline in infectivity and propagule number has been shown to occur between harvest and sowing of consecutive wheat crops, probably associated with the microbial degradation of colonised host debris during the warm-moist conditions in late autumn or early winter (Cotterill and Sivasithamparam, 1988). In contrast, increases in infectivity of *G. graminis* were observed when soils were hot and dry, conditions which do not support large populations of antagonistic microbiota. The nature of the microbial populations is a major factor which determines whether soils are conducive or suppressive to pathogens. In general, nutrient-poor, sandy, alkaline soils are very favourable for saprophytic growth of *G. graminis* and are relatively conducive for pathogenic growth of the fungus (Yarham, 1981). Severe take-all has also been reported on sandy acidic soils, characteristic of much of the Western Australian cereal belt (Glenn and Parker, 1988).

Microbial antagonism of *G. graminis* is thought to be prevalent in temperate climates in soils which are more organically rich and support relatively high populations of soil microbiota (for a review see Rovira and Wildermuth, 1981). In these environments continuous cereal cropping often results in a build up of antagonistic soil bacteria which act to reduce the severity of the disease. This suppression of fungal infectivity is known as take-all decline (T.A.D.). In the southern Australian cereal belt, however, the Mediterranean climate combined with the prevalence of leached, nutrient-poor soils results in lower numbers of antagonistic soil microrganisms and a perpetuation of *G. graminis* inoculum. Consequently, saprophytic survival, in the absence of microbial antagonists, is thought to be a significant aspect of disease build-up, commonly observed in the Australian grain belt.

The broad host range and widespread distribution of *G. graminis* throughout the cereal belt of southern Australia imply that the fungus is expected to exhibit high levels of genetic variability among isolates recovered from different locations or host species. This is supported by studies of other phytopathogenic fungi where pathogens which lack host specialisation, such as *Atkinsonella hypoxylon* (Leuchtmann and Clay, 1989) and *Peridermium harknessii* (Tuskan *et al.*, 1990) showed considerable genetic heterogeneity. In contrast, two host-specialised pathogens, *Magnaporthe grisea* (Leung and Williams, 1986) and *Erysiphe graminis* f. sp. *hordei* (Koch and Kohler, 1991), were genetically homogeneous.

2.3 Factors responsible for the generation and maintenance of genetic diversity

Fundamental to understanding the evolution of *G. graminis* is information about the amount and nature of genetic variation in populations and why such variation exists. There are five major factors which contribute to changes in gene frequency in phytopathogen populations; mutation, migration, finite population size, genetic drift and natural selection.

2.3.1 Mutation

G. graminis is haploid throughout the majority of its life cycle (Asher, 1981) and consequently any mutations which do arise will be expressed in the phenotype of the organism and if non-lethal, will directly contribute to the overall diversity within populations. Mutation may involve simple point mutations and/or relatively large scale genomic rearrangements. The nuclear genomes of eukaryotes are subject to continual turnover through unequal exchange, gene conversion and DNA transposition, processes which are known to operate both within and between chromosomes. These molecular mechanisms of genome rearrangement constitute a mechanism for the generation of genetic diversity and the origin of evolutionary novelties (Dover, 1982).

Some families of repetitive DNA sequences are subject to continual changes in their composition, copy number and chromosomal location and significantly contribute to overall levels of genetic diversity within species (Flavell, 1986). Some of these families consist of sequences which are primarily heterochromatic regions of DNA (Doolittle and Sapienza, 1980) and are not under strong selective pressure. Consequently, large changes in their numbers or sequence may have little or no effect on the survival of the organism and genomic variation can accumulate. Other families of repeat sequences are transcribed into functional RNA, such as the ribosomal or transfer RNAs. Variation in these repeats may arise via duplication of entire families of sequence, or through accumulation of mutations in the intergenic spacer regions between the tandemly repeated ribosomal genes (King and Schaal, 1989).

2.3.2 Migration

The epidemiological consequences of long-distance migration have been well documented in some foliar pathogens where disease is seasonal and episodic. For example, in Australia, populations of *Puccinia graminis* f.sp. *tritici* have periodically undergone drastic changes when a new exotic race supplants the existing endemic races of the pathogen (Burdon *et al.*, 1982; Burdon *et al.*, 1983). Where diseases are persistent, however, the importance of migration may not be so readily apparent. This

may be the case with *G. graminis* where inoculum is soil-borne and can survive saprophytically between successive cereal crops.

Wind-borne inoculum may provide for migration of genetically diverse isolates between populations and may account for some of the variation within populations of *G. graminis*. Ascospores have been implicated as a source of inoculum to account for the spread of take-all to locations which were previously disease free (Garrett, 1981). In contrast, Brooks (1965) found that ascospores were unlikely to infect roots because of competition by antagonistic microflora for root exudates, which provide a food resource for the germinating ascospore. Furthermore, ascospores become inviable after a few days of dry conditions and are considered to be epidemiologically insignificant within the cereal belt of southern Australia (Hornby, 1981). Migration of individuals between populations may occur via wind dispersal of fine particles of infected plant materials, but the frequency of successful establishment of isolates in new locations is unknown.

2.3.3 Population size

When a limited number of individuals establish a new population, the gene frequencies in the founding population may differ significantly from those in the population from which they were originally derived (Hartyl and Clark, 1989). The new population generally shows reduced genetic diversity compared with the population of origin and is said to exhibit a founder effect. Changes in the genetic composition of the Australian stem rust population are thought to have resulted from founder effects due to migration, establishment and asexual proliferation of a restricted number of pathogen genotypes (Burdon *et al.*, 1982).

The supposed infrequency of successful establishment of *G. graminis* by dispersal of wind-borne inoculum implies that pathogen populations only rarely arise from founder effects (Hornby, 1981). The widespread distribution of endemic populations implies that *G. graminis* is and has been a common component of the Australian soil mycoflora (Garrett, 1981; Cotterill and Sivasithamparam, 1989).

Since phytopathogenic fungi are largely dependent upon their host plants, the density and distribution of host species may be important factors affecting population size and therefore, genetic diversity within *G. graminis*. For example, greater numbers of *Ggt* propagules were recorded in soils which had been in pasture the previous season in comparison to those under wheat (Cotterill and Sivasithamparam, 1988). This was attributable to a better distribution and greater biomass of grass roots relative to that of wheat roots. Greater densities of susceptible host species should provide reduced competition between individuals for host resources, leading to the maintenance of larger, potentially more variable, pathogen populations.

Exposure of *G. graminis* to non-susceptible hosts would restrict the fungus to surviving saprophytically on host debris from the previous crop. In this state isolates would be susceptible to microbial antagonism and autolysis, leading to the decreases in the size and genetic diversity of pathogen populations. Non-susceptible crops (e.g. grain legumes) are often used as a means of reducing the inoculum of *G. graminis* within the soil to prevent high incidences of take-all in successive cereal crops (Cotterill and Sivasithamparam, 1989).

2.3.4 Genetic drift and natural selection

Genetic differentiation between populations is thought to be primarily associated with the evolutionary forces of genetic drift and natural selection. The neutral theory of molecular evolution proposes that genetic drift is the primary force driving molecular evolution (Kimura, 1983). Genetic drift occurs in all natural populations and is a function of population size and structure. Neutral mutations arise at random, and chance determines which mutations go to fixation and which are lost within and between populations. In large populations, many neutral mutations arise and slowly drift to fixation or loss. High levels of genetic diversity are thus expected to accumulate in these large populations. In small populations, however, drift is rapid, new mutations are quickly fixed or lost and consequently little diversity persists (Ayala, 1982; Kimura, 1983).

Natural selection affects levels of intra-population variation at selected and linked neutral sites within the genome. If the intensity of selection varies over space and time it can lead to considerable genetic differentiation between populations. For example, directional selection tends to sweep favoured variants through a population to fixation, eliminating much of the variation attributable to neutral mutations. Selection, therefore, tends to cause reductions in levels of genetic variation below those which may result from genetic drift alone (Aquadro, 1992).

Studies of the plant-pathogenic fungi *Erysiphe graminis* on cereals (O'Dell *et al.*, 1989), *Leptographium wageneri* on conifers (Zambino and Harrington, 1989) and *Magnaporthe grisea* on rice and grasses (Levy *et al.*, 1991) have indicated a division of pathogen populations into host-based groups, presumably resulting from host-mediated selection and infrequent sexual recombination between pathotypes specialised on different hosts. These results implied that the host genotype applies varying degrees of selection pressures and if intense, as would be expected in the case of tolerant or resistant hosts, can have significant effects on the genetic composition of pathogen populations.

Host-mediated selection of plant pathogens requires variation within the pathogen population for virulence on their respective hosts. *G. graminis* exhibits considerable variation in virulence in naturally occurring populations isolated from field crops (Asher, 1981). Intensive cereal production and the use of hosts which differ in their susceptibility to infection (e.g. rye, oats and possibly barley) may be expected to apply varying intensities of selection pressure upon *G. graminis* populations. This may result in the selection and subsequent accumulation of pathogen genotypes which are better adapted to parasitising different hosts. For example, cultivation of oats would be expected to select out *Gga* pathotypes from a taxonomically mixed population of *G. graminis* and suppress *Ggt* and *Ggg*. The inoculum density of *Gga* pathotypes should increase relative to that of the other two varieties, leading to significant changes in the overall genetic composition of the population. Consequently, intense host-mediated selection may force pathogen populations through evolutionary bottlenecks resulting in

reductions in population size and decreased genetic diversity in the recovering populations (Ayala, 1982; Hartyl and Clark, 1989).

2.4 The genetic bases of variation within *G. graminis*

The breeding biology determines the relationship between the frequencies of genes, genotypes and phenotypes, their rates of change and the generation of new gene combinations by recombination. Therefore, consideration of the breeding system is crucial to determine factors responsible for the generation and maintenance of genetic variation within populations of *G. graminis*.

G. graminis is a homothallic ascomycete which is haploid throughout the vegetative phase of its life cycle. The mycelium is composed of two cell types; young, hyaline infective cells which are generally uninucleate and older pigmented cells which form the ectotrophic runner hyphae and may be multinucleate. Since the fungus is homothallic, an isolate which arises from a single uninucleate cell can produce a perithecium (fruiting body) and undergo recombination resulting in identical selfed progeny (ascospores) (Blanch *et al.*, 1981). Asexual phialidic conidia are produced in soil but are apparently inviable, whereas those produced in culture are larger and have been shown to germinate (Asher, 1981).

2.4.1 Parasexual Recombination

Laboratory studies have shown that several fungi have the potential to generate asexual variation through parasexuality (Crawford *et al.*, 1986; Loftus *et al.*, 1988). Parasexuality involves heterokaryosis, karyogamy, mitotic recombination and segregation (Michelmore and Hubert, 1987), processes which can affect the rate of gene frequency change within populations. Vegetative compatibility genes have been characterised in several ascomycetes, and identical alleles at several loci are required for vegetative heterokaryon formation (Fincham *et al.*, 1979). Consequently, only closely related isolates are likely to anastomose. The existence of these hyphal

incompatibility systems may break up populations into reproductively isolated, genetically diverse sub-populations.

Somatic hybrids of *G. graminis* have been induced in the laboratory, resulting in the formation of heterokaryotic isolates (Asher, 1981), but no information is available as to the frequency of these events in the field. Rawlinson *et al.* (1973) have shown that hyphal anastomosis occurs in culture between isolates sampled from a population of *G. graminis* but its frequency is isolate-dependent, with many of the pairings between isolates failing to produce somatic hybrids. The occurrence of limited somatic hybridisation does indicate that the development of genetically diverse isolates could result from mitotic recombination between different nuclei. The frequency of mitotic recombination in heterokaryons, however, is apparently low and therefore is unlikely to generate much genotypic variation within populations over short periods of time (Blanch *et al.*, 1981).

2.4.2 Sexual Recombination

Within *G. graminis*, novel combinations of genes could be produced by sexual recombination between genetically diverse isolates. The frequency of the occurrence of outcrossing can have profound effects on the rates of change in gene frequencies and accordingly on the rate of genetic response to selection pressures.

G. graminis is a homothallic fungus and is, therefore, self-fertile but Blanch *et al.* (1981) were able to cross isolates of *Ggt.* The numbers of hybrid perithecia were observed to be low in relation to those that had arisen through selfing, indicating that the primary mode of reproduction may still have been self-fertilisation. Examination of hybrid perithecia which had formed upon wheat roots indicated that sexual recombination had occurred between these isolates. Where crossing had taken place in a perithecium all asci within that perithecium were observed to have arisen from heterozygous diploid nuclei. Therefore, *G. graminis* is potentially capable of outbreeding and genetic variation within the species may be generated from the processes of sexual recombination.

Crossing between genetically diverse isolates requires hyphal anastomosis within the developing perithecia for the exchange of nuclei and the generation of hybrid progeny. Given the assumption that somatic hybridisation within *G. graminis* is rare, it is unlikely that sexual recombination between isolates occurs frequently enough to generate high levels of recombination and genotypic diversity within a limited number of generations. This implies that mutation may be a major source of genetic variation and that heterogeneous populations may be composed of a number of independent vegetative units. The use of molecular markers to assess levels of genetic diversity within and between populations of *G. graminis* should provide information on the genetic structure of populations, which may be useful in determining the pathogen's primary mode of reproduction in the field (Brown and Weir, 1983).

2.5 Molecular markers for analysis of *G. graminis* populations

Genetic studies of any organism require precise, easily scored characters or markers. Molecular markers have several advantages over morphological markers, for example (a) numerous molecular markers can be identified; (b) most are codominant (i.e. heterozygotes can be distinguished from homozygotes) and (c) numerous alleles may exist for many markers (Michelmore and Hubert, 1987).

Molecular techniques have provided three classes of markers that allow detailed genetic analyses of phytopathogenic fungi. These are isozymes, DNA restriction fragment length polymorphisms (RFLPs) and DNA sequences amplified by the Polymerase Chain Reaction (PCR).

2.5.1 Isozymes

Isozyme analysis offers one method of quantifying genetic diversity and has been used in population genetic analyses of a number of phytopathogenic fungi (Leung and Williams, 1986; Leuchtmann and Clay, 1989; Tuskan *et al.*, 1990). Isozyme analyses have proved useful in developing an understanding of disease epidemiology and can aid in identifying factors affecting the dynamics of pathogen populations. For example,

isozyme frequency data have provided assessments of the frequency of sexual reproduction within populations of *Puccinia graminis* (Burdon and Roelfs, 1985) and its importance in generating genetically variable pathogen genotypes within *Phytophthora infestans* (Tooley *et al.*, 1985). These data may also be useful in determining the evolutionary history of pathogen populations, with low levels of genetic diversity often being related to the founding of new populations (Spieth, 1975), or to the passage of populations through bottlenecks, resulting in significant decreases in effective population size (Hartyl and Clark, 1989).

2.5.2 Restriction fragment length polymorphisms

Studies of genetic variation using restriction fragment length polymorphisms (RFLPs) have clarified taxonomic divisions between closely related phytopathogenic fungi which were difficult to discriminate by other means (Braithwaite *et al.*, 1990; Vilgalyis and Gonzalez, 1990). Studies of mitochondrial DNA diversity have allowed the discrimination of *G. graminis* from other closely related fungi and indicated that *Ggt* and *Gga* are more closely related to each other than either is to *Ggg* (Henson, 1989; Bateman *et al.*, 1992). Nevertheless, these studies did not clearly differentiate between these taxonomic varieties.

RFLP analyses have been reported to detect greater diversity than isozymes within a number of plant pathogenic fungi (Hamer *et al.*, 1989; O'Dell *et al.*, 1989; Goodwin *et al.*, 1992). DNA repeat-sequences have proved particularly useful in detecting variation within populations which were relatively homogeneous at isozyme loci. For example, repeat-sequence diversity has provided differentiation between closely related isolates in a number of plant pathogens including *Globodera pallida* (Schnick *et al.*, 1990), *Magnaporthe grisea* (Levy *et al.*, 1991) and *Cryphonectria parasitica* (Milgroom *et al.*, 1992). Therefore, RFLPs should prove useful in analyses of *G. graminis* populations which may exhibit low levels of genetic diversity as a result of extensive self-fertilisation.

Recent studies examining the diversity of repetitive sequences within *G. graminis* have indicated genetic differentiation both within and between varieties of the pathogen. In particular, ribosomal DNA (rDNA) repeats appeared useful in variety identification (Ward and Gray, 1992) and were also capable of differentiating between rye and wheat pathotypes within *Ggt* (O'Dell *et al.*, 1992). Nevertheless, the banding patterns resolved in these hybridisation experiments did not provide a discrete variety-specific marker, classifications being based on the overall similarities observed within varieties relative to the variation detected between varieties.

2.5.3 Polymerase chain reaction

The polymerase chain reaction (PCR) provides an alternative means of generating genetic markers and has advantages over RFLP analyses in that it is generally less expensive and is technically simpler to use. The sequence of the DNA amplified is dependent upon the type of primer used and the conditions of the reactions (Saiki, 1989). If sequence information is available, specific primers can be designed and highly diagnostic DNA products amplified. For example, a cloned fragment of *Ggt* mitochondrial DNA, known to be diagnostic for the pathogen (Henson, 1989), was partially sequenced and primers designed to amplify an internal region of this cloned sequence (Schesser *et al.*, 1991). The resulting products were unique to *G. graminis* and have proved useful in the accurate diagnosis of disease. This approach has been applied successfully in the detection of *G. graminis* from infected plant tissue (Henson, 1992). The PCR method circumvents the laborious process of DNA hybridisation experiments to detect *G. graminis* and provides a more rapid method for pathogen identification. The PCR products were, however, monomorphic between varieties of *G. graminis* and could not be used to differentiate them.

Polymorphisms identified by the PCR are in the length of DNA amplified between two short target sequences, so if that region is highly variable the likelihood of detecting polymorphisms increases (Saiki, 1989). One method of increasing the yield of polymorphic products involves targeting highly conserved sequences which are known

to flank variable spacer regions, e.g. the consensus sequences for intron splice junctions. Introns are common to many eukaryotic genes and the junctions to introns are highly conserved sequences (Hawkins, 1988) which should facilitate consistent amplification of sequences from a diversity of taxa. The introns, however, are generally thought to be highly variable in sequence and length and provide ideal sources for polymorphic markers (Weining and Langridge, 1991). Use of primers specific for intron splice junctions may resolve numerous polymorphisms between closely related isolates of *G. graminis*. Additionally, as introns are thought to be present in many genes it may be possible to target numerous genetic loci over many chromosomal locations.

2.6 Applications

2.6.1 Taxonomy and Phylogenetic Relationships

Molecular markers may make a significant contribution to classification at the species level, when morphological differences are subtle and ambiguous. Within *G. graminis*, only slight morphological differences exist between varieties and there is significant intra-specific variation for these characters (Walker, 1981). Taxonomic identification requires the detection of markers that are conserved within but differ between varieties of *G. graminis*.

Multilocus electrophoretic phenotypes of isozyme polymorphisms have been used in the taxonomic identification of intra-specific varieties within some plant pathogenic fungi (Burdon and Marshall, 1981; Zambino and Harrington, 1989; Simcox *et al.*, 1992). These analyses, however, require the examination of numerous loci and intra-specific variation may be too low to resolve taxonomic divisions within some species (Leung and Williams, 1986). In contrast, a small number of RFLP and/or PCR markers may provide virtually unambiguous identification of the taxonomic status of isolates (O'Dell *et al.*, 1989; Chen *et al.*, 1992) and may prove useful for discriminating between varieties of *G. graminis*.

Classification of isolates is most difficult, but also most critical, when a single host is parasitised by several varieties of the pathogen, e.g. wheat and barley are susceptible to both *Gga* and *Ggt*. The ability to distinguish between these varieties and to determine the frequency of the respective pathotypes within populations may be crucial in limiting the severity of disease expression over time, as it has implications for the timing and choice of which non-susceptible hosts should be used in controlling the pathogen. If the different varieties coexist within populations, inter-variety hybridisation events might provide pathogens with a ready source of variation for adaptation to a new host, for example, *Ggt* may acquire from *Gga* the capacity to infect oats. Variety-specific markers should allow the detection of inter-variety hybrids within populations of *G. graminis*. It is likely, however, that the frequency of inter-varietal hybridisation is low, due to the restrictions on anastomosis and heterokaryon formation between genetically diverse isolates discussed above.

Many families of repetitive DNA tend to be uniform in sequence within and between individuals of an interbreeding taxon, but a family that is shared between different taxa often reveals considerable diversity (Dover, 1982). This suggests that there are mechanisms which maintain homogeneity of repeat sequences within a species and when a new variant arises within a family it is either corrected or spreads throughout the family. These variants may eventually become fixed within an interbreeding population as a consequence of stochastic and directional changes operating within the genome by a process known as molecular drive (Dover, 1982). Consequently, some repetitive DNA sequences may provide a source of markers for identification of taxonomic varieties within *G. graminis*, as has been shown by O'Dell *et al.* (1992).

For a character to be useful in elucidating evolutionary relationships within species, it must exhibit diversity but also be conserved to some extent. The degree of conservation versus diversity dictates the taxonomic level for which a marker will be useful. Repeated DNA elements have been shown to accumulate polymorphisms at a high rate, probably due to processes of genome rearrangement resulting from

mechanisms of inter-chromosomal exchange and amplification of sequences (Flavell, 1986). Sequence variants, however, can become fixed within interbreeding or genetically isolated populations in relatively short periods of evolutionary time and result in differentiation between populations (Dover, 1982). Therefore, molecular drive may be a contributing factor in speciation events since maximising homogeneity within a population tends to maximise the discontinuities between populations.

Analyses of repeat-sequence variation among poultry strains (Kuhnlein *et al.*, 1989) and island populations of foxes (Gilbert *et al.*, 1990) were found to reflect the known genetic relationships of these different populations and were used to reconstruct their recent evolutionary histories. Repetitive sequences may be good markers for determining phylogenetic relationships within *G. graminis* due to the conservation of different variants within the reproductively isolated varieties (O'Dell *et al.*, 1992). Consequently, the distribution of repeat-sequence variants within and between populations of *G. graminis* may provide clues to the mechanisms and processes that have affected the recent evolution of the species.

2.6.2 Epidemiology and Population Genetics

Both evolution of and differentiation between, natural populations appear to be governed by a combination of factors including the prevailing mode of reproduction, the intensity of natural selection and the degree of genetic drift, the latter being modified by the extent of inter-population gene flow. Population genetic models make it possible to ascertain the primary mode of reproduction and the extent of gene flow, by examining the distribution of genetic variation within and between populations (Tibayrenc *et al.*, 1991). The type of evidence required is the frequency distribution of genotypes, which can be obtained by isozyme, RFLP and PCR analyses.

Isozyme studies of natural plant populations have demonstrated a general trend of high variability in outcrossing populations and lower levels of variation in selfing populations (Hamrick *et al.*, 1979). This observation was confirmed in studies of the plant pathogenic fungi *Puccinia graminis* (Burdon and Roelfs, 1985) and *Phytophthora*

infestans (Goodwin *et al.*, 1992) which indicated that asexual or self-reproducing populations exhibited less variation at isozyme loci than did populations which were thought to be reproducing sexually. Therefore, examination of the overall levels of genetic diversity within populations of *G. graminis* may provide an indication of the relative frequency of sexual reproduction within populations.

A survey by Loveless and Hamrick (1984) found that species which reproduce by self-fertilisation had a greater proportion of their genetic variation distributed between rather than within populations. This distribution of variation would also be expected to be found between populations of phytopathogenic fungi which reproduce asexually. Therefore, the distribution of variation between populations may be used to determine the predominant mode of reproduction and may indicate the extent of inter-population gene flow. For example, if there are significant levels of gene flow, the various populations will effectively act as one large panmictic population and exhibit relatively low levels of genetic differentiation (Hartyl and Clark, 1989). If, however, there are restrictions on levels of inter-population gene flow, the individual populations may be expected to show considerable genetic differentiation.

As discussed previously, self-fertilisation and asexual reproduction are widely accepted to be the primary modes of reproduction within *G. graminis*. Since the pathogen is haploid, all of the progeny resulting from self-fertilisation will be identical to the parental strain. This implies that populations may be composed of a series of clonal lineages of isolates. The presence of a particular multilocus genotype in high frequency is reported to be the most robust and significant evidence of clonal reproduction (Tibayrenc *et al.*, 1991). For example, molecular analysis of repeat-sequence variation within a population of *Septoria tritici* indicated that clonal reproduction was extensive, the population being composed of a limited number of RFLP multilocus genotypes compared to the numbers expected under random mating (McDonald and Martinez, 1991). Clonal population structure has also been reported in *Phytophthora infestans* (Goodwin *et al.*, 1992), where analyses of isozyme, RFLP and mating type variation detected a limited number of multilocus genotypes within some

populations, even though both mating types were present. These results indicated that the genotypes were replicating as separate units, without the genetic reorganisation that results from sexual recombination.

When genetic data have been derived from high resolution methods, such as DNA fingerprinting with repeat-sequence probes, the multiple occurrence of an identical genotype implies that sexual recombination is absent, an observation that does not require statistical testing (Tibayrenc *et al.*, 1991). It is possible, however, to use statistical tests which examine for non-random associations between loci (linkage disequilibrium), known to be indicative of clonally propagated populations (Weir, 1990).

2.7 Summary

Detailed population genetic studies of a number of phytopathogenic fungi indicate that levels of genetic diversity can vary considerably between species. The main factors affecting genetic diversity within and between populations are associated with the rates of mutation and inter-population migration, the intensities of environmental and host-mediated selection and the breeding biology of the pathogen.

G. graminis is known to exhibit considerable variation in virulence in naturally occurring populations isolated from field crops. This observation, combined with the broad ecogeographic and host ranges of the pathogen, implies that the species should be highly variable. In terms of the breeding biology, most results suggest an extremely low frequency of hyphal anastomosis and out-crossing. Consequently, the opportunities for the exchange and re-assortment of genetic material between strains would appear to be limited. The implication is that genetically heterogeneous populations, generated by mutation, may be composed primarily of clonally propagated lineages resulting from asexual or self-reproduction.

Within clonally reproducing populations, variation may be maintained as a result of relatively low host-mediated selection pressures, provided by exposure to and coevolution with a diversity of host genotypes, most of which are highly susceptible to

the pathogen. In contrast, exposure of populations to tolerant or resistant hosts, such as rye or oats, would be expected to exert intense selection upon the population resulting in the suppression of isolates poorly adapted to these hosts. Consequently, intense host-mediated selection is expected to have dramatic effects on the genetic composition of *G. graminis* populations, probably resulting in significant decreases in genetic diversity.

The development of variety-specific markers will make it possible to ascertain the effects of host-mediated selection upon the taxonomic composition of *G. graminis* populations and may also aid in determining if the barriers to inter-variety hybridisation observed in the laboratory also occur in nature. These aspects are of considerable importance in understanding take-all epidemiology and the evolution of pathotype diversity within *G. graminis*.

The use of molecular markers to assess levels of genetic diversity within and between populations of *G. graminis* should provide information on the genetic structure of populations, which may be useful in determining the predominant mode of reproduction in the field and the extent of inter-population gene flow. It is likely that isozymes will be easier to use than RFLP or PCR markers due to the more involved procedures for DNA extraction and manipulation. However, recent studies have indicated that RFLP and PCR markers will be more applicable than isozymes for developing variety-specific markers and in studies of populations which are expected to be genetically homogeneous.

Chapter 3 Isozyme variation within and among varieties of *G. graminis*

3.1 Introduction

Intra-specific classification of *G. graminis* by pathogenicity testing and inducing perithecial production is a slow and laborious process and often proves to be inconclusive. For example, many Australian isolates have been reported to parasitise oats (only susceptible to *Gga*), but have spore lengths in the range characteristic of *Ggt* (Yeats, 1986). Furthermore, many *G. graminis* isolates lose their pathogenic and reproductive abilities after prolonged periods in culture (Cunningham, 1981). The genetic basis of the similarities and differences between the pathotypes is not understood, so a greater knowledge of the genetic variation within and between the varieties is required for reliable classification of isolates.

Attempts to differentiate the three varieties of *G. graminis* by electrophoresis of total protein profiles indicated that *Ggt* and *Gga* were more closely related to each other than to *Ggg*. The differences in the banding patterns were too slight, however, to provide reliable separation of the wheat and oat take-all fungi (Abbott and Holland, 1975; Maas *et al.*, 1990). Gel electrophoresis of isozymes, in contrast, has been used successfully to determine taxonomic relationships (Burdon and Marshall, 1981; Liu *et al.*, 1990; Simcox *et al.*, 1992) and the population biology (Burdon and Roelfs, 1985; Leuchtmann and Clay, 1989) of related fungi. Furthermore, isozymes provide markers useful in the genetic analyses of phytopathogens (Linde *et al.*, 1990; Spielman *et al.*, 1990).

The objectives of this research were to evaluate the amount of genetic diversity at isozyme loci within and between varieties of *G. graminis*. The distribution of diversity between varieties will aid in identifying loci and/or alleles which may be useful for

discriminating taxa. Assessments of intra-variety variation will also determine those loci likely to be most useful for analyses of *G. graminis* populations.

3.2 Materials and methods

3.2.1 Origin of isolates and culture conditions

A collection of 24 isolates representing the three varieties of *G. graminis* was used in this study (Table 3.1). Isolates were selected on the basis of ecogeographic origin and host diversity and also to provide a representative sample of the genetic diversity within the species in southern Australia.

Wheat (cv. Spear) and oats (cv. Echidna) were exposed to each isolate, firstly to asses the pathogenic potential of each isolate and secondly to provide fresh, vigorous fungal cultures for subsequent experiments. Small pots (10 cm diam. x 15 cm deep) were half-filled with sterile sand and the fungal inoculum was introduced as pieces of agar culture (Hornby *et al.*, 1977). The inoculum was covered with a thin layer of sand and 5 seedlings of each host species were planted into each pot. Each of the host x isolate treatments was replicated twice. After 3-4 weeks growth in a glasshouse (constant temperature 18°C) plants were harvested and the roots scored for the presence or absence of take-all symptoms. No attempt was made to score the severity of infection.

All isolates were reisolated from infected root material before being prepared for electrophoresis. Root segments were surface-sterilised in 1% silver nitrate ($AgNO_3$) for 1 minute, washed in 5% sodium chloride (NaCl), rinsed twice in sterile distilled water, air dried and plated onto potato dextrose agar (PDA) supplemented with 100 mg/litre streptomycin sulphate. Plates were incubated in the dark at 22°C. Upon emergence from the root, hyphal tips were removed and subcultured onto fresh PDA and stored at 4°C until required.

Table 3.1. Fungal isolates: Origins and pathogenicity to wheat and oats.

	Host	Location	Source	pathogenicity	
				wheat	oats
Ggt					
KM69	*Triticum aestivum* L.	Tamworth, N.S.W.	P.T.W. Wong	+	
WUF143	*Hordeum murinum* L.	Beverley, W.A.	O. Glenn	+	+
23152	*Triticum aestivum*	Gerogery, N.S.W.	J. Walker	+	
23125	*Triticum aestivum*	Urana, N.S.W.	J. Walker	+	
C3	*Hordeum murinum*	Cowra, N.S.W.	P.T.W. Wong	+	
500	*Triticum aestivum*	W.A.	C.A. Parker	+	+
800	*Triticum aestivum*	Avon, S.A.	A.D. Rovira	+	
23580	*Triticum aestivum*	W.A.	P.T.W. Wong	+	
EBI	*Hordeum vulgare* L.	Eire	P.T.W. Wong	+	
17916	*Triticum aestivum*	Dubbo, N.S.W.	J. Walker	+	
KSI	*Triticum aestivum*	Kapunda, S.A.	A.D. Rovira	+	
51463	*Triticum aestivum*	Palmer, S.A.	J. Walker	+	
Gga					
137T	*Agrostis* sp.	N.S.W.	P.T.W. Wong	+	+
23144	*Agrostis* sp.	Concord, N.S.W.	P.T.W. Wong	+	+
192M	*Agrostis* sp.	N.S.W.	P.T.W. Wong	+	+
35793	*Avena sativa* L	Yerong Creek, N.S.W.	J. Walker	+	+
Ggg					
30577	*Stenotaphrum secundatum* (Walt.) Kuntze	Artarmon, N.S.W.	J. Walker	+	
30584	*Paspalidium* sp.	Inverell, N.S.W.	J. Walker	+	
23148	*Pennisetum clandestinum* Hochst. ex Chiov	Turramurra, N.S.W.	J. Walker	+	
51652	*Cynodon dactylon* Pers	Perth, W.A.	J. Walker	+	
H4	*Eragrostis setifolis* Nees	Warialda, N.S.W.	P.T.W. Wong	+	
W2P	*Paspalidium* sp.	Walcha, N.S.W.	P.T.W. Wong	+	
ADI	*Aristida* sp.	Tamworth, N.S.W.	P.T.W. Wong	+	
23147	*Bromus catharticus* Vahl.	Rydalmere, N.S.W.	J. Walker	+	+

3.2.2 Protein extraction for electrophoresis

Mycelium of *G. graminis* for enzyme extraction was grown in 100 ml flasks containing 25 ml of sterile NDY medium (Appendix A) inoculated with small pieces of stock culture. Flasks were incubated at 22°C on a rotary shaker (100 rpm) for 2-3 weeks. Mycelium was harvested and blotted dry on chromatography paper (Whatman 3MM) and was processed immediately or frozen at -80°C until required. Small pieces of mycelium (approx. 200 mg fresh weight) were frozen in liquid nitrogen and ground into a viscous slurry in the presence of 0.2 ml extraction buffer (Appendix A). Samples were placed on ice for 10 minutes before being centrifuged at 12,000 rpm for 5 minutes to remove cell wall debris. The clear supernatant was transferred to 1.50 ml polypropylene tubes and chilled on ice before being loaded onto the gel.

3.2.3 Electrophoresis

Cellogel (Chemetron:Milan) electrophoresis was conducted as described by Richardson *et al.* (1986). Gel slices (20 x 15 cm) were soaked in 300 ml of electrophoresis buffer for 3 hours at 4°C before being placed in gel trays containing 700 ml cold (4°C) electrode buffer. The gel trays were designed such that the gel made direct contact with the electrode buffer. Loading wells (0.5 x 0.2 cm) were indented upon the surface of the gel using a drawing pen. Approximately 5 µl of sample was pipetted into the wells and allowed to soak into the gel prior to electrophoresis. All gels were run at 4°C under the conditions listed in Table 3.2. Enzyme names with enzyme commission (EC) numbers, abbreviations, buffer systems for each enzyme and modifications to the enzyme staining solutions (from Richardson *et al.*, 1986) are shown in Table 3.3.

Alleles for each of the enzymes were determined by repeated side by side comparisons. In the final analysis all isolates were run on a single series of gels for a complete inter/intra-variety comparison.

Table 3.2. Electrophoretic conditions used in isozyme analysis.

System	Buffer	Electric current	Duration of Electrophoresis
1	11.6 mM Na_2HPO_4 8.4 mM NaH_2PO_4	220 V	1.5 h. at 4°C
2	15 mM Tris 5 mM Na_2EDTA 10 mM $MgCl_2$	220 V	1.5 - 2.0 h. at 4°C
3	100 mM Tris 1 mM Na_2EDTA 1 mM $MgCl_2$	220 V	1.5 h. at 4°C

3.2.4 Scoring of gels and data analyses

Relative mobility (R_f) values were assigned to each band of enzyme activity. *Ggg* isolate 51652 was used as a standard and was included on all gels, the bands resolved at each locus being assigned an R_f value of 1.0. The total number of alleles per locus was determined and each isolate assigned a binary value indicating the presence (1) or absence (0) of a particular allele within each locus. An electrophoretic phenotype was determined for each isolate, defined as the presence or absence of a band of activity for each allele at each locus

As crossing experiments were not conducted between any of the isolates, the actual genetic basis of electrophoretic variation in enzymes could not be determined, so an analysis of phenotypic, rather than genotypic, diversity was employed. The frequencies of the electrophoretic variants were calculated and phenotypic diversity was estimated using Shannon's Index $H_0 = \Sigma p_i \log_2 p_i$, where p_i is the phenotypic frequency at a particular enzyme locus (Peet, 1974; M^c Cain *et al.*, 1992). Nei's gene diversity, which treats the variants as alleles at separate loci, was also computed and the different measures compared. Gene diversity (Nei, 1973; Weir, 1990) was calculated from allele frequencies at the respective enzyme loci as follows. If p_{lu} is the frequency of the u th allele at the lth locus the gene diversity at this locus is $D_l = 1 - \Sigma p^2_{lu}$.

Table 3.3. Composition of staining solutions for detection of isozymes.

Enzyme	Abbreviation	EC No.	Buffer system	Stain composition
Aconitase	ACON	4.2.1.3	1	2 ml 0.1 M Tris-HCl buffer pH8.0, 0.25 ml aconitate (100 mg per ml pH 8), 0.1 ml NADP (20 mg per ml), 0.1 ml MTT (20 mg per ml), 0.1 ml PMS (20 mg per ml), 0.2 ml $MgCl_2$ (0.2 M), 50 µl IDH (10 mg per ml)
Fumarase	FUM	4.2.1.2	1	2 ml 0.1 M Tris-HCl buffer pH 8.0, 0.25 ml fumarate (75 mg per ml pH 8), 0.1 ml NAD (25 mg per ml), 0.1 ml MTT (20 mg per ml), 0.1 ml PMS (20 mg per ml), 10 µl MDH (10 mg per ml)
Glutamate-oxaloacetate transaminase	GOT	2.6.1.1	1	10 mg fast garnet GBC salt, 2 ml 0.1 M Tris-HCl buffer pH 8.0, 0.25 ml α - ketoglutarate (75 mg per ml pH 8) 0.25ml L - aspartate (75 mg per ml pH 8)
Glucose-6-phosphate dehydrogenase	G6PD	1.1.1.49	3	20 mg glucose-6-phosphate, 2 ml 0.1 M Tris-HCl buffer pH 8.0, 0.1 ml NADP (20 mg per ml), 0.1 ml MTT (20 mg per ml), 0.1 ml PMS (20 mg per ml), 0.2 ml $MgCl_2$ (0.2 M)
Glucose-phosphate isomerase	GPI	5.3.1.9	2	15 mg fructose-6-phosphate, 2 ml 0.1 M Tris-HCl buffer pH 8.0, 0.1 ml NADP (20 mg per ml), 0.1 ml MTT (20 mg per ml), 0.1 ml PMS (20 mg per ml), 0.2 ml $MgCl_2$ (0.2 M), 10 µl G-6PD (10 mg per ml)
Glutathione reductase	GSR	1.6.4.2	2	35 mg oxidized glutathione, 6 mg NADPH, 2 ml 0.1 M Tris-HCl buffer pH 8.6, 0.1 ml MTT (20 mg per ml), 0.1 ml DCIP (20 mg per ml)
Hexokinase	HK	2.7.1.1	1	15 mg glucose, 15 mg ATP, 2 ml 0.1 M Tris-HCl buffer pH 8.6, 0.1 ml NADP (20 mg per ml), 0.1 ml MTT (20 mg per ml), 0.1 ml PMS (20 mg per ml), 10 µl G-6PD (10 mg per ml)
Isocitrate dehydrogenase	IDH	1.1.1.42	1	40 mg DL-isocitric acid, 2 ml 0.1 M Tris-HCl buffer pH 8.0, 0.1 ml NADP (20 mg per ml), 0.1 ml MTT (20 mg per ml), 0.1 ml PMS (20 mg per ml), 0.2 ml $MgCl_2$ (0.2 M)
Malate dehydrogenase	MDH	1.1.1.37	2	2 ml 0.1 M Tris-HCl buffer pH 8.0, 0.25ml malate (75 mg per ml pH8), 0.1 ml NAD (25 mg per ml), 0.1 ml MTT (20 mg per ml), 0.1 ml PMS (20 mg per ml)
Malic enzyme	ME	1.1.1.40	2	2 ml 0.1 M Tris-HCl buffer pH 7.4, 0.25 ml malate (75 mg per ml pH 8), 0.1 ml NADP (25 mg per ml), 0.1 ml MTT (20 mg per ml), 0.1 ml PMS (20 mg per ml), 0.2 ml $MgCl_2$ (0.2 M)
6-Phosphogluconate dehydrogenase	6PGD	1.1.1.44	3	20 mg 6-phosphogluconic acid, 2 ml 0.1 M Tris-HCl buffer pH 8.0, 0.1 ml NADP (20 mg per ml), 0.1 ml MTT (20 mg per ml), 0.1 ml PMS (20 mg per ml), 0.2 ml $MgCl_2$ (0.2 M)
Phosphoglucomutase	PGM	2.7.5.1	2	90 mg glucose-1-phosphate, 2 ml 0.1 m Tris-HCl buffer pH 8.0, 0.1 ml NADP (20 mg per ml), 0.1 ml MTT (20 mg per ml), 0.1 ml PMS (20 mg per ml), 0.2 ml $MgCl_2$ (0.2 M), 20 µl G-6PD (10 mg per ml)

To complement the analyses based on diversity indices, similarities among the electrophoretic phenotypes were calculated using the simple similarity statistic (S), $S_{xy} = 2N_{xy}/(N_x + N_y)$, where N_x and N_y are the number of bands in individual x and y respectively, and N_{xy} is the number shared by both (Lynch, 1990). Pairwise comparisons were made between all isolates and the values used to generate a similarity matrix. To infer genetic identities between isolates a cluster analysis was performed on these similarity values using the group average method (Sneath and Sokal, 1973). The similarity between two groups is defined as the average similarity of all points of unit involving a member of each group. These results were used to generate a dendrogram displaying the hierachical associations between all isolates.

GENSTAT 5, release 1.3 (Rothamsted Experimental Station, 1989) was used for data analysis.

3.3 Results

3.3.1 Pathogenicity tests

Each of the isolates listed in Table 3.1 was observed to infect wheat roots. Isolates classified as *Ggt* and *Gga* produced stelar lesions and ectotrophic runner hyphae, whereas those classified as *Ggg* heavily colonised the epidermal layer of the roots with no apparent penetration of the stele. *Gga* isolates infected oat roots although the level of infection was lower than that observed in wheat. *Ggt* and *Ggg* produced only very slight symptoms upon oats relative to wheat, with very few stelar lesions.

There were few disagreements with the original classification of isolates. Exceptions were *Ggt* isolate 500 and *Ggg* isolate 23147 which produced more stelar lesions and ectotrophic runner hyphae on oats compared with the other non-*Gga* type isolates. *Ggg* isolates 23147 and 23148 also produced a few small stelar lesions upon wheat roots but to a lesser extent than those classified as *Ggt* and *Gga*.

3.3.2 Isozyme variability

All of the enzymes showed well resolved bands and revealed electrophoretic variation between the isolates of *G. graminis*. In 9 of the 12 enzymes a single band per isolate was resolved, whereas glutamate-oxaloacetate transaminase (GOT), hexokinase (HK) and malate dehydrogenase (MDH) resolved 2 bands per isolate. The respective bands showed large differences in relative mobility and varied independently. Consequently, these bands were presumed to be the products of separate loci and were scored accordingly, giving a total of 15 scorable enzymes. Electrophoretic variants at these loci were assumed to be allelic forms of the enzymes.

The number of alleles resolved at each of the enzyme loci ranged from 2 in both 6PGD and MDH to 5 in ME (Table 3.4). Isolates of *Ggt* and *Ggg* accounted for the

Table 3.4. List of enzymes showing number of putative loci and number of alleles resolved per locus.

Enzyme	No. Loci	No. Alleles
ACON	1	3
FUM	1	3
GOT	2	4 (GOT-1)
		4 (GOT-2)
G6PD	1	3
GPI	1	3
GSR	1	3
HK	2	3 (HK-1)
		3 (HK-2)
IDH	1	3
MDH	2	2 (MDH-1)
		3 (MDH-2)
ME	1	5
6PGD	1	2
PGM	1	3

Table 3.5. Descriptive isozyme data for isolates representing the 3 varieties of *G. graminis*.

Variety	Polymorphic loci (%)	Average No. alleles/locus
Ggt	87	2.13
Gga	33	1.33
Ggg	87	2.33
G. graminis	100	3.13

majority of variation within the collection with 87% of their loci exhibiting polymorphisms (Table 3.5). The number of alleles per locus was slightly higher in *Ggg*. In contrast *Gga* isolates showed a much lower level of variation with only 33% of the loci identified as polymorphic.

3.3.3 Estimates of genetic diversity within and between varieties of *G. graminis*.

Gga exhibited low levels of variability, with the 3 isolates being invariant at 10 of the 15 loci (Tables 3.6 and 3.7). In contrast *Ggt* and *Ggg* showed high levels of diversity with only 2 loci being monomorphic within each of these varieties. *Ggg* was found to be the most diverse of the 3 varieties. Both measures of diversity supported these observations.

Diversity indices were used to partition diversity into within- and between-variety components (Tables 3.6 and 3.7). More of the diversity (54%) occurred within varieties of *G. graminis* than between them (Table 3.6). Nei's gene diversity indicated the same partitioning of diversity, with an average of 59% of the diversity found to reside within varieties (Table 3.7).

The proportion of variation within and between varieties differed between loci with ME detecting the most and MDH-1 the least within-variety diversity. The enzyme ME was found to be the most variable across all *G. graminis* isolates. Both diversity indices indicated that the loci GOT-1, GOT-2, GSR and 6PGD partitioned more diversity

Table 3.6. Estimates of genetic diversity (H_0) within and between varieties of *G. graminis*.

Locus	Within varieties			Average within varieties	Total within collection	Proportion within varieties	Proportion between varieties
	Htritici	*Havenae*	*Hgraminis*	Hvar	Hcoll	$\frac{Hvar}{Hcoll}$	$\frac{Hcoll-Hvar}{Hcoll}$
ACON	1.430	0.000	0.811	0.747	1.279	0.584	0.416
FUM	0.760	0.000	1.310	0.690	1.236	0.558	0.442
GOT-1	0.500	0.000	1.310	0.604	1.883	0.321	0.680
GOT-2	0.500	0.000	0.557	0.352	1.648	0.214	0.786
G6PD	0.500	0.000	0.811	0.437	0.748	0.585	0.415
GPI	0.760	0.915	1.417	1.031	1.513	0.681	0.319
GSR	0.500	0.915	0.000	0.472	1.458	0.324	0.677
HK-1	0.000	0.000	1.500	0.500	0.922	0.542	0.458
HK-2	0.760	0.000	0.811	0.524	0.922	0.568	0.432
IDH	1.430	0.915	0.958	1.101	1.581	0.696	0.304
MDH-1	0.760	0.000	0.000	0.253	0.469	0.540	0.458
MDH-2	0.760	0.915	1.413	1.030	1.353	0.761	0.239
ME	1.747	0.915	1.500	1.387	1.983	0.699	0.301
6PGD	0.000	0.000	0.958	0.319	0.881	0.362	0.638
PGM	0.990	0.000	1.310	0.767	1.188	0.645	0.355
Mean	0.760	0.305	0.978	0.681	1.271	0.539	0.461

between rather than within varieties of *G. graminis* and therefore could provide markers to discriminate between varieties. Locus GOT-2 clearly differentiated the 3 varieties with the exception of *Ggg* isolate H4 which had the 1.07 *Ggt* allele (Figure 3.1). Isolate 51463 (*Ggt*) had a unique allele (R_f = 1.11). Loci GOT-1 and GSR appeared to clearly differentiate *Ggg* (R_f=1.00) but there was considerable conservation of alleles between *Ggt* and *Gga*. The enzyme 6PGD discriminated between the *Ggt* and *Gga* isolates, but there was no clear distinction between these and isolates classified as *Ggg*. The majority of *Ggt* and *Ggg* isolates showed the 1.00 fumarase (FUM) allele. *Gga* was

Table 3.7. Estimates of Nei's gene diversity (D) within and between varieties of *G. graminis*.

Locus	Within varieties			Average within varieties	Total within collection	Proportion within varieties	Proportion between varieties
	D*tritici*	D*avenae*	D*graminis*	Dvar	Dcoll	$\frac{Dvar}{Dcoll}$	$\frac{Dcoll-Dvar}{Dcoll}$
ACON	0.592	0.000	0.375	0.322	0.515	0.626	0.374
FUM	0.345	0.000	0.531	0.243	0.505	0.481	0.519
GOT-1	0.197	0.000	0.531	0.292	0.715	0.409	0.591
GOT-2	0.197	0.000	0.219	0.139	0.635	0.218	0.782
G6PD	0.197	0.000	0.375	0.191	0.265	0.720	0.280
GPI	0.345	0.444	0.584	0.461	0.635	0.726	0.274
GSR	0.197	0.444	0.000	0.214	0.615	0.348	0.652
HK-1	0.000	0.000	0.625	0.208	0.340	0.613	0.387
HK-2	0.345	0.000	0.375	0.240	0.340	0.706	0.294
IDH	0.592	0.444	0.469	0.502	0.665	0.755	0.245
MDH-1	0.345	0.000	0.000	0.115	0.180	0.639	0.361
MDH-2	0.345	0.444	0.584	0.461	0.555	0.831	0.169
ME	0.666	0.444	0.625	0.579	0.720	0.804	0.197
6PGD	0.000	0.000	0.469	0.156	0.420	0.372	0.628
PGM	0.494	0.000	0.531	0.342	0.515	0.664	0.337
Mean	0.324	0.119	0.420	0.298	0.508	0.594	0.406

invariant for the 1.06 allele which was also present within a few *Ggt* and *Ggg* isolates, therefore indicating no clear differentiation between the three varieties at this locus.

To complement the diversity analyses, estimates of similarity between isolates were conducted based upon the common occurrence of alleles at each locus. All isolates were observed to be electrophoretically distinct. All pairs of isolates were compared and a similarity matrix was constructed (Table 3.8). Those found to be the most similar were *Gga* isolates 192M and 35793 (95.7%) and those least similar *Ggt* 51463 and *Ggg*

23147 (46.8%). The overall average similarity between all isolates was approximately 60%.

Figure 3.1. Electrophoretic phenotypes of putative taxonomic markers identified for *G. graminis*.

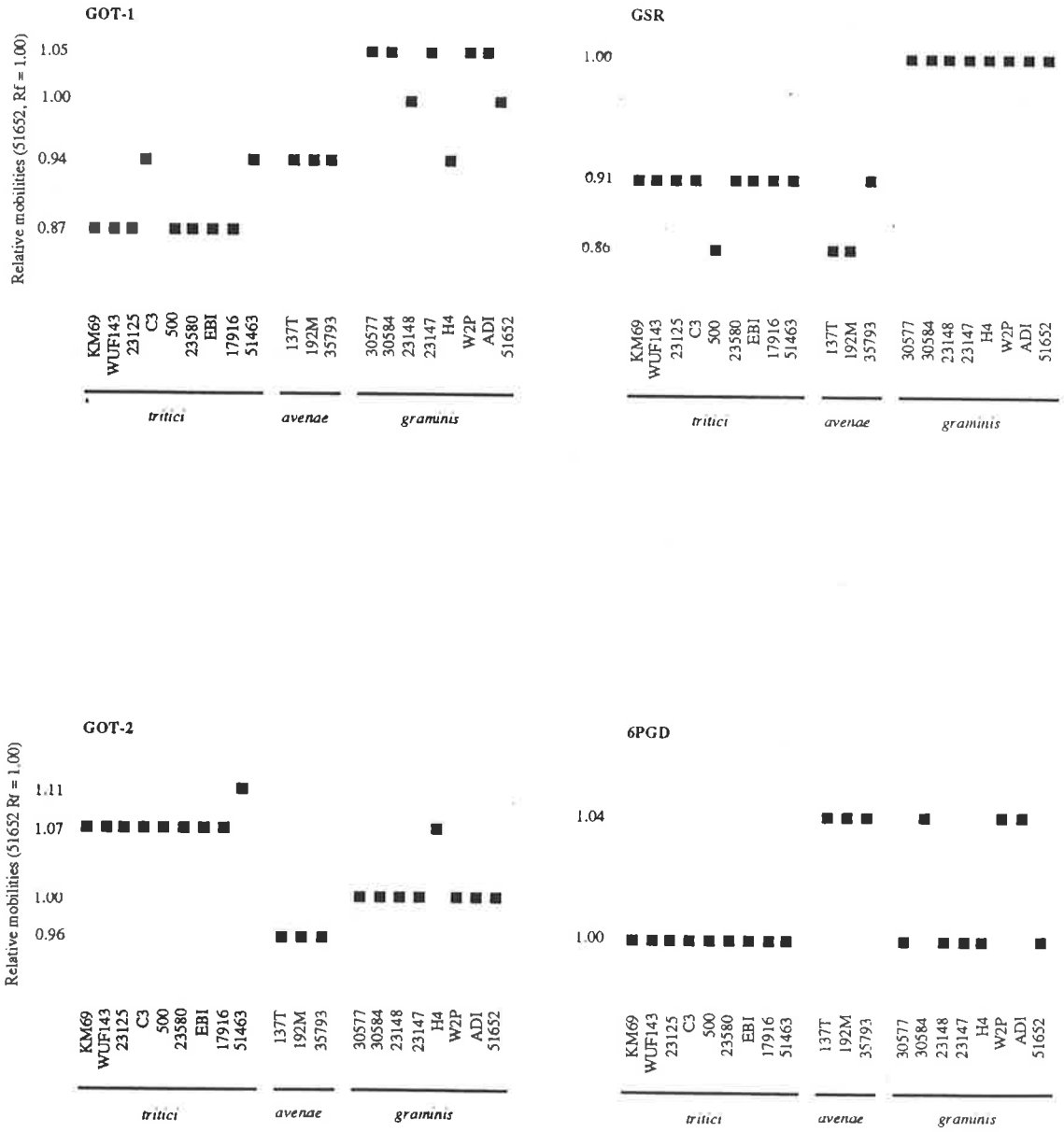
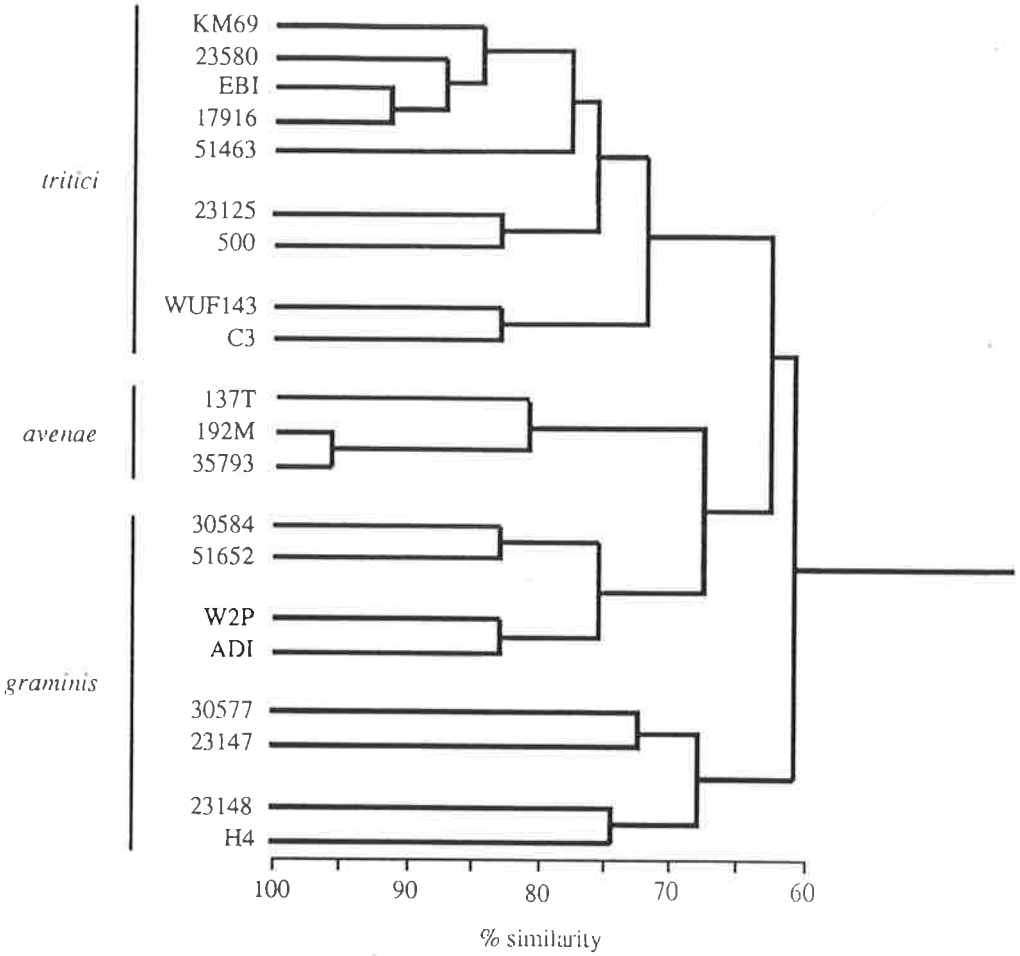


Table 3.8. Genetic similarities among isolates of G. graminis based on the number of shared alleles at the 15 isozyme loci. Figures indicate the percentage similarity between isolates.

KM69										
WUF143	70.2									
23125	74.5	78.7								
C3	70.2	83.0	74.5							
500	78.7	70.2	83.0	74.5						
23580	78.7	70.2	78.7	78.7	74.5					
EBI	87.2	70.2	83.0	78.7	78.7	91.5				
17916	87.2	61.7	74.5	70.2	70.2	83.0	91.5			
51463	70.2	61.7	74.5	78.7	70.2	74.5	83.0	83.0		
137T	61.7	57.4	61.7	66.0	66.0	70.2	74.5	66.0	70.2	
192M	57.4	61.7	57.4	61.7	66.0	57.4	61.7	53.2	57.4	83.0
35793	61.7	66.0	61.7	66.0	61.7	61.7	66.0	57.4	61.7	78.7
30577	57.4	61.7	48.9	61.7	48.9	53.2	61.7	57.4	53.2	57.4
30584	57.4	61.7	66.0	66.0	66.0	61.7	61.7	53.2	61.7	61.7
23148	57.4	57.4	61.7	61.7	61.7	66.0	61.7	53.2	53.2	53.2
51652	66.0	66.0	70.2	70.2	70.2	70.2	70.2	61.7	66.0	61.7
H4	70.2	61.7	66.0	74.5	66.0	70.2	78.7	70.2	70.2	66.0
W2P	61.7	57.4	57.4	70.2	66.0	66.0	70.2	61.7	61.7	70.2
ADI	48.9	70.2	61.7	70.2	57.4	57.4	57.4	48.9	57.4	61.7
23147	59.6	51.1	51.1	51.1	59.6	55.3	55.3	51.1	46.8	51.1
	KM69	WUF143	23125	C3	500	23580	EBI	17916	51463	137T

192M										
35793	95.7									
30577	57.4	57.4								
30584	66.0	66.0	61.7							
23148	48.9	48.9	70.2	66.0						
51652	66.0	66.0	61.7	83.0	74.5					
H4	61.7	61.7	74.5	70.2	74.5	70.2				
W2P	74.5	74.5	70.2	74.5	66.0	70.2	70.2			
ADI	70.2	70.2	66.0	83.0	61.7	74.5	61.7	83.0		
23147	63.8	63.8	72.3	59.6	68.1	63.8	59.6	72.3	68.1	
	192M	35793	30577	30584	23148	51652	H4	W2P	ADI	23147

Figure 3.2. Dendrogram showing phenetic relationships within *G. graminis*, generated by group average cluster analysis of genetic similarities at isozyme loci.



A cluster analysis of the similarity values grouped the isolates and produced a dendrogram (Figure 3.2) showing the hierarchical associations between isolates. The dendrogram had 2 primary clusters, one containing only *Ggg* isolates, shown to have an average similarity of approximately 68%. The other primary cluster contained isolates from all three varieties with an overall average similarity of approximately 62%. Within this cluster there were two subgroups, one specific for all the *Ggt* isolates and the other further subdivided into two distinct groupings of *Gga* and *Ggg*. The *Ggt* isolates showed an overall similarity of over 70%, whereas the *Gga* isolates formed a discrete group with an average similarity of 80%. The *Ggg* isolates within this cluster had an

overall similarity of 75% and were apparently more closely related to *Gga* and *Ggt* than to the remaining *Ggg* isolates.

3.4 Discussion

The electrophoresis of soluble enzymes can be used to determine the extent of genetic differentiation within and between varieties of *G. graminis*. Isozymes revealed greater genetic similarities within varieties than between varieties and provided a suite of markers to discriminate between these groups. The degree of isozyme differentiation between varieties generally agreed with the separation of the species into different taxa according to the taxonomic criteria of morphology and host range.

The isolates used in this study were originally classified on the basis of ascospore length and pathogenicity upon their original host. This study generally supported these classifications by testing their pathogenicity upon wheat and oats. Two *Ggg* isolates (23147 and 23148), however, were observed to parasitise wheat but to a lesser extent than the *Ggt* and *Gga* isolates. Additionally, 23147 and isolate 500 (*Ggt)* were capable of infecting oats.

The combination of the genetic resistance of oats to *Ggt* and *Ggg* and its susceptibility to *Gga* is a more reliable criterion for separating the varieties than is ascospore length (Chambers and Flentje, 1967). The use of a continuous variable such as spore length, is ambiguous since it involves a subjective determination of acceptable levels of variation within a variety. This is emphasised by the fact that many Western Australian isolates, with ascospore lengths in the range attributed to *Ggt*, are capable of parasitising oats (Yeats, 1986). Isolate 500 originated from a region of West Australia known to have a high incidence of these types (Yeats *et al.*, 1986). Consequently, isolates 500 and 23147 might have required reclassification to *Gga*. Isozyme analysis, in contrast, indicated that these isolates were more related to *Ggt* and *Ggg*, respectively, and taxonomic revision was not required.

Extensive polymorphisms were revealed within and between varieties of *G. graminis* at the isozyme loci used in this study. Only two of the fifteen loci were

invariant within *Ggt* and *Ggg*, whereas the majority were monomorphic within *Gga*. On average *Ggg* showed the highest levels of genetic diversity followed by *Ggt* and *Gga*. Clearly, a greater number of isolates needs to be examined for accurate determination of the levels of genetic diversity within the respective varieties.

Electrophoresis is useful in discriminating between taxonomic groups only when the within-group differences are smaller than those observed between groups (Burdon and Marshall, 1981). Electrophoretic variation detected in this study indicated that on average a greater proportion of variation was found within rather than between varieties, indicating that there is a continuum of isozyme variation within the pathogen. This tends to imply that studies of isozyme variability may not be useful in identification of the taxonomic status of isolates. Nevertheless, some enzymes resolved a greater proportion of diversity between varieties and may provide the key information for the separation of the taxa. These loci were GOT-1, GOT-2, GSR and 6PGD and with a few exceptions, the alleles detected at these loci were specific to a variety of *G. graminis* (Figure 3.1). Consequently, these markers may be useful for the taxonomic identification of isolates.

Genetic similarities between isolates, averaged across all isozyme loci, generally indicated that isolates within a particular variety showed closer relationships to each other than they did to isolates from other varieties (Figure 3.2). All of the *Ggt* isolates formed a discrete group with an average similarity of approximately 70%. *Gga* isolates also formed one group (80% similarity) whereas those from *Ggg* were subdivided into two distinct groups (60% similarity). Inter-variety comparisons, therefore, indicated that *Gga* isolates showed the greatest similarities followed by isolates within *Ggt* and *Ggg*, respectively.

Hierarchical clustering of the isolates according to their isozyme similarities implied that *Gga* isolates showed a closer relationship to a subset of the *Ggg* isolates than they did to the *Ggt* isolates. It was notable that *Ggg* isolate 23147, shown to be weakly pathogenic to oats, was more remotely related to the *Gga* isolates than the other *Ggg* isolates were (Figure 3.2). Isolate 500, also shown to be weakly pathogenic to oats,

clustered with the *Ggt* isolates. This distinction between isozyme similarities and ability to parasitise oats implied that isozyme variation could not be used to clearly predict pathogenicity of isolates. Nevertheless, isozymes could be used to provide an overall estimate of phenetic relationships within *G. graminis*.

The genetic relationships between varieties at isozyme loci were similar to those previously reported with electrophoresis of total protein profiles (Abbott and Holland, 1975; Maas *et al.*, 1991). These earlier studies however, had not indicated a subdivision of *Ggg* into two groups, which implied a closer genetic relatedness of some of these isolates to those identified as *Ggt* and *Gga*. This observation may be important in understanding the development of pathogenic variability within the fungus and possibly reflects the evolutionary history of the different varieties of *G. graminis*.

These data have shown that isozyme variability should provide a useful tool in the analysis of pathogen populations to provide insight into the genetic diversity and evolutionary biology of *G. graminis*. Cluster analysis of the genetic similarities at isozyme loci grouped isolates according to their taxonomic identities, which generally agreed with the separation of the species into three distinct varieties. Consequently, isozymes may be useful in determining the taxonomic composition of pathogen populations.

The extent of isozyme polymorphism revealed in this study will also be useful in genetic analysis of *G. graminis*. Isozyme polymorphisms can be used in genome mapping and as neutral genetic markers to determine the inheritance of specific traits such as virulence (Spielman *et al.*, 1990). Additionally, they can complement the use of DNA markers (e.g. RFLPs) in phenetic analyses by providing an independent set of data when determining relationships between taxa.

Chapter 4 Intraspecific classification of isolates of *G. graminis* using restriction fragment length polymorphisms

4.1 Introduction

Isozyme analysis revealed high levels of diversity both within and between varieties of *G. graminis*, indicating the usefulness of this marker system for a population genetic study of the pathogen. Nevertheless, on average more diversity was found within rather than between varieties implying limitations to the use of isozymes as variety-specific markers. A more reliable method for the identification of isolates is needed.

Molecular probes developed to detect RFLPs have been successfully used as genetic markers in a number of phytopathogenic fungi (Michelmore and Hubert, 1987) and have determined taxonomic relationships between groups of closely related fungi (Braithwaite *et al.*, 1990; Vilgalyis and Gonzalez, 1990). Some progress has been made towards the identification of *G. graminis* which has helped clarify intra-specific relationships within the species (Henson, 1989; Bateman *et al.*, 1992). The probes used, however, did not clearly differentiate between the taxonomic varieties.

Examination of repetitive DNA sequences has indicated their usefulness in determining taxonomic relationships between and within species of fungi (Hintz *et al.*, 1989; O'Dell *et al.*, 1989). Ribosomal DNA (rDNA) repeat sequences have been used to discriminate between varieties of *G. graminis* (Ward and Gray, 1992) and were also capable of differentiating between rye and wheat pathotypes within *Ggt* (O'Dell *et al.*, 1992). These experiments, however, did not provide definitive variety-specific markers.

It is likely that low-copy sequences may provide the best means of clearly differentiating the varieties of *G. graminis*. To achieve this, a random genomic library was constructed using a *Ggt* isolate as the source of clones. These clones were then used as probes to search for variety-specific markers and to assess levels of genetic diversity within and between varieties of the pathogen. By these methods a number of

genetic markers were developed to examine basic questions on the variation, distribution and evolution of *G. graminis* populations.

4.2 Methods and materials

4.2.1 Growth of fungi for DNA analysis

The identities of isolates used in this study are listed in Table 3.1, Chapter 3. Mycelium was grown as described in Chapter 3. Replicate cultures were grown without agitation and were incubated for 4-5 weeks at 22°C until a thick mycelial mat had formed on the liquid surface.

4.2.2 DNA extraction

Mycelium was filtered, washed twice in sterile distilled water and blotted dry on chromatography paper (Whatman 3MM) before being frozen in liquid nitrogen, ground to a fine powder in a mortar, resuspended in two volumes of DNA extraction buffer (Appendix A) and further homogenised by gentle grinding. The icy slurry was then immediately transferred to a polypropylene tube and an equal volume of phenol/chloroform/iso-amyl alcohol (25:24:1) added. The solution was extracted by gentle mixing at 4°C on a rotary mixer for 30 minutes.

The two phases were separated by centrifugation at 5,000 rpm and 4°C for 10 minutes. The aqueous phase was transferred to a fresh tube, an equal volume of phenol/chloroform/iso-amyl alcohol added and the process repeated. The aqueous phase was then transferred to a fresh tube and the DNA precipitated with ethanol as described below.

4.2.3 Ethanol precipitation of DNA

One tenth volume of 3 M sodium acetate pH 4.8 was mixed with the DNA solution and 2.5 volumes of absolute ethanol added, followed by gentle mixing. To recover the DNA, samples were centrifuged at 12,000 rpm for 10-15 minutes. The supernatant was

removed and the pellet washed twice in 70% ethanol. The sample was then dried under vacuum for 5-10 minutes before being resuspended in TE buffer (Appendix A).

4.2.4 DNA purification via caesium chloride (CsCl) centrifugation

DNA was resuspended in 7.0 ml of TE buffer. CsCl (7.5 g) was added and dissolved with gentle agitation. The solution was then transferred to an ultracentrifuge tube and 0.5 ml ethidium bromide (10 mg/ml) added. After thorough mixing the sample was centrifuged at 40,000 rpm for 40 hours (rotor 70.1 Ti, Beckman L2-65B). The DNA band was transferred to a 20 ml screw top tube and extracted with water saturated butanol until no pink colour was visible. The DNA solution was transferred to dialysis tubing and dialysed against three changes of 2 litres TE buffer for 16 hours.

The concentration of the DNA was determined by spectroscopy. A 1:50 dilution of the DNA solution was made and its optical density (OD) measured at a wavelength of 260 nm.

4.2.5 Digestion of DNA with restriction enzymes

DNA (1.5-5.0 µg) was digested in a reaction containing 10 µl DNA solution, 1µl RNAase (10 mg/ml, DNAase free), 20 units of restriction enzyme and 2 µl of the appropriate 10x restriction enzyme buffer (Appendix A). The reaction was made up to a total volume of 20 µl by the addition of sterile water. In the case where the DNA was purified by caesium chloride gradients the RNAase was omitted from the reaction. All reactions were incubated at 37°C for 6-12 hours. The enzymes commonly used were BamH I, Dra I, Hae III, Hpa II, Pst I and Pvu II (Boehringer Mannheim).

4.2.6 Construction of *G. graminis* genomic clones

Ten micrograms of vector DNA (plasmid pUC19) was digested to completion with the restriction enzyme Pst I. The DNA was ethanol precipitated and frozen in liquid nitrogen. Upon thawing the DNA was collected by centrifugation (15,000 rpm, 10 minutes) washed twice with 70% ethanol and dried under vacuum. The sample was resuspended in 40 µl water.

Dephosphorylation of the vector was carried out in a reaction volume of 50 µl with 5 µl 10x phosphatase buffer (Appendix A), 0.15 units calf intestinal alkaline phosphatase (CIP) and incubation at 37°C for 30 minutes, followed by 65°C for 10 minutes. Addition of another 0.10 unit CIP was followed by a further incubation at 37°C for 30 minutes. The reaction was then extracted twice with an equal volume of phenol/chloroform and precipitated with ethanol. The DNA was recovered and resuspended in TE buffer to a final concentration of 0.5 µg/µl.

4.2.7 Ligation of genomic DNA into plasmid vector

DNA (5.0 µg) of *Ggt* isolate C3 was digested with 20 units of the restriction enzyme Pst I at 37°C for 3 hours. The DNA was precipitated with ethanol and resuspended in TE buffer to a final concentration of 0.5 µg/µl.

The ligation reaction was performed in a 10 µl volume containing 2.0 µg digested fungal DNA, 0.5 µg of vector, 1.0 µl 10x ligation buffer (Appendix A), 1.0 µl 10 mM ATP pH 7.0, 1.0 µl T4 DNA ligase and 2.0 µl water. The reaction was incubated at 12°C for 12 hours. The ligated DNA was then precipitated with ethanol, recovered and resuspended in 20 µl TE buffer.

4.2.8 Transformation of *Escherichia coli*

Preparation of competent cells

An overnight culture of *E. coli*, strain DH5α, was prepared by inoculating cells into 2.0 ml LB media and incubating at 37°C. A 0.50 ml aliquot was added to 50 ml SOB medium (Appendix A) and incubated at 37°C until the OD_{600} nm had reached 0.45-0.55. The culture was then chilled on ice for 10 minutes and transferred to a sterile centrifuge tube. Cells were pelleted at 2,500 rpm for 12 minutes at 4°C, resuspended in 8.5 ml of TFB buffer (Appendix A) and chilled on ice for a further 10 minutes. The centrifugation step was repeated, the supernatant discarded and the cells resuspended in 2.0 ml TFB buffer + 70 µl redistilled dimethylsulfoxide (DMSO). Cells were then

chilled on ice for 20 minutes, with the addition of 160 µl of 1 M dithiothreitol (DTT) after 5 minutes and 75 µl DMSO after a further 10 minutes.

Transformation of competent cells

Competent cells (0.20 ml) were transferred to a sterile glass tube and 10.0 µl of the ligated plasmid DNA added, mixed gently and placed on ice for 30 minutes. The cells were heat shocked at 42°C for 2 mins and 800 µl SOC medium (Appendix A) added prior to incubation in a 37°C shaker for 45 minutes.

Cells were plated out as 100 µl aliquots onto LB plates containing Ampicillin, X-gal and IPTG (Appendix A) and incubated at 37°C overnight. Recombinant colonies (containing inserts of *G. graminis* DNA) were identified as white colonies on this medium, non-recombinant colonies as blue.

4.2.9 Isolation of recombinant plasmids

The alkaline lysis method for mini-scale plasmid preparations was used as described in Sambrook *et al.* (1989). A sterile culture tube containing 3.0 ml LB medium (+ 50 µg/ml ampicillin) was inoculated with a single colony and grown overnight in a 37°C shaker. Cells were pelleted by centrifugation at 6,000 rpm for 5 minutes and the media aspirated off. The cells were resuspended in 0.10 ml plasmid I solution (50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA pH 8.0) and placed on ice for 10 minutes. Cells were lysed by the addition of 0.2 ml plasmid II solution (0.2 N NaOH, 1.0% SDS) and incubated on ice for a further 10 minutes. Following the addition of 0.15 ml 3 M sodium acetate (pH 4.8), the solution was incubated at -20°C for 10 minutes and centrifuged at 12,000 rpm for 15 minutes. The supernatant was transferred to a fresh 1.5 ml polypropylene tube and extracted twice with equal volumes of phenol/chloroform/iso-amyl alcohol (25:24:1). Plasmid DNA was precipitated with ethanol and frozen in liquid nitrogen. The DNA was recovered by centrifugation at 12,000 rpm, washed twice in 70% ethanol, dried under vacuum and resuspended in 50µl TE buffer.

4.2.10 Radioactive labelling of plasmid DNA

Recombinant plasmid DNA was digested with the restriction enzyme Pvu II to excise the *G. graminis* DNA fragment. This digestion provided an efficient method of isolating the fungal DNA insert from within pUC19 as the Pvu II restriction sites flank the Pst I cloning site at either end. An aliquot of the digested sample was fractionated by electrophoresis in a 1% agarose gel in TAE buffer (Appendix A), stained with ethidium bromide (10μg/ml) and viewed under UV light. Concentration of the DNA was approximated by comparison with plasmid DNA of known concentrations.

Samples of DNA (approximately 0.05 μg, total volume 10 μl), were denatured by boiling in water for 5 minutes and chilled on ice to prevent reannealing of the separate strands, then (2 x)pUC19 specific oligolabelling buffer (12.5 μl), 3 μl [α-^{32}P]-dCTP (~50 μCi) and 1.50 units Klenow enzyme were added, mixed and incubated at 37°C for 45-60 minutes. Labelled DNA was separated from unincorporated nucleotides on a G-100 Sephadex column.

The specific oligolabelling buffer contained primers with sequences complementary to the vector DNA flanking the fungal DNA insert (Appendix A). This provided a highly efficient means of priming the labelling reactions.

4.2.11 Electrophoresis and transfer of DNA to nylon membranes

Digestion of DNA was carried out as described previously. Digested DNA was mixed with 1.0 μl 10x gel loading buffer and fractionated in 1% agarose gels in TAE buffer at 20-25 mA overnight. After electrophoresis, gels were stained in 10 μg/ml ethidium bromide for 20 minutes and viewed under UV light. Gels were soaked for 30 minutes in denaturing solution followed by 20 minutes in neutralising solution (Appendix A). The DNA was transferred to a nylon membrane (Hybond-N$^+$, Amersham) using 20x SSC for 12-16 hours (Appendix A). Transfer was by capillary blotting as described by Southern (1975). Upon completion of the transfer, the membrane was rinsed briefly in 2x SSC and placed in a vacuum oven at 80°C for 20 minutes. DNA was fixed to the membrane by soaking in 0.4 M NaOH for 30 minutes

(DNA side up). The membrane was rinsed sequentially in neutralising solution and 5x SSC before being blotted dry with Whatman 3MM paper.

4.2.12 Hybridisation and autoradiography

Prehybridisation of membranes was performed in a hybridisation buffer containing 2.0 ml sterile water, 3.0 ml 5x HSB, 1.0 ml 10x Denhardts III solution, 4.0 ml 25% dextran sulphate and 0.20 ml salmon sperm DNA (5 µg/ml) (Appendix A). Salmon sperm DNA was boiled in water for 5 minutes and chilled on ice before being added to the buffer. The membrane was placed in a hybridisation bottle (Hybaid) and the solution (pre warmed to 65°C) was added. Membranes were prehybridised at 65°C overnight.

The radiolabelled probe was denatured in boiling water for 5 minutes in a tube containing 200 µl salmon sperm DNA (5 µg/ml) and added to a fresh, pre-warmed solution of hybridisation buffer. The prehybridisation mix was drained off and the hybridisation buffer added to the membrane. Hybridisation was conducted at 65°C overnight.

The membranes were then washed in 2x SSC, 0.1% SDS for 30 minutes at 65°C to remove unbound DNA. Washes were repeated in 1x SSC, 0.1% SDS and 0.5x SSC, 0.1% SDS. The membranes were sealed in plastic and X ray film exposed to them at -80°C for 1 hour to 5 days, depending on signal strength.

4.2.13 Collection and analysis of data

Variations in hybridisation patterns were distinguished visually from the autoradiographs. Each clearly resolved band was assigned a value in ascending numerical order, the largest restriction fragment having a value of 1. By comparing the banding profiles, a restriction phenotype was assigned to each isolate. These data were scored in a binary format (i.e. 1 = presence of a specific band, 0 = absence of that band). Phenotypes for each of the restriction enzyme/probe combinations were checked by using replicated membranes and side by side comparisons.

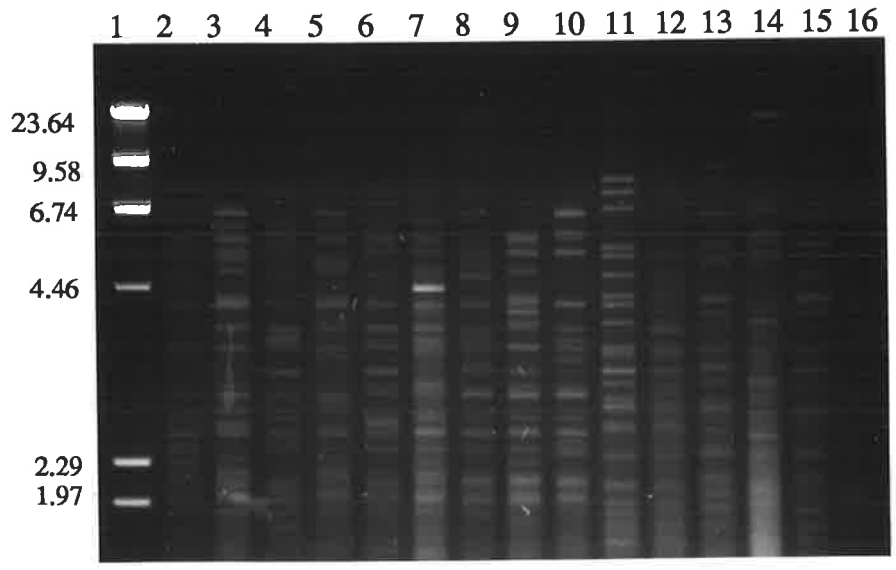
The phenotypic frequencies within the collection of isolates were calculated and used to estimate levels of diversity both within and between the varieties of *G. graminis* using Shannon's diversity index (Chapter 3). Estimates of the genetic similarities between isolates were determined by combining the restriction phenotypes from each hybridisation experiment and calculated as described in Chapter 3.

4.3 Results

4.3.1 Repetitive DNA sequences within *G. graminis*

DNA was extracted from 18 isolates which were taken as representative of the three varieties of *G. graminis*. Digestion of the DNA with the restriction enzymes BamH I, Dra I, Hind III, Hae III and Hpa II revealed many restriction fragment length polymorphisms (RFLPs) between the isolates. Many of these bands stained intensely

Figure 4.1. Agarose (1.5%) gel of *G. graminis* DNA digested with restriction enzyme Hpa II, fractionated by electrophoresis and stained with ethidium bromide. Lanes: 1, λ Hind III standard molecular weight markers (kilobase pairs); 2-7, *Ggt* -C3, 51463, EBI, 17916, 800, KM69; 8-10, *Gga*- 35793, 137T, 192M; 11-15, *Ggg*- 30584, 23148, H4, 30577, ADI; 16, *Gga*- 23144.



with ethidium bromide and were thought to represent repetitive sequences (O'Dell *et al.*, 1992). The differences in the size of the fragments provided a means of documenting variation between isolates. The enzymes BamH I, Dra I and Hind III revealed a greater number of high molecular weight fragments than did Hae III and Hpa II and their resolution on the gels was generally poorer than those seen with the latter enzymes. Consequently, only the Hae III and Hpa II digests were analysed by visual interpretation of the gels. An example of an Hpa II digest is shown in Figure 4.1.

Of the 18 isolates examined, five, 23580, 23125, 23152, 500 (*Ggt*) and 23144 (*Gga*), yielded restriction profiles that were difficult to resolve clearly and were not included in the final analysis. Each isolate had an individual phenotype (DNA fingerprint) upon digestion with Hae III and Hpa II. No one band or group of bands within the restriction digest profiles could be used for accurate discrimination between the three varieties of *G. graminis*.

A similarity matrix was generated by pairwise comparisons of restriction profiles between isolates (Table 4.1). Isolates found to be most similar in their banding patterns were KM69 and C3 (*Ggt*) with an overall similarity of 86%. Those isolates showing the least similarity (42%) were 23148 and 30577 (*Ggg*). The average overall similarity between isolates was approximately 57%.

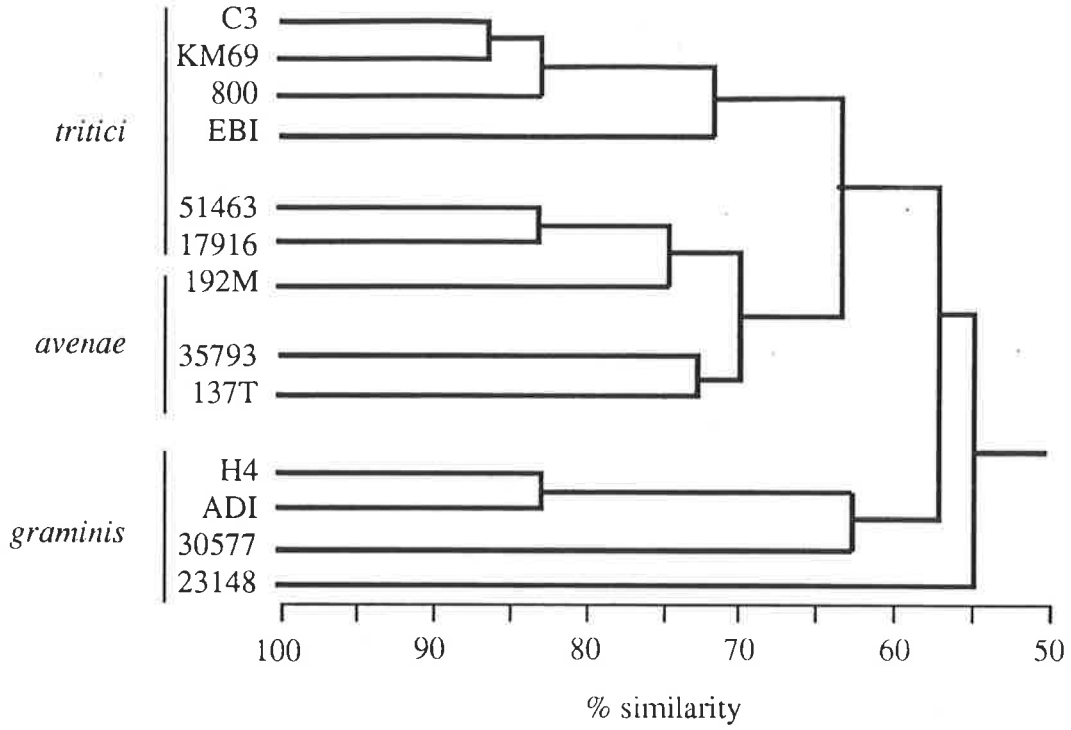
The dendrogram (Figure 4.2), showed the overall relationships between isolates and had two primary clusters, one specific for *Ggg*, the other grouping *Ggt* and *Gga*. The latter cluster is subdivided into three discrete groups, one each for *Ggt* and *Gga* and one containing isolates from both varieties. *Ggg* isolate 23148 was observed to be very different from the other isolates.

Table 4.1. Genetic similarities (%) among isolates of *G. graminis* based on DNA repeat-sequence phenotypes detected following digestion with the restriction enzymes Hae III and Hpa II.

C3						
51463	62.7					
EBI	67.8	61.0				
17916	62.7	83.1	64.4			
800	81.4	57.6	79.7	57.6		
KM69	86.4	59.3	67.8	62.7	84.7	
35793	64.4	71.2	66.1	67.8	72.9	71.2
137T	61.0	71.2	55.9	71.2	66.1	74.6
192M	61.0	71.2	55.9	78.0	62.7	67.8
23148	54.2	64.4	55.9	54.2	52.9	54.2
H4	50.8	61.0	55.9	67.8	55.9	57.6
30577	50.8	61.0	45.8	54.2	55.9	54.2
ADI	50.8	61.0	55.9	64.4	52.9	50.8
	C3	51463	EBI	17916	800	KM69

35793						
137T	72.9					
192M	66.1	52.5				
23148	52.5	55.9	52.5			
H4	59.3	62.7	62.7	55.9		
30577	62.7	59.3	59.3	42.4	69.5	
ADI	52.5	59.3	59.3	62.7	83.1	55.9
	35793	137T	192M	23148	H4	30577

Figure 4.2. Dendrogram generated by group average cluster analysis of the genetic similarities between DNA repeat-sequence phenotypes within *G. graminis*.



4.3.2 Genetic diversity within *G. graminis* detected by DNA hybridisation experiments

Fifty clones were selected at random and were found to contain recombinant DNA ranging in size from 60 base pairs (bp) to over 4.0 kilobase pairs (kbp). Initially, pairs of isolates C3, 800, 192M, 23144, ADI and W2P were selected for analysis as being representative of *Ggt*, *Gga* and *Ggg*, respectively. DNA from each isolate was digested with the restriction enzymes BamH I, Dra I and Hind III and screened with each of the 50 clones. These probe/enzyme combinations are hereafter labelled with the probe number and the first letter of the enzyme name, e.g. combination 35B represents probe 35 with restriction enzyme BamH I. Some combinations were monomorphic in *Ggt* (T, Table 4.2), others in *Gga* (A) and others in *Ggg* (G) and were polymorphic across varieties. Some combinations were monomorphic across two or all of the varieties (blank). Finally, many combinations resolved RFLP phenotypes which were isolate-

Table 4.2. Summary of RFLP patterns observed within and between varieties of *G. graminis*.

Insert	size	Hind III							BamH I							Dra I						
	(kbp)	number	*tritici*		*avenae*		*graminis*		number	*tritici*		*avenae*		*graminis*		number	*tritici*		*avenae*		*graminis*	
			C3	800	192M	23144	ADI	W2P		C3	800	192M	23144	ADI	W2P		C3	800	192M	23144	ADI	W2P
1	0.55	S							S	T	T	A	A		U	S						
3	1.51	S							S	T	T	A	A		U	S	U		A	A		
4	0.50	M	U	U	U	U	U	U	S							M	U	U	U	U	U	U
5	0.71	L					U	U	L	T	T	A	A	U	U	L						
6	0.18	L	T	T	A	A	U	U	L					U	U	L					U	U
7	0.34	L	T	T	A	A	U	U	L	T	T	A	A	U	U	S					G	G
8	0.34	S					G	G	S	U	U	A	A	U	U	S						
10	0.36	M	T	T	A	A	U	U	M	T	T	A	A	U	U	L						
12	0.80	M	T	T	A	A	*	*	L	T	T	A	A	*	*	L	T	T	A	A	*	*
14	0.73	L							L					U	U	M					U	U
16	4.57	M	T	T	A	A	*	U	L					G	G	L	T	T	A	A	U	U
23	3.90	M	U	U	A	A	U	U	M	U	U	A	A	U	U	S	T	T	A	A	G	G
24	4.67	M	T	T	A	A	*	U	L					G	G	L	T	T	A	A	U	U
26	0.42	S	T	T	A	A	U	U	S	U	U	A	A	U	U	S						
28	0.40	L	U	U	A	A	*	U	L	U	U	A	A	U	U	M	T	T	A	A	U	U
30	0.48	S	T	T	A	A	U	U	S					U	U	S					U	U
34	0.35	M	U	U	A	A	U	U	L	U	U	A	A	G	G	S	U	U	A	A	U	U
35	1.33	L	T	T	A	A	*	U	L	T	T	A	A	G	G	S	T	T	A	A	G	G
38	0.95	L	T	T	A	A	*	U	L					G	G	L						
54	2.30	S					U	U	L					U	U	L					U	
55	2.20	L	T	T	A	A	G	G	L	T	T	A	A	G	G	S	T	T	A	A	U	U
60	0.34	L	T	T	A	A	*	*	L	T	T	A	A	U	U	L	U	U	A	A	U	U
62	0.26	L	T	T	A	A	*	*	L	U	U	A	A	*	*	L	T	T	A	A	*	*
66	1.13	L	T	T	A	A	U	U	L	T	T	A	U	U	U	L					*	U
75	3.46	L	T	T	A	A	U	U	L					U	U	L					U	U
76	1.33	L	T	T	A	A	G	G	L	T	T	A	A	U	U	L	T	T	A	A	U	U
140	1.16	L	T	T	A	A	*	U	L	T	T	A	A	U	U	L					G	G

Table 4.2 continued

Insert	size	Hind III							BamH I							Dra I						
	(kbp)	number	*tritici*		*avenae*		*graminis*		number	*tritici*		*avenae*		*graminis*		number	*tritici*		*avenae*		*graminis*	
			C3	800	192M	23144	ADI	W2P		C3	800	192M	23144	ADI	W2P		C3	800	192M	23144	ADI	W2P
158	3.98	M	U	U	U	U	U	U	M	U	U	U	U	U	U	M	U	U	U	U	U	U
179	0.18	L	T	T	A	A	U	U	L	T	T	A	A	U	U	L	T	T	A	A	U	U
181	0.18	L	T	T	A	A	G	G	L	T	T	A	A	U	U	S						
182	0.06	S	T	T	A	A	G	G	S						U	S						
183	0.71	L					*	U	L	T	T	A	A	U	U	L	T	T	A	A	*	U
184	0.07	L	T	T	A	A	*	U	S	T	T	A	A	U	U	S	T	T	A	A	U	U
185	1.13	L					*	U	L						U	S					G	G
186	0.08	M					*	U	M	T	T	A	A	U	U	M					U	U
188	0.13	M	T	T	A	A	*	*	L							L						
192	1.16	S							S					G	G	S	T	T	A	A	U	U
199	0.48	S	T	T	A	A	*	*	S	T	T	A	A	*	*	S						
201	0.63	L	T	T	A	A	*	U	S	T	T	A	A	U	U	S	T	T	A	A	*	*
202	2.28	L	T	T	A	A	U	U	L					U	U	S						
203	0.08	L	T	T	A	A	U	U	L	T	T	A	A	U	U	S						
205	1.68	L	T	T	A	A	U	U	L	T	T	A	A	U	U	L	T	T	A	A	U	U
206	0.13	L	T	T	A	A	*	U	L	T	T	A	A	U	U	S	T	T	A	A	U	U
208	0.30	L					*	U	S	T	T	A	A	G	G	L						
210	1.44	L	T	T	A	A	*	U	L					*	U	S	T	T	A	A	U	U
211	0.24	S							S						U	L	T	T	A	A		U
212	1.53	L					*	U	L					G	G	L		U				
216	2.38	L					*	*	L	T	T	A	A	U	U	L	T	T	A	A	U	U
217	0.79	L	T	T	A	A	U	U	S	T	T	A	A	U	U	S						
229	1.40	S							S					U	U	S						

Number: Number of bands hybridising to a particular probe. S, single band per isolate; L, low copy (2-4 fragments per isolate); M, multiple copy (≥ 5 fragments per isolate). U- unique phenotype; A, phenotype specific to *Gga*; G, specific to *Ggg*; T, specific to *Ggt*; *, missing value. Blank spaces indicate monomorphism between varieties.

specific (U). Of the 150 probe/enzyme combinations 9 (6%) detected inter-variety polymorphisms whilst being monomorphic within a variety (e.g. 35/BamH I). Eighty five (57%) of the combinations gave clear differentiation between *Ggt* and *Gga* whilst exhibiting variation within *Ggg*. These two categories represented the most likely candidates for variety-specific markers and were investigated further.

The combinations which resolved isolate-specific RFLP phenotypes should be useful to document variation within varieties of *G. graminis*. Fourteen (9%) of the combinations gave individual phenotypes for *Ggt* and *Ggg*, whereas only 4 (3%) revealed discrete phenotypes among all isolates. *Ggg* was the most variable variety, with 91 (60%) of the combinations polymorphic between isolates. This is in sharp contrast to *Ggt* and *Gga* where variation between isolates within a variety was detected in only 11% and 4% of the combinations, respectively. Only 21 (14%) of the combinations were invariant among the 6 isolates studied.

Twenty two of the combinations were selected to examine diversity within the set of isolates used in this study. They were selected on the criterion of having clearly resolved restriction fragments which would either be useful in discriminating the varieties of *G. graminis* or revealed high levels of diversity which could be used to document intra-variety variability. Each of these probes revealed unique, non-overlapping combinations of hybridisation fragments (RFLP phenotypes) when screened against the *G. graminis* isolates. This implies that they were targeting different regions of the genome.

Polymorphisms were identified within *Ggt* and *Ggg* with each of the 22 combinations, with the average number of RFLP phenotypes being slightly greater in *Ggt* (Table 4.3). Many of these differences within *Ggt* were attributed to only one or two isolates (usually 23580 and 500), with the majority of isolates having common phenotypes. In contrast, *Gga* showed variation at only 48% of the probe/enzyme combinations with the average number of phenotypes per combination being approximately half of that revealed in *Ggt* and *Ggg*.

Table 4.3. Descriptive statistics of genetic diversity within the 3 varieties of *G. graminis* detected with the 22 probe/enzyme combinations listed in Table 4.4. A probe/enzyme combination was considered polymorphic when at least two RFLP phenotypes occurred within a variety of *G. graminis*.

Variety	*Polymorphic RFLP markers (%)	Average No. phenotypes/RFLP marker
Ggt	100	3.71
Gga	48	1.57
Ggg	100	3.05
G. graminis	100	7.43

Levels of diversity within *Ggg* and *Ggt* were approximately the same, with mean values of 1.506 and 1.458, respectively (Table 4.4). In contrast, *Gga* exhibited relatively low levels of variation, with a mean diversity of 0.466. *Gga* isolates were monomorphic at 13 of the probe/enzyme combinations, whereas all resolved variation within *Ggt* and *Ggg*. Combinations 4D, 4H, 23B, 34D and 158H resolved the most variation between isolates, irrespective of their taxonomic status, and revealed multiple fragments in all isolates indicating that they were hybridising with repetitive sequences (Figures 4.3 and 4.4). Combination 4H produced fewer hybridisation fragments than the other repeat sequence probes (5-8 fragments, ranging from 1.20 kbp to 11.0 kbp) while 158H produced the most (greater than 20, ranging from 0.50 kbp to 14.60 kbp). The majority of isolates showed unique phenotypes (DNA fingerprints) at each of the combinations. Only *Ggt* isolates C3 and KM69 were identical with 4D whereas *Ggt* isolates 800, 23125 and EBI were identical with 4H and 23B. *Gga* 192M and *Ggt* 23580 were also identical with 4H. The lowest levels of diversity were shown by the combinations 26H and 30H which resolved only a single hybridisation fragment per isolate.

Most of the diversity (54%) occurred between varieties of *G. graminis* (Table 4.4). If the highly variable repeat-sequence probes (4D, 4H, 23B etc.) were removed from this analysis this figure would be greater. The proportion of diversity partitioned within

Table 4.4. Estimates of genetic diversity (H_0) within and between varieties of *G. graminis* calculated from RFLP phenotype frequencies determined with 22 probe/enzyme combinations.

Locus	Within varieties			Average within varieties	Total within collection	Proportion within varieties	Proportion between varieties
	$H_{tritici}$	H_{avenae}	$H_{graminis}$	Hvar	Hcoll	$\frac{Hvar}{Hcoll}$	$\frac{Hcoll-Hvar}{Hcoll}$
4D	2.493	0.915	2.000	1.803	3.479	0.518	0.482
4H	2.940	1.585	2.000	2.175	3.833	0.567	0.433
5B	0.980	0.915	2.000	1.298	2.606	0.498	0.502
10H	0.980	1.585	0.811	1.125	2.069	0.544	0.456
16D	1.747	0.000	1.500	1.082	1.939	0.551	0.449
23B	2.631	0.915	2.000	1.849	3.567	0.518	0.482
26B	1.347	0.000	0.811	0.719	1.889	0.381	0.619
26H	0.500	0.000	1.500	0.667	1.375	0.485	0.515
30H	0.500	0.000	1.000	0.450	1.187	0.379	0.621
34D	2.050	0.915	1.500	1.488	2.938	0.507	0.494
60B	1.438	0.000	1.500	0.979	2.134	0.459	0.541
60H	1.438	0.000	1.000	0.813	2.472	0.329	0.671
75Hp	0.980	0.000	1.500	1.132	2.504	0.452	0.548
140B	0.915	0.000	2.000	1.277	2.586	0.494	0.506
158H	3.169	1.585	2.000	2.251	3.999	0.563	0.437
179D	1.870	0.000	1.000	0.957	2.718	0.352	0.648
179H	0.980	0.000	1.500	0.829	2.229	0.372	0.628
181H	0.500	0.915	1.500	0.972	1.866	0.521	0.479
202H	0.980	0.000	1.500	0.827	2.333	0.354	0.646
205B	1.438	0.000	2.000	1.146	2.690	0.426	0.574
216D	1.438	0.915	1.500	1.284	2.504	0.513	0.487
217B	0.760	0.000	1.000	0.587	2.075	0.287	0.714
Mean	1.458	0.466	1.506	1.169	2.500	0.458	0.542

Probe: No. refers to identity of genomic clone. Refer to Table 2 for insert sizes. Letter refers to restriction enzyme, eg. B-BamH I, D-Dra I, H-Hind III, Hp-Hpa II.

Figure 4.3. Hybridisation of clone 23 with *G. graminis* DNA digested with *Bam*H I (ie. combination 23B). Lanes: 1-10, *Ggt*- C3, EBI, 23580, 17916, KM69, 51463, 800, 23152, 23125, 500; 11-12, *Gga*- 137T, 192M; 13-17, *Ggg*- H4, 30577, 30584, ADI, 23148. Size of fragments in kilobase pairs.

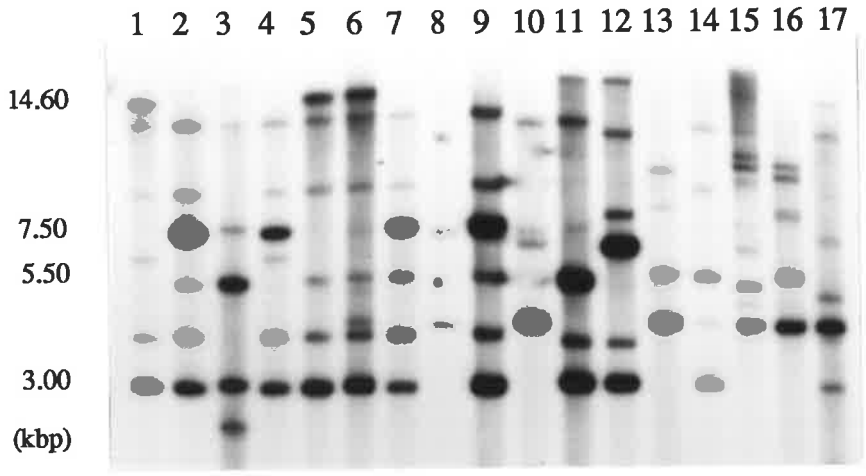
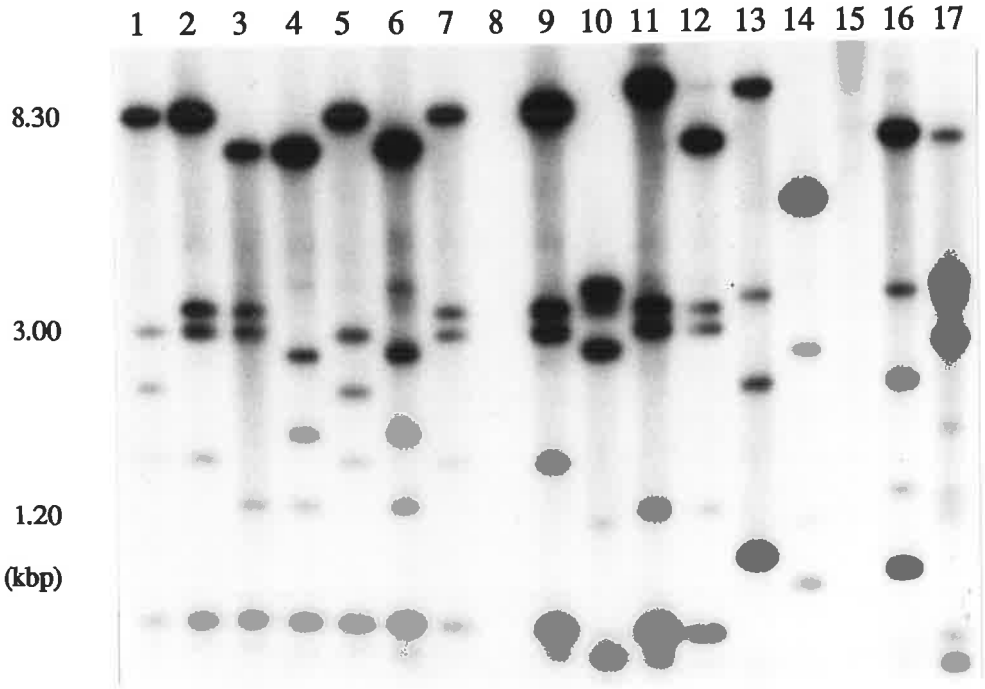


Figure 4.4. Hybridisation of clone 4 with *G. graminis* DNA digested with *Hind* III (ie. combination 4H). Lanes: 1-10, *Ggt*- C3, EBI, 23580, 17916, KM69, 51463, 800, degraded DNA, 23125, 500; 11-12, *Gga*- 137T, 192M; 13-17, *Ggg*- H4, 30577, degraded DNA, ADI, 23148. Size of fragments in kilobase pairs.



and between varieties varied depending on the combination used. Thirteen of the combinations exhibited a greater proportion of diversity between varieties of *G. graminis* (Table 4.4). In general these combinations showed conservation of a RFLP phenotype within a particular variety, which was distinct from the phenotypes in the other varieties.

Correlations between the original classification of the isolates and their classifications at putative RFLP markers are shown in Table 4.5. Combination 181H

Table 4.5. Taxonomic classifications of isolates based upon RFLP phenotypes detected between varieties of *G. graminis*.

Isolate	5B	26B	26H	30H	60H	75Hp	140B	179H	179D	181H	202H	205B	216D	217B
Ggt														
KM69	T	T	T		T	T	T	T	T	T	T	T	T	T
23125	T	T	T	U	T	T	T	T	T	T	T	T	T	T
C3	T		T		T	T	T	T	T	T	T	T	T	T
800	T	T	T		T	T	T	T	T	T	T	T	T	T
23580	U		T		U	T	T	T	U	T	U	U	T	U
EBI	U		T		T	T	T		U	T	T	T	T	U
17916	T	T	T		T	T	T	T	U	T	T	T	T	T
51463	T	T	T		T	T	T	T	T	T	T	T	T	T
500	T	A	A		T	*	T		U	G	A		U	T
Gga														
137T	U	A	A	A	A	A	A	A	A	U	A	A	U	A
192M	A	A	A	A	A	A	A	A	A	A	A	A	A	A
35793	U	A	A		*	A	*	*	*	*	A	A	*	T
23144	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Ggg														
30577	U		A			U		U	U	T	A		U	G
23148	T				U	U		G		G	T		T	G
H4	G		G	A	G	G		G	G	G	G	G	G	G
ADI	G		G	A	G	G		G	G	G	G	G	G	G
23147	U	A	A		*	*		*	*	*	U	A	*	U

T- *Ggt*, A- *Gga*, G- *Ggg*, U- unique RFLP phenotype; * - RFLP phenotype not determined, Blank- RFLP phenotype shared between *Ggt* and *Ggg*.

was also included as it showed within-variety diversity (Hvar, Table 4.4) comparable with the other 13 combinations. Many combinations resolved phenotypes which were common to *Ggt* and *Ggg* (e.g. 26B and 30H) and it proved difficult to separate these varieties. The majority of combinations clearly distinguished *Gga* isolates from *Ggt* and *Ggg*, exceptions being 5B, 181H and 216D (isolate 137T showed unique phenotypes with these combinations). Overall, there was agreement between these RFLP phenotypes and the existing classifications of isolates as either *Ggt* or *Gga*. *Ggg* however, proved to be highly variable, as indicated by the abundance of unique RFLP phenotypes within this variety. Those combinations which gave the clearest separation of the three varieties were 217B, 60H, 179D, 179H and 202H.

Probe 179 showed variation between the three varieties, but the distinctions were clearer when DNA was digested with enzyme Hind III rather than Dra I. Combination 179H clearly differentiated *Ggt* from *Gga* by their respective hybridisation fragments of 10.10 and 12.96 kbp. *Ggg* was highly variable with this combination but did not have any hybridisation fragments in common with the other two varieties (Figure 4.5). Combination 179D resolved more complex patterns, with the combination of 12.96 and 4.37 kbp fragments being unique to *Gga*. The most common pattern within *Ggt* was a doublet of bands of 14.45 and 3.25 kbp, isolates 23580 and EBI showed one of the *Gga* fragments but not both and therefore were distinguishable from the other two varieties (Figure 4.5).

Combination 217B clearly separated all three varieties of *G. graminis* by the hybridisation fragments 5.84 kbp (*Ggt*), 5.25 kbp (*Gga*) and a variety of bands within *Ggg* of approximately 3.4 kbp. *Ggt* isolates 23580 and EBI were unique, resolving a 15.34 kbp fragment (Figure 4.5).

A doublet of fragments (10.00 and 6.49 kbp) was common to all *Ggt* isolates with 60H, except 23580. This combination clearly separated *Gga* isolates which showed a RFLP phenotype with fragments of 14.10, 2.82 and 2.20 kbp (Figure 4.5). *Ggg* isolates were highly variable at this combination but were distinct from isolates within the other varieties.

Figure 4.5. RFLP phenotypes of use as taxonomic markers within *G. graminis*. Sizes of hybridisation fragments are given in kilo base pairs (kbp). Missing lanes indicate RFLP phenotype not determined.

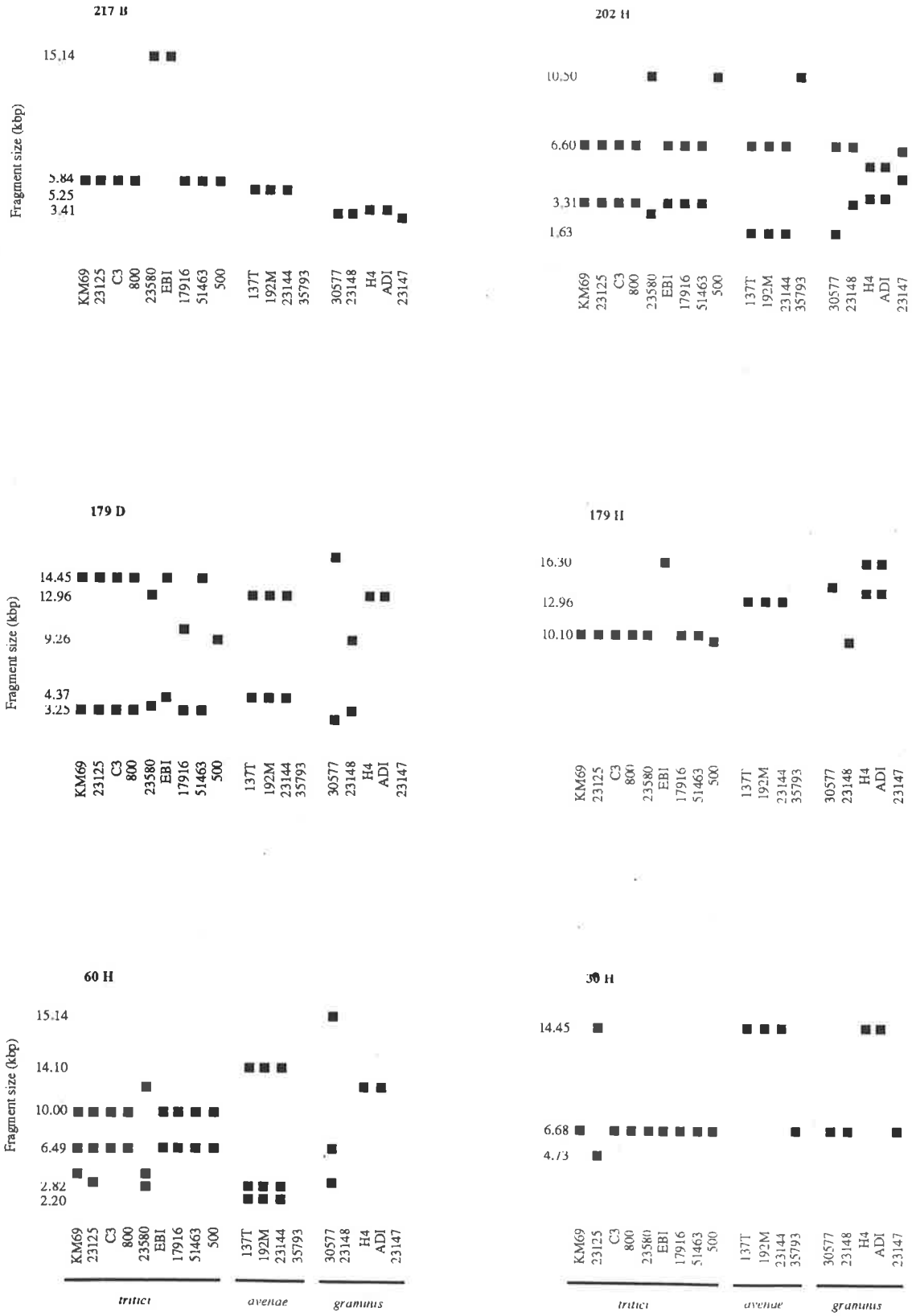
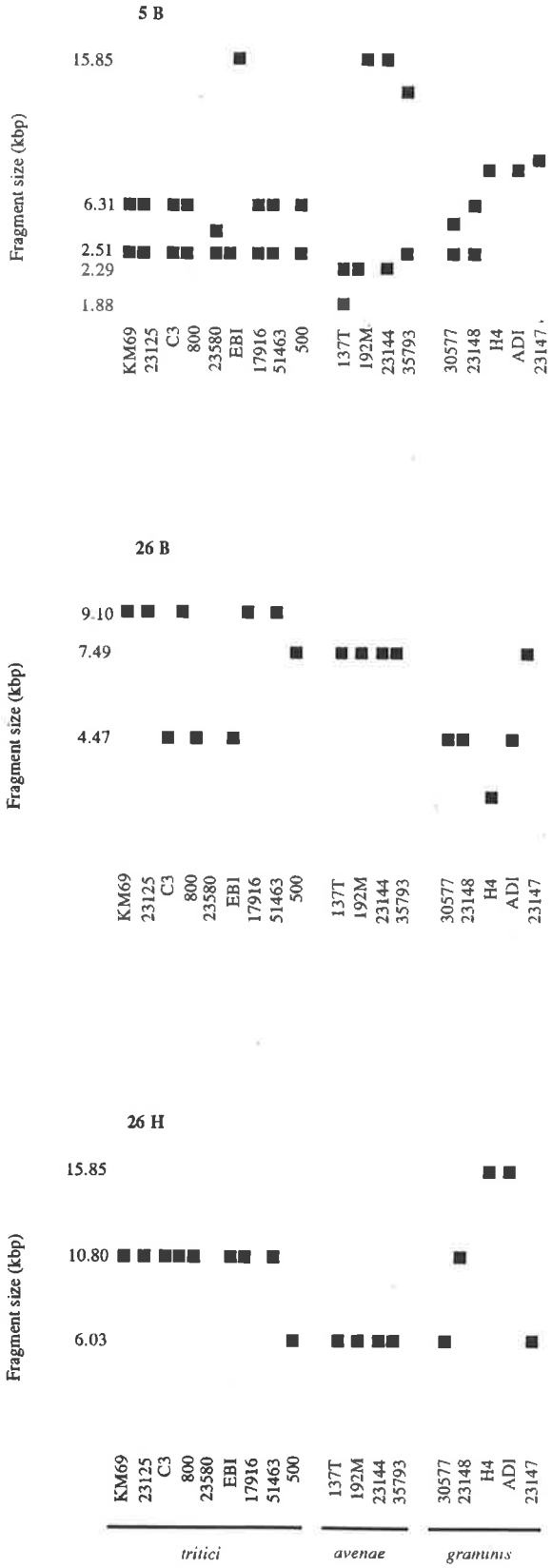


Figure 4.5. continued.



With 202H, *Ggt* isolates (except 23580) showed a doublet of fragments of 6.60 and 3.31 kbp, while *Gga* isolates were distinguished by the presence of a 1.63 kbp fragment in place of the 3.31 kbp *Ggt* fragment. *Ggg* showed a diversity of RFLP phenotypes, including the *Gga* type in *Ggg* 23147 and the *Ggt* type in 23148 (Figure 4.5).

With the combination 26H, a fragment of 10.80 kbp was common to all *Ggt* isolates (except 500) and a fragment of 6.03 kbp was common to *Gga* isolates (Figure 4.5). *Ggg* isolates 23147 and 30577 also had this 6.03 kbp fragment. Isolate 23148 (*Ggg*) showed the fragment characteristic of *Ggt*, whereas isolates H4 and ADI contained a 15.85 kbp fragment.

Combination 26B revealed a fragment of 7.49 kbp common to all *Gga* isolates. This fragment was also found in isolates 500 and 23147 and clearly differentiated *Gga* from the remaining *Ggt* and *Ggg* isolates. The presence of a 9.10 kbp fragment was common to the majority of *Ggt* isolates (Figure 4.5). Therefore, probe 26 appeared useful at differentiating *Gga* from *Ggt* and *Ggg* but could not separate the latter two varieties.

Combination 5B revealed a doublet of bands (6.31 and 2.51 kbp) which were present in most *Ggt* isolates and also in *Ggg* isolate 23148 (Figure 4.5). Isolates 23580 and EBI had unique RFLP phenotypes with this combination. Both *Ggg* and *Gga* were highly variable, with only two isolates from each variety having common RFLP phenotypes. Isolates 192M and 23144 (*Gga*) showed a doublet of fragments (15.85 and 2.29 kbp) whereas H4 and ADI (*Ggg*) showed a single fragment of approximately 8.0 kbp. All other isolates within these two varieties had unique RFLP phenotypes. Consequently, combination 5B may be useful only for the identification of *Ggt*.

Probe/enzyme combination 30H also showed considerable diversity within *Ggg* and *Gga*, with isolates in both varieties showing either a 14.45 kbp or 6.68 kbp fragment (Figure 4.5). All *Ggt* isolates (except 23125) had the 6.68 kbp fragment. While this probe is not useful for variety identification, it could be applied in studies of diversity between populations of *G. graminis* as it revealed considerable diversity within two of the varieties.

Isolates which showed anomalies between their original identification and putative RFLP markers were 23580, EBI, 500, and 23147. Isolate 23580 was classified as *Ggt* with 7 of the 14 combinations. This isolate showed unique phenotypes with 5 and could not be separated from *Ggg* with 26B and 30H. EBI showed homology with *Ggg* with three of the markers, although the remainder, with the exception of 5B, grouped it with *Ggt*. Isolates 500 (*Ggt*) and 23147 (*Ggg*) showed a diversity of RFLP phenotypes making positive identification difficult (Table 4.5).

The similarity coefficient was used to quantify the similarities between isolates by comparing the RFLP phenotypes at each of the 22 probe/enzyme combinations (Table 4.6). Isolates 35793 and 23147 were not examined at each of the combinations and were excluded from the analysis. The most similar isolates were *Ggt* isolates 23125 and 800 (97%) and those least alike *Ggt* 17916 and *Ggg* H4 (55%). The average similarity between all isolates was approximately 60%.

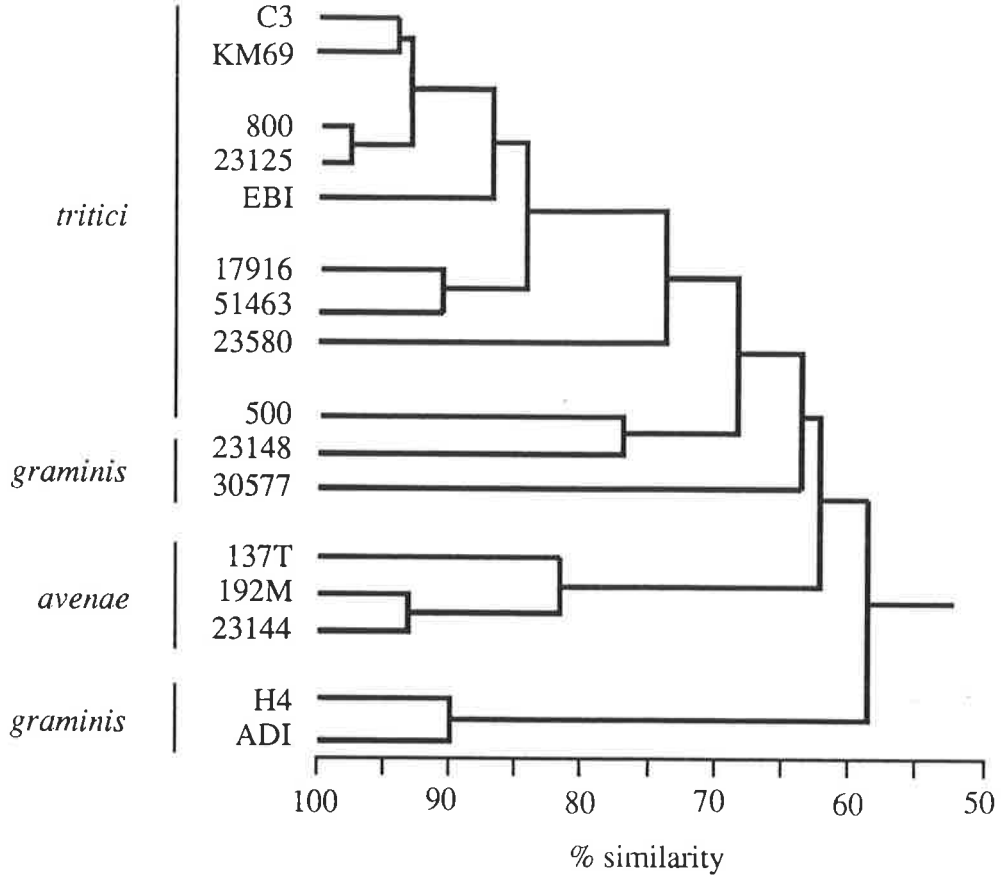
Group average cluster analysis separated the isolates into two groups (Figure 4.6). *Ggg* isolates H4 and ADI formed one of the groups and showed 90% similarity to each other, but were markedly different from the other *G. graminis* isolates. The other group was further subdivided into two, one exclusively of *Gga*, the other containing *Ggt* and *Ggg*. The *Gga* isolates had an average similarity of over 80% but were clearly differentiated from the *Ggt* isolates. Most *Ggt* isolates formed a discrete group with an average similarity of approximately 75%. This excluded isolate 500 which was more similar to *Ggg* isolate 23148. Nevertheless, both of these isolates showed a greater relationship to *Ggt* and *Gga* than to *Ggg* isolates H4 and ADI.

Table 4.6. Genetic similarities (%) among isolates of *G. graminis*, based on RFLP phenotypes detected with 22 probe/enzyme combinations.

C3								
EBI	85.7							
23580	71.4	75.1						
17916	85.2	78.3	73.5					
KM69	93.7	84.7	72.5	86.2				
51463	84.1	78.3	74.6	90.5	86.2			
800	94.2	89.4	75.1	86.8	94.2	86.8		
23125	91.5	86.8	73.5	84.1	91.5	84.1	97.4	
500	67.7	64.0	60.3	66.7	65.6	67.7	69.3	66.7
137T	58.2	61.9	65.6	58.2	59.3	59.3	63.0	63.5
192M	61.9	64.6	68.3	66.1	60.8	61.9	64.6	65.1
H4	58.2	59.8	59.3	55.0	58.2	55.0	59.8	59.3
30577	62.4	65.1	60.3	62.4	62.4	65.6	64.0	62.4
ADI	56.6	59.3	60.8	54.5	55.6	55.6	57.1	56.6
23148	70.9	67.2	64.6	75.1	68.8	79.4	70.4	68.8
23144	61.4	64.0	66.7	68.8	64.1	64.6	64.0	63.5
	C3	EBI	23580	17916	KM69	51463	800	23125

500								
137T	57.7							
192M	58.2	83.6						
H4	67.2	59.8	60.3					
30577	65.1	58.7	56.1	60.8				
ADI	62.4	58.2	59.8	89.9	62.4			
23148	76.7	57.7	60.3	56.6	66.1	57.1		
23144	58.7	79.9	93.1	57.7	57.7	59.3	61.9	
	500	137T	192M	H4	30577	ADI	23148	23144

Figure 4.6. Dendrogram showing phenetic relationships within *G. graminis* generated by group average cluster analysis of similarities between RFLP phenotypes.



4.4 Discussion

4.4.1 Repetitive DNA sequences within *G. graminis*

The presence of many high molecular weight, intensely staining restriction fragments in digests of *G. graminis* DNA indicated that there were many repeated sequences within the genome. The identity of these restriction fragments has recently been confirmed in a study of isolates of *G. graminis* from the United Kingdom (O'Dell *et al.*, 1992). A high proportion of repetitive DNA has also been reported to occur in other phytopathogens including *Erysiphe graminis* (O'Dell *et al.*, 1989) and *Colletotrichum gloeosporioides* (Braithwaite *et al.*, 1990). Some of these repeat

sequences have been identified as specifying the ribosomal RNAs, but these represent only a fraction of the total repetitive DNA (O'Dell *et al.*, 1992). Most of the repeated DNA is thought to represent either tandem arrays of sequence or multiple copies which are randomly dispersed throughout the genome. Many hypotheses have been raised concerning the amplification of these sequences including unequal crossing over, gene conversion and duplicative transposition (Dover, 1982; Flavell, 1986). To determine the extent of homology between families of repeat sequences within *G. graminis* it will be necessary to purify individual repeats and use them as probes in DNA hybridisation experiments.

In this study most isolates had their own unique phenotype of repeat sequences with the restriction enzymes Hae III and Hpa II. The only exceptions were *Ggt* isolates KM69 and C3 which were identical upon digestion with Hae III. This indicated that these restriction enzymes produce DNA fingerprints of *G. graminis* isolates and therefore should be useful in documenting variation between closely related isolates e.g. within interbreeding populations of *G. graminis*.

The amount of variation in repeat sequences detected between isolates was too great for a visual interpretation of inter-variety relationships, but cluster analysis revealed conservation of repeat-sequence phenotypes within varieties, implying that *Ggt* and *Gga* are more related to each other than either is to *Ggg*. It is notable that there was no clear distinction between *Ggt* and *Gga*, since two of the *Ggt* isolates (51463 and 17916) showed a greater similarity to *Gga* than to the remaining *Ggt* isolates. This is in contrast to the isozyme results which implied a closer relationship between *Gga* and some *Ggg* isolates. The overall average similarity between isolates in their repetitive DNA profiles was approximately 55% which was in agreement with the average similarity from isozyme loci (60%). With the exception of the two *Ggt* isolates each variety was observed to form a discrete group based upon similarities in size of their repetitive DNA restriction fragments.

Other reports of repetitive DNA sequences being used to fingerprint plant pathogens have involved hybridisation experiments using cloned genomic sequences.

These sequences were found to be taxon-specific and therefore, could be used in assessing the taxonomic composition of pathogen populations (O'Dell *et al.*, 1989; Levy *et al.*, 1991).

The analysis of repetitive sequences has indicated that the overall similarities within varieties of *G. graminis* are generally greater than those observed between varieties. Consequently, these repeat sequences may be useful in differentiating the three varieties of the pathogen. These results were consistent with those of O'Dell *et al.* (1992). The presence of repeat-sequence polymorphisms characteristic of *Gga* within some *Ggt* isolates (e.g. 51463, 17916) may suggest that these two groups have not diverged sufficiently for complete separation of the varieties. It is unlikely that repeat-sequence similarities are due to recombination between *Ggt* and *Gga*, due to the lack of hyphal anastomosis between the varieties (Asher, 1981). It is probable that similar polymorphisms have arisen independently within the varieties by random processes of genome rearrangement (Dover, 1982; Flavell, 1986).

4.4.2 Genetic diversity within *G. graminis* detected with genomic probes

These results demonstrate the successful application of RFLP analysis to document variation and evaluate relationships within the take-all fungus *G. graminis*. The probes were derived from a genomic library constructed from *Ggt* isolate C3. Fifty clones were randomly selected from the library and each was observed to have homology to isolates representing each variety of *G. graminis*. Some probes had reduced homology to *Ggg* as was indicated by the lower intensity of the hybridisation signal. Nevertheless, clearly resolved fragments were observed in each isolate. Assessments of the levels of diversity among the three varieties showed that only 14% of the probe/enzyme combinations failed to reveal variation. Extensive polymorphism was revealed between varieties, with 57% of the combinations yielding RFLP phenotypes with potential to differentiate the taxonomic groups.

The 22 combinations shown to detect the most diversity were used for further studies. Each of the combinations revealed polymorphisms within *Ggt* and *Ggg* but in

general only one or two isolates (e.g. 23580 and 500) accounted for most of the diversity within *Ggt*. *Gga* exhibited far less diversity than the other varieties.

Most of the diversity detected by the RFLP analyses was apportioned between varieties which implied that these markers should be useful in variety classification of *G. graminis*. In contrast, isozyme analyses (Chapter 3) apportioned more diversity within rather than between varieties of the pathogen. The differences between the genetic markers probably reflect the evolutionary stability of isozyme loci which are under strong selective pressure to maintain metabolic function in the organism. Furthermore, isozymes are restricted to analysing only a small portion of the genome and do not have the potential to detect sequences which may be specific to a variety, so the variation at these loci appears as a continuum between varieties. Random genomic clones, in contrast, provided a diversity of markers, likely to be scattered over many chromosomal locations and representing some sequences which may not be under strong selection. Variants of these sequences may be maintained through reproductive or geographical isolation and eventually become fixed within a variety, therefore providing specific markers. Studies of mitochondrial (Henson, 1989) and ribosomal (Bateman *et al.*, 1992; O'Dell *et al.*, 1992) DNA variation have indicated the value of RFLP markers in identification of *G. graminis*.

The 22 probe/enzyme combinations differed in their capacity to detect diversity within and between varieties of *G. graminis*. This indicated that some combinations could be used to detect genetic variation between closely related groups of individuals (e.g. within interbreeding populations), whereas others can be used to determine the taxonomic identity of isolates. Thirteen combinations exhibited a greater proportion of diversity between varieties and revealed RFLP phenotypes which were useful as variety specific markers (Table 4.4). Most of the combinations proved reliable in identifying the taxonomic status of isolates. However, a number of probes could not clearly differentiate all *Ggt* isolates from *Ggg*, indicating that these varieties may show a closer relationship to each other than either does to *Gga*. Each of the 13 combinations (except 30H) provided differentiation of *Gga* from *Ggt* and *Ggg*.

There were some anomalies between the original classification of isolates and their identification with the RFLP taxonomic markers. *Ggg* isolate 23148 could not be distinguished from *Ggt* with most of the combinations and was observed to be weakly pathogenic upon wheat (Table 4.5). This isolate may require reclassification as *Ggt*. Isolates 23147 and 500 also showed a combination of variety-specific RFLP phenotypes. The presence of the highly conserved *Gga* markers in these isolates coincided with their weak pathogenicity upon oats. Isolates 500 and 35793 (*Gga*) shared a 202H phenotype which was not found in any other isolate and further illustrates the relationship of 500 to the *Gga* isolates. It is notable that isolate 500 originated from the south-west of Western Australia, a region which is known to contain many oat-parasitic fungi previously classified as *Ggt* (Yeats *et al.*, 1986). This isolate also had many *Ggt* type RFLP markers and may represent a rare hybrid isolate between the two varieties.

The overall similarity between isolates was approximately 60% and was similar to that revealed in the analysis of isozyme diversity. These results indicated that *Gga* isolates exhibited the greatest within-variety similarities followed by *Ggt* and *Ggg*, respectively. Additionally, *Ggt* and some *Ggg* isolates appeared to be more closely related to each other than either group was to *Gga*, as indicated by the clustering of isolates from these two former varieties within the one group and the separation of *Gga* isolates. Nevertheless, a subset of *Ggg* isolates (H4 and ADI) were only distantly related to *Ggt* and *Gga* which indicated that these varieties may have been derived from *Ggg*, an hypothesis originally raised by Walker (1981) on the basis of pathogenic capacity and shared morphological characteristics between *Ggt* and *Gga*.

RFLP analyses clearly indicated the degree of differentiation between the three varieties of *G. graminis* and provided a means of distinguishing the varieties which is faster and less ambiguous than pathogenicity testing and morphological classification. The use of molecular taxonomic markers provides a means of determining the taxonomic composition of pathogen populations. This is of considerable importance since it has been reported that populations of *G. graminis* within Australia often contain

isolates which parasitise oats but are morphologically classified as *Ggt* (Yeats, 1986). The taxonomic identity of these isolates can now be determined as can their relative frequencies within the populations. It may be possible to increase the speed and sensitivity of variety identification by developing polymerase chain reaction (PCR) techniques. This is the subject of the next chapter.

Chapter 5 Intraspecific classification of isolates of *G. graminis* using the polymerase chain reaction

5.1 Introduction

The previous chapters have shown the value of genetic markers in estimating phenetic relationships between varieties of *G. graminis* and should aid in determining the evolutionary dynamics of host-pathogen interactions. The high levels of genetic diversity within the species, revealed by isozyme and RFLP analyses, have indicated the complexity of these relationships. Comparisons between varieties of *G. graminis*, using a range of genetic markers which cover diverse sites within the genome, may reveal patterns of similarity within this background of high genetic diversity. This approach should clarify intra-specific relationships, whilst developing a range of markers useful in genetic research.

The polymerase chain reaction (PCR) provides an alternative means of generating genetic markers and has been used to detect *G. graminis* in infected wheat plants from the field (Schesser *et al.*, 1991; Henson, 1992). This provided a method for pathogen identification which was faster than previous DNA hybridisation experiments (Henson, 1989). The amplified sequences were, however, conserved between varieties of *G. graminis* and could not be used to differentiate between them.

One method of generating polymorphisms with PCR involves targeting highly conserved sequences which are known to flank variable spacer regions e.g. the consensus sequences for intron splice junctions (ISJ) (Hawkins, 1988). Introns are present within many fungal genes (Scazzacchio, 1989) and are generally highly variable in sequence and length. Recently, PCR amplification of ISJ sequences has proved to be an ideal source of polymorphic markers for the generation of genetic maps in cereals (Weining and Langridge, 1991) and therefore may provide markers for a genetic analyses of *G. graminis*.

The purpose of this study is to assess if amplification of intron splice junction sequences will provide genetic markers to differentiate between varieties of *G. graminis*. The use of an additional set of independent genetic markers will aid in assessing phenetic relationships within the species and will broaden the range of loci useful in other genetic analyses.

5.2 Materials and Methods

Fungal isolates, culture conditions and isolation of DNA have been described in Chapters 3 and 4 respectively.

5.2.1 PCR and product analyses

PCR reactions were conducted in a 25 µl volume containing 0.1-0.2 µg fungal DNA, 25 pmol of each primer, 5.0 µmol each of dATP, dCTP, dGTP and dTTP, 2.5 µl 10x reaction buffer (Promega, Appendix A) and 0.5 units *Taq* polymerase (Promega). The solution was mixed, overlayed with approximately 50 µl paraffin oil and placed in a Programmable Thermal Controller (MJ Research). Control reactions were included with each run and consisted of (i) the complete reaction mix without template DNA and (ii) the reaction mix with template but lacking primers. *Ggt* isolate 23580 was included as a positive control in each of the amplification experiments.

The primers used were specific for the intron-exon splice junctions and their sequences were based on the consensus sequences reported for plants (Weining and Langridge, 1991). Primer R1 generates products from the exon regions whereas primer E4 produces products from the intron regions. The sequences of these primers are

R1 5' TCGTGGCTGACTTACCTG

E4 5' GGAATTCCACCTGCA

A two step program was used in PCR reactions with these primers, the first 6 cycles used a low annealing temperature (40°C) to permit amplification from targets which had poor homology with the plant intron-exon junctions. The annealing temperature was then raised to 58°C for the remaining 28 cycles, as this has been reported to

increase both reproduceability in banding patterns and resolution of the amplified products (Weining and Langridge, 1991). The full program consisted of an initial denaturing step of 94°C for 4 minutes followed by 6 cycles of 1 min. denaturation at 94°C, 2 min. annealing at 40°C and 2 min. extension at 72°C. This was followed by a further 28 cycles of 94°C for 1 min., 58°C for 2 min. and 72°C for 2 min. A final extension for 7 min. at 72°C was performed before cooling to ambient temperature. Amplified products were analysed by electrophoresis in 3% agarose gels in TBE buffer by loading 7.0 µl of the reaction mix, mixed with 1.0 µl 10x gel loading buffer (Appendix A).

5.2.2 Scoring of gels and data analysis

Variation between isolates was assessed by comparing the profiles of their amplification products. In this way a primer-specific PCR phenotype was assigned to each isolate, the data being scored in a binary format (as in previous chapters). PCR phenotypes for each of the isolates were checked by using four replicates of DNA samples isolated at separate occasions, and by repeated side-by-side comparisons. In the final analysis all isolates were run on a single series of gels for complete inter/intra-variety comparisons.

Banding profiles generated by amplification of DNA with primer R1 were used to provide estimates of genetic similarities between isolates using the similarity coefficient. The results of this analysis were used to generate a similarity matrix indicating the genetic identities between isolates based upon their total amplification products. A cluster analysis was then conducted on this matrix to illustrate the relationships between isolates. The methods used are as described in previous chapters.

5.3 Results

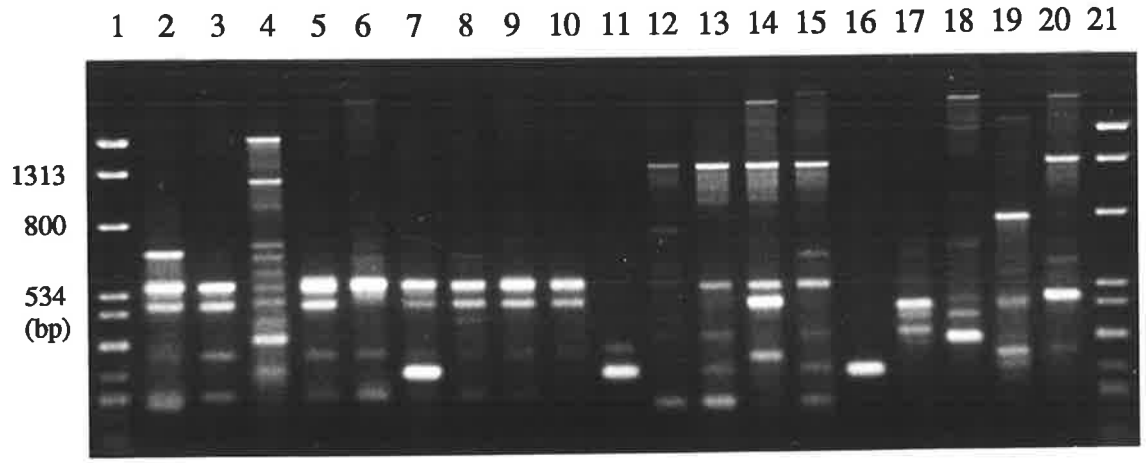
Primer E4 yielded amplification products in a size range of approximately 80 bp to 2000 bp and each isolate had its own specific phenotype, the products being highly

variable both in size and number between isolates. No single band or series of bands could be used to group isolates according to their taxonomic classification.

Primer R1 revealed variability both within and between varieties of *G. graminis*, with 18 phenotypes detected among the 20 isolates. Isolates within a particular variety, however, often differed by the presence or absence of a single amplification product or pair of products. A comparison of the phenotypes (Figure 5.1) showed that a band of 550 bp was amplified in all isolates except *Ggg* 51652, 30584 and ADI, although amplification was weak in some isolates. A band of 500 bp was amplified in all *Ggt* isolates except 23580 and 23152 (Figure 5.1). This product was also detected in *Gga* isolate 35793 and *Ggg* isolate 23147. A 1.30 kbp product was common to all *Gga* isolates together with *Ggt* isolates WUF143 and 500 and *Ggg* isolates 23147 and ADI (weak).

Banding profiles generated with R1 were identical in three *Ggt* isolates (Table 5.1) and isolate 800 showed 95% similarity with 5 other *Ggt* isolates. Isolate WUF143 was most dissimilar to the other isolates, showing only 37% similarity with four of them. *Ggt* isolates 500 and WUF143 were as similar to the *Gga* isolates as they were to the

Figure 5.1. PCR products generated with primer R1 from 19 *G. graminis* isolates. Lanes: 1 and 21, λ dVI *Hae* III molecular size standards; 2-11, *Ggt*- 800, EBI, WUF143, 17916, 23580, KSI, 51463, 23125, KM69, 23152; 12-15, *Gga*- 137T, 192M, 35793, 23144; 16-20, *Ggg*- 51652, 23148, 30584, ADI, 23147. Sizes of products are in base pairs (bp).



rest of the *Ggt* isolates. In several cases, similarities between isolates of different varieties were greater than similarities within varieties. For example, *Gga* 35793 and *Ggt* KM69 were 84% similar while *Gga* 35793 and 23144 were only 58% similar.

Table 5.1. Genetic similarities (%) among isolates of *G. graminis* based on the total amplification products resolved with primer R1.

800										
EBI	94.7									
WUF143	42.1	36.8								
17916	94.7	100.0	36.8							
23580	94.7	89.5	36.8	89.5						
KSI	78.9	84.2	52.6	84.2	73.7					
51463	94.7	89.5	47.4	89.5	89.5	73.7				
23125	94.7	100.0	36.8	100.0	89.5	84.2	89.5			
KM69	84.2	89.5	47.4	89.5	78.9	94.7	78.9	89.5		
23152	73.7	78.9	47.4	78.9	78.9	94.7	68.4	78.9	89.5	
500	68.4	73.7	42.1	73.7	73.7	68.4	63.2	73.7	73.7	73.7
137T	57.9	63.2	52.6	63.2	63.2	57.9	52.6	63.2	63.2	63.2
192M	63.2	68.4	47.4	68.4	68.4	63.2	57.9	68.4	57.9	68.4
35793	68.4	73.7	42.1	73.7	63.2	78.9	63.2	73.7	84.2	73.7
23144	68.4	63.2	52.6	63.2	73.7	57.9	63.2	63.2	52.6	63.2
51652	63.2	68.4	47.4	68.4	68.4	84.2	57.9	68.4	78.9	89.5
23148	68.4	73.7	52.6	73.7	63.2	78.9	73.7	73.7	84.2	73.7
30584	57.9	63.2	52.6	63.2	52.6	68.4	63.2	63.2	73.7	63.2
ADI	57.9	63.2	42.1	63.2	52.6	78.9	52.6	63.2	73.7	73.7
23147	57.9	63.2	42.1	63.2	52.6	68.4	52.6	63.2	73.7	63.2
	800	EBI	WUF143	17916	23580	KSI	51463	23125	KM69	23152

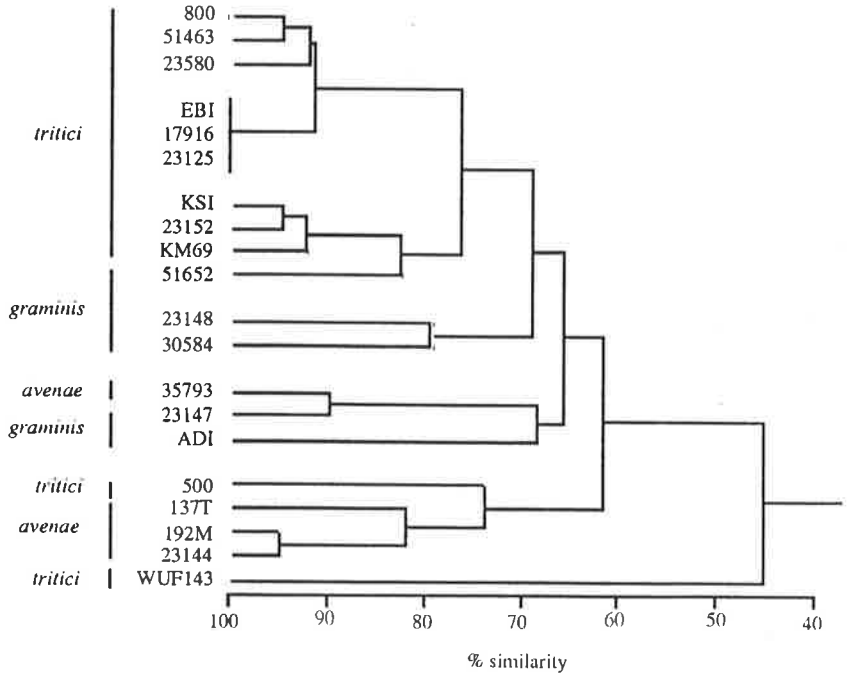
137T	78.9									
192M	73.7	84.2								
35793	68.4	68.4	63.2							
23144	68.4	78.9	94.7	57.9						
51652	73.7	63.2	68.4	63.2	63.2					
23148	68.4	57.9	52.6	68.4	47.4	73.7				
30584	57.9	47.4	42.1	68.4	36.8	73.7	78.9			
ADI	68.4	47.4	52.6	68.4	47.4	73.7	57.9	57.9		
23147	57.9	57.9	52.6	89.5	47.4	63.2	57.9	68.4	68.4	
	500	137T	192M	35793	23144	51652	23148	30584	ADI	23147

A cluster analysis, based upon the similarity matrix, was used to show the overall genetic similarities between *G. graminis* isolates. Isolate WUF143 showed a low genetic similarity to most isolates. The remaining isolates formed two separate groups with an overall average similarity of approximately 60% (Figure 5.2). One group contained three of the four *Gga* isolates and, slightly removed from them, *Ggt* 500. The average similarity within this group was approximately 72%. The second group consisted of three main clusters, one containing the majority of *Ggt* isolates and a single *Ggg* isolate. The average similarity within this cluster was approximately 75%. The other two clusters mainly consisted of *Ggg* isolates, but one of these contained *Gga* isolate 35793.

5.4 Discussion

The use of intron splice junction primers in PCR analyses revealed high levels of diversity between isolates of *G. graminis*. As predicted, E4, which primed amplification

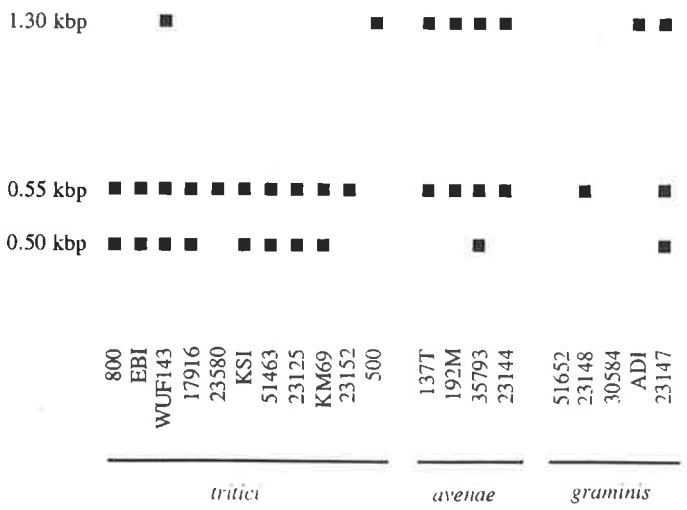
Figure 5.2. Dendrogram of genetic similarities within *G. graminis* derived from the total amplification products resolved with primer R1.



from the intron regions, produced a greater diversity of products between isolates than R1, which primarily amplified exon sequences. Since the introns are not expected to be under strong selection, they tend to accumulate mutations and show greater sequence variation relative to the exons.

High levels of diversity were revealed with primer R1 (18 different phenotypes detected between the 20 isolates) indicating its potential for examining diversity between closely related isolates of *G. graminis*. Furthermore, R1 proved useful in taxonomic identification of isolates (Figure 5.3). A 1.30 kbp product was observed in all *Gga* isolates and also in *Ggt* isolates WUF143 and 500, both of which originated from the south west of Western Australia and were classified by having spore lengths characteristic of *Ggt*. Populations of the pathogen within this geographical region have been reported to contain high frequencies of isolates which can parasitise oats but cannot be distinguished from *Ggt* on the basis of morphological characteristics (Yeats *et al.*, 1986). In inter-isolate comparisons based on the total R1 amplified products, both 500 and WUF143 showed closer genetic similarities to *Gga* isolates compared with *Ggt* isolates. Pathogenicity tests indicated that isolate 500 was capable of infecting oats although WUF143 could not. Isolate 500 also resolved some of the *Gga*-type RFLP

Figure 5.3. Representation of PCR products generated with primer R1 for use as molecular taxonomic markers within *G. graminis*.



markers (Chapter 4), but WUF143 was not included in that analysis. The evidence from these independent markers implies that isolate 500 may require reclassification as *Gga*. Analyses of WUF143 with RFLP markers may aid in the clarification of this isolate's taxonomic identity.

Ggg isolate 23147 showed the *Gga*-type 1.30 kbp product and was capable of infecting oats, albeit to a lesser extent than the *Gga* isolates. Isolate 23147 had a high genetic similarity with *Gga* isolate 35793 (89.5%) and was also shown to have many of the *Gga*-type variety-specific RFLP markers (Chapter 4). A more thorough analysis with more RFLP markers may indicate whether isolate 23147 requires reclassification as *Gga*.

The 500 and 550 bp R1 amplification products appeared to be *Ggt*-specific in the absence of the 1.30 kbp *Gga* type band. *Ggg* isolate 23148 not only resolved the 500 and 550 bp products but also had its greatest genetic similarity with *Ggt* isolate KM69 (85%). Furthermore, 23148 showed many of the *Ggt* varietal specific RFLP markers as well as being weakly pathogenic upon wheat. This implied that 23148 may require reclassification as *Ggt*.

Ggg isolates 51652 and 30584 did not show any of the *Ggt*-specific or *Gga*-specific PCR products. Isolate ADI showed weak amplification of the 1.30 kbp *Gga* type marker and therefore presented an anomaly to the classification of isolates based upon their amplification products. ADI was the only *Ggg* isolate which produced a variety-specific PCR product which did not correlate with the presence of RFLP variety-specific markers or pathogenicity.

Gga isolate 35793 and *Ggg* isolates 23147 and ADI formed a group which showed a greater similarity to *Ggt* and *Ggg* isolates compared with the remaining *Gga* isolates. In this analysis, 35793 was the only *Gga* isolate to have originated from oats, the others having originated from turf grass (*Agrostis* spp.). RFLP analyses confirmed the identity of 35793 as *Gga* but also indicated that it showed a closer relationship to *Ggt* isolates than to the turf isolates of *Gga*. Previous studies of inter-varietal relationships within *G. graminis* using electrophoresis of protein profiles (Abbot and Holland, 1975; Maas *et*

al., 1991), mitochondrial DNA probes (Bateman *et al.*, 1992) and ribosomal DNA probes (Ward and Gray, 1992; O'Dell *et al.*, 1992) have indicated that *Ggt* and *Gga* show a closer relationship to each other than either does to *Ggg*. In contrast, RFLP and PCR analyses of isolates used in this study have indicated a clear separation of *Gga* from *Ggt* and *Ggg* and a differentiation of *Ggg* into two subgroups, one showing a closer similarity to *Ggt* isolates. The separation of isolate 35793 from the other *Gga* isolates and its greater similarity to *Ggt* agrees with RFLP analyses and may indicate that there is a differentiation between *Gga* isolates within Australia. Two pathotypes may have evolved which are differentially specialised in parasitising cereals and turf grass species. A study of the *Gga* isolates present in cereal crops, using RFLP markers which distinguish oat and turf grass isolates, may determine if there is a separation of this variety into two pathotypes.

The overall genetic similarity between R1 phenotypes of *Ggt* isolates was approximately 75% (excluding WUF143). This is the same as that observed at isozyme and RFLP loci. Isolate EBI, originating from Eire, was identical to the two Australian isolates 17916 and 23125, which shows a high degree of sequence conservation within *Ggt*. Consequently, this technique may be applicable to the identification of take-all isolates from outside Australia.

Ggg isolate 51652 showed high genetic similarity to the *Ggt* isolates. This did not correlate with similarities at RFLP loci. Given that the sequences amplified with R1 are from exon regions flanking the intron splice junction, there may be amplification of some sequences which are highly conserved throughout the species (e.g. 300 bp products, Figure 5.1) resulting in the high similarities between otherwise divergent isolates. Individual isolates, however, can be assigned to a variety by the presence or absence of a few amplification products and 51652 can be distinguished from *Ggt* by its lack of the 500 and 550 bp products.

The overall genetic similarity of isolates, excluding the strongly divergent *Ggt* WUF143, was 60% as was found for isozyme and RFLP analyses. This correspondence among independent sets of genetic data provides confidence in the accuracy of intra-

specific relationships determined with these markers. PCR with primer R1 is capable of differentiating between the three varieties of *G. graminis* and taxonomic identifications by this method generally agreed with those based upon pathogenicity and RFLP variety specific markers. Consequently, these techniques should aid in the classification of isolates to their taxonomic status.

It is possible that the variety-specific PCR products may have been amplified from sequences which were already represented by a variety-specific RFLP phenotype. To determine the independence of the markers the specific PCR products could be isolated and cross-hybridised with the clones to determine if there is any sequence homology between them. Alternatively, they could be used as probes in genomic hybridisations and the resulting RFLP phenotypes compared with those resolved by the genomic clones.

The levels of genetic diversity revealed by the two intron splice junction primers indicate that they will prove useful at examining variation between closely related isolates, e.g. within interbreeding populations of *G. graminis*. Primer E4 revealed a unique PCR phenotype for each isolate whereas R1 phenotypes were identical in only three *Ggt* isolates. In this respect they were similar to the repetitive DNA phenotypes which were useful in fingerprinting individual isolates (Chapter 4). Use of these primers may indicate if pathogen populations are composed of clonal lineages of isolates. This may be common due to the restrictions to outcrossing within *G. graminis* (Chambers and Flentje, 1967; Rawlinson *et al.*, 1973). Consequently each pathogen population may have evolved specific genotypes detectable by PCR, and if so, this technique could prove useful in determining rates of migration and gene flow between populations, factors which are crucial in understanding the spread of disease and the evolution of diverse pathogen genotypes. Thus, PCR using intron splice junction primers provides not only a means of determining the taxonomic composition of *G. graminis* populations but also should prove useful in population genetic studies of the pathogen.

Chapter 6 Isozyme variation within and between populations of the take-all fungus *G. graminis*

6.1 Introduction

Genetic diversity within *G. graminis* has been poorly quantified, with previous studies being restricted to describing variation within small collections of isolates of diverse origins (Maas *et al.*, 1990; Ward and Gray, 1992; O'Dell *et al.*, 1992). As yet no attempts have been made to assess levels of genetic diversity within pathogen populations or to compare levels of diversity between populations.

Previous experiments to estimate levels of isozyme diversity within *G. graminis* have indicated that the technique should be useful for the genetic analysis of pathogen populations (Chapter 3). Considerable diversity was detected both within and between varieties of the fungus. Some loci resolved variety-specific alleles, which may be useful in determining the taxonomic composition of pathogen populations. This provides a means of estimating the frequencies of the different varieties in regions of cereal production and may aid in identifying the factors responsible for their distribution and relative abundance. Examination of whether the different varieties coexist within populations may prove useful in determining the frequency of sexual hybridisation between varieties and consequently, may aid in detecting the evolution of pathogenic variability.

Given the extent of genetic differentiation between the taxonomic varieties, analysis of genetic similarities between populations should determine whether fungi isolated from different cereal hosts are genetically distinct and if so, may provide evidence for the evolution of host-specific pathogen genotypes within *G. graminis*.

G. graminis has a discontinuous distribution within southern Australia due to separation of the Western Australian cereal belt from regions in eastern Australia. Deserts provide probable effective barriers to migration between these geographical

regions and consequently, pathogen populations from the separate areas may exhibit considerable genetic differentiation due to a lack of gene flow between them. Phenetic analysis of genetic diversity between populations may determine if there has been evolution of geographically restricted pathotypes of *G. graminis* within southern Australia. Similar analyses have indicated correlations between genetic and geographical identities in a number of plant pathogenic fungi including *Peridermium harknessii* (Tuskan *et al.*, 1990) and *Phytophthora infestans* (Goodwin *et al.*, 1992).

The primary objective of this study was to evaluate genetic variability at isozyme loci within and between populations of *G. graminis* present in regions of intensive cereal production. The distribution of pathogen variation between ecogeographically diverse locations may aid in identifying factors important in the generation and maintenance of genetic diversity within the pathogen. This research should provide much needed information on the genetic structuring of pathogen populations and the evolutionary consequences of genetic interactions between pathogen and host.

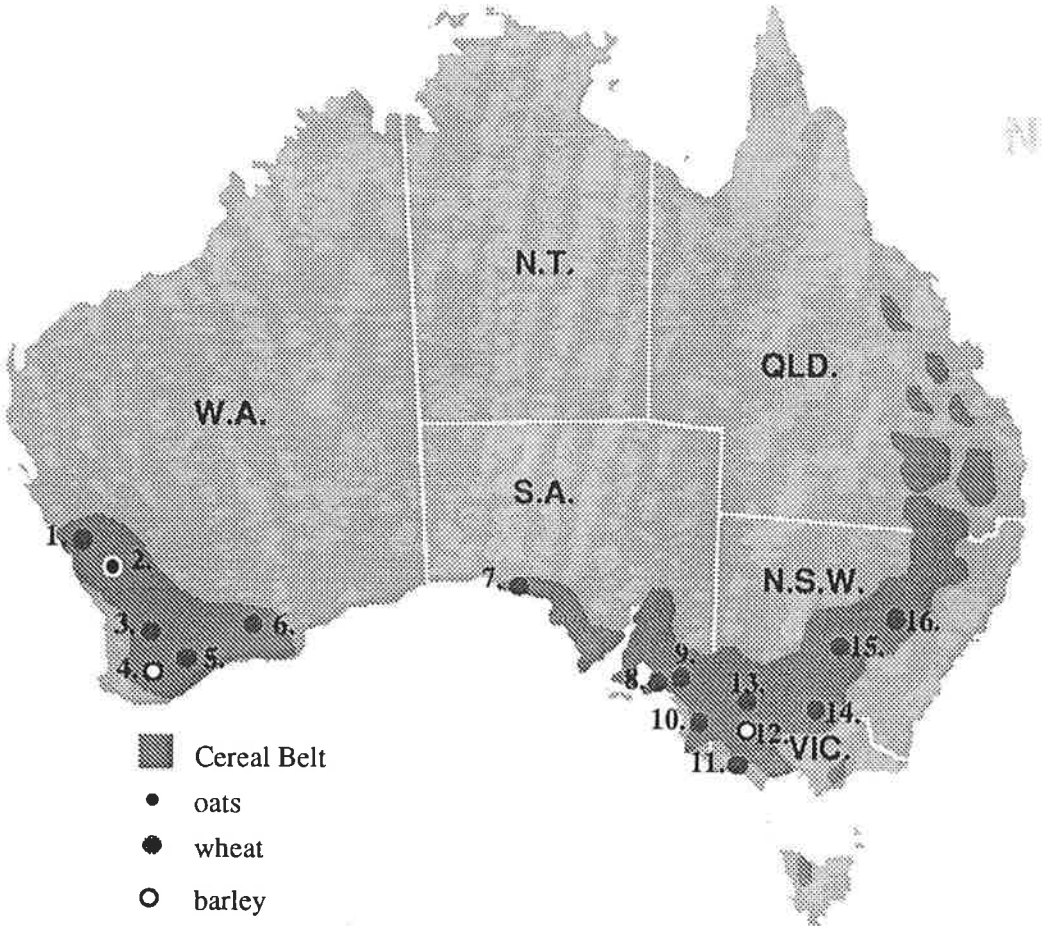
6.2 Materials and methods

6.2.1 Population sampling and isolation of *G. graminis*

Populations were sampled from a diversity of cereal hosts over a broad ecogeographic range throughout the cereal growing regions of southern Australia (Figure 6.1, Table 6.1). Approximately 100 plants were sampled from each location by running random transects through fields and sampling at 10-metre intervals.

All *G. graminis* isolates were obtained from infected roots. Root systems were washed and small segments (2-3 cm) showing symptoms of *G. graminis* infection were removed and placed on moist filter paper. Root segments were surface sterilised (Chapter 3) and two segments per plant were plated on to the *G. graminis* selective medium SM- GGT3 (Juhnke and Mathre, 1984) (Appendix A). Plates were incubated in darkness at 22°C and were checked periodically for the emergence of hyphae from the roots. Isolates identified as *G. graminis* were transferred to PDA plates to obtain pure

Figure 6.1. Geographical distribution of *G. graminis* collection sites throughout the cereal belt of southern Australia.



cultures. Only one isolate per plant was placed into pure culture. Isolates were recovered by these methods until a collection of at least 30 individuals was obtained for each of the 16 populations. All isolates were stored on these plates at 4°C. Subculturing to fresh plates was kept to a minimum to avoid instability of cultural and virulence characteristics often observed with repeated transfers (Cunningham, 1981).

6.2.2 Growth of isolates, sample preparation and isozyme electrophoresis

The 30 isolates from each of the 16 populations were transferred to liquid culture and grown by the methods described in Chapter 3. Sample preparation, isozyme

Table 6.1. Cereal hosts, hosts of previous year and locations of *G. graminis* populations examined in this study.

Number	Abbrev.	Crop	Previous Crop	Location
1	CV	wheat	pasture grasses	Chapman Valley, Geraldton, W.A.
2	NN	oats	wheat	New Norcia, W.A.
3	WN	wheat	wheat	Wandering, W.A.
4	BB	barley	wheat	Boyup Brook, W.A.
5	DY	wheat	wheat	Dumbleyoung, W.A.
6	JM	wheat	wheat	Jerramungup, W.A.
7	MD	wheat	pasture grasses	Mudamuckla, S.A.
8	WI	wheat	pasture grasses	Waite Institute, Adelaide, S.A.
9	MN	wheat	barley	Monarto, S.A.
10	BT	wheat	pasture grasses	Bordertown, S.A.
11	HM	wheat	pasture grasses	Hamilton, Vic.
12	HS	barley	pasture grasses	Horsham, Vic.
13	WP	wheat	pasture grasses	Walpeup, Vic.
14	GM	wheat	oats	Ganmain, N.S.W.
15	TM	wheat	wheat	Temora, N.S.W.
16	TW	wheat	wheat	Terrawinda, Coonabarabran, N.S.W.

electrophoresis and scoring of gels were as described previously (Chapter 3). *Ggg* isolate 51652 was included on each gel and was used as a reference standard in calculating the relative mobility (R_f) values of the alleles at each enzyme locus. In the final analysis one isolate from each population was included on a series of gels: (a) to confirm their allelic identities at each of the enzyme loci and (b) to provide another means for comparing alleles across populations.

6.2.3 Data analysis

Allele frequencies at each enzyme locus were calculated for each of the 16 populations, and within the collection of isolates as a whole. Statistical analyses of the electrophoretic data were performed on the allele frequencies. Estimates of genetic diversity were derived from Nei's gene identity (D) (Nei, 1973; Weir, 1990) and

Shannon's diversity index (H) (Peet, 1974; McCain *et al.*, 1992). Genetic identity provides an estimate of the number of identical alleles between two populations. Identities between pairwise combinations of populations were estimated from the data of polymorphic enzyme loci (Nei, 1972). Data were analysed using the computer program Gendist (J. Felsenstein, Univ. of Washington, 1989).

Genetic identity (I) was calculated as follows

$$I = \frac{\sum_l \sum_u p_{lu1}\, p_{lu2}}{\sqrt{\sum_l \sum_u p^2_{lu1}\, p^2_{lu2}}}$$

where l is summed over loci, u over alleles at the l-th locus, and where p_{lu1} is the frequency of the u-th allele at the l-th locus in population 1.

Identity values were used to construct an identity matrix between the populations. Hierarchical cluster analysis (group average method) was conducted on the matrix and the results used to group populations according to their genetic identities. Similarities between populations are presented in the form of a dendrogram.

6.3 Results

The fifteen enzyme loci used in this study showed clear banding patterns for all of the 480 *G. graminis* isolates. Only one clear band was resolved per isolate at each of these loci, indicating the absence of heterozygous individuals. Of the 15 loci examined only HK-1 and MDH-1 were found to be monomorphic. Between two and five alleles were resolved at each polymorphic locus (Table 6.2).

Allele frequencies of the thirteen polymorphic loci within each *G. graminis* population are shown in Table 6.3. Two alleles were unique to two of the populations i.e. ACON allele 0.94 was only present in the HM population and GOT-2 allele 1.11 only in the WI population. All other alleles were observed in at least two populations.

The total number of polymorphic loci and the average number of alleles per locus were both found to vary between populations. The percentage of polymorphic loci ranged from 7% in NN to 73% within the HM, TW and WI populations (Table 6.4).

Table 6.2. List of enzymes with number of putative loci and number of alleles resolved per locus.

Enzyme	No. Loci	No. Alleles
ACON	1	3
FUM	1	3
GOT	2	3 (GOT-1)
		3 (GOT-2)
G6PD	1	3
GPI	1	3
GSR	1	4
HK	2	1 (HK-1)
		2 (HK-2)
IDH	1	5
MDH	2	1 (MDH-1)
		2 (MDH-2)
ME	1	4
6PGD	1	3
PGM	1	4

The average number of alleles per locus ranged from 1.07 within NN to 2.00 within HM.

6.3.1 Partitioning of genetic diversity within and between populations of *G. graminis*

The Western Australian populations NN, JM and CV exhibited low levels of diversity within populations, with 12, 11 and 10 of their respective loci observed to be monomorphic (Tables 6.5 and 6.6). The HM, WI, MN and TW populations exhibited high levels of genetic diversity within populations, with only two of the enzyme loci in each population found to be monomorphic.

The diversity indices of Nei and Shannon were used to partition the diversity into within- and between-population components. Shannon's index showed that on average 60% of the diversity occurred between *G. graminis* populations. A slightly lower figure

Table 6.3. Allele frequencies at 15 isozyme loci in populations of *G. graminis*.

Locus	Allele	CV	WN	DY	JM	MD	WI	MN	BT
ACON	1.04	1.00	0.20	0.03		0.53	0.57	0.47	0.10
	1.00		0.80	0.97	1.00	0.47	0.43	0.53	0.90
	0.94								
FUM	1.04		0.07		0.07				
	1.00	1.00	0.93	0.77	0.93	1.00		0.63	
	0.86			0.23			1.00	0.37	1.00
GOT-1	1.00								
	0.94		0.83	1.00	1.00		0.17	0.20	0.30
	0.87	1.00	0.17			1.00	0.83	0.80	0.70
GOT-2	1.11						0.67		
	1.07	0.83	0.83	0.37	1.00	1.00	0.23	1.00	1.00
	1.00	0.17	0.17	0.63			0.10		
G6PD	1.04		0.07	0.03					
	1.00	1.00	0.93	0.97	1.00	1.00	0.93	0.80	1.00
	0.94						0.07	0.20	
GPI	1.00								
	0.94	1.00	1.00	1.00	1.00	0.23	0.37	0.80	0.17
	0.88					0.77	0.63	0.20	0.83
GSR	1.00							0.20	
	0.91	0.93	0.17					0.67	0.93
	0.86			0.80		0.07	0.83	0.13	0.07
	0.82	0.07	0.83	0.20	1.00	0.93	0.17		
HK-1	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
HK-2	1.00	1.00	1.00	1.00	1.00	0.90	0.77	0.80	0.63
	0.96					0.10	0.23	0.20	0.37
IDH	1.07								
	1.00					0.40			0.20
	0.96			0.20			0.20		
	0.91	1.00	1.00	0.80	0.90	0.60	0.80	0.87	0.80
	0.86				0.10			0.13	
MDH-1	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
MDH-2	1.00		1.00				1.00		0.60
	0.93	1.00		1.00	1.00	1.00		1.00	0.40
ME	1.30		0.87		1.00				
	1.22	0.07		0.93					
	1.00	0.93	0.13	0.07			0.57	0.10	0.30
	0.96					1.00	0.43	0.90	0.70
6PGD	1.04		1.00	1.00	1.00		0.23		
	1.00	1.00				1.00	0.77	0.80	1.00
	0.96							0.20	
PGM	1.13		0.80	0.70	1.00		0.07		
	1.07	1.00	0.20	0.30		1.00	0.93	0.83	0.07
	1.00							0.17	
	0.91								0.93

Table 6.3. continued.

Locus	Allele	HM	WP	GM	TM	TW	BB	HS	NN	Sum
ACON	1.04	0.17	0.13	0.37		0.17	0.17			0.24
	1.00	0.73	0.87	0.63	1.00	0.83	0.83	1.00	1.00	0.75
	0.94	0.10								0.01
FUM	1.04				0.10		0.07			0.02
	1.00	0.63		1.00	0.90	0.83	0.93	1.00	1.00	0.72
	0.86	0.37	1.00			0.17				0.26
GOT-1	1.00					0.13	0.10			0.01
	0.94	1.00	1.00	1.00	1.00	0.20	0.90		1.00	0.60
	0.87					0.67		1.00		0.39
GOT-2	1.11									0.04
	1.07	0.80	1.00	1.00	0.83	1.00	0.90	1.00	0.83	0.85
	1.00	0.20			0.17		0.10		0.17	0.11
G6PD	1.04					0.17				0.02
	1.00	0.90	1.00	0.93	1.00	0.83	1.00	1.00	1.00	0.95
	0.94	0.10		0.07						0.03
GPI	1.00	0.17	0.23	0.17				0.10		0.04
	0.94	0.63	0.70	0.83	1.00	0.73	1.00	0.83	1.00	0.77
	0.88	0.20	0.07			0.27		0.07		0.19
GSR	1.00	0.20		0.07						0.03
	0.91	0.43	1.00				0.90	1.00		0.38
	0.86				0.23	0.87	0.10			0.19
	0.82	0.37		0.93	0.77	0.13			1.00	0.40
HK-1	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
HK-2	1.00	1.00	1.00	1.00	1.00	0.93	1.00	1.00	1.00	0.94
	0.96					0.07				0.06
IDH	1.07					0.20		0.10		0.02
	1.00		0.27			0.13		0.67		0.10
	0.96						0.23			0.04
	0.91	0.90	0.73	0.90	1.00	0.67	0.77	0.23	1.00	0.81
	0.86	0.10		0.10						0.03
MDH-1	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
MDH-2	1.00	0.23	0.90							0.23
	0.93	0.77	0.10	1.00	1.00	1.00	1.00	1.00	1.00	0.77
ME	1.30			1.00	1.00				1.00	0.30
	1.22	0.10					0.07	0.20		0.09
	1.00	0.90				0.23	0.93	0.80		0.31
	0.96		1.00			0.77				0.30
6PGD	1.04	0.43		0.90		0.13	0.07		1.00	0.36
	1.00	0.47	1.00		1.00	0.87	0.93	1.00		0.61
	0.96	0.10		0.10						0.03
PGM	1.13	0.90			1.00		0.10		1.00	0.35
	1.07		1.00	1.00		0.53	0.90	1.00		0.55
	1.00	0.10								0.01
	0.91					0.47				0.09

Table 6.4. Descriptive isozyme data for the 16 populations of *G. graminis* examined in this study.

Population	Polymorphic loci (%)	Average No. alleles/locus
CV	20	1.20
WN	53	1.53
DY	53	1.47
JM	13	1.13
MD	33	1.33
WI	73	1.80
MN	67	1.80
BT	60	1.60
HM	73	2.00
WP	27	1.30
GM	40	1.40
TM	20	1.20
TW	73	1.87
BB	60	1.60
HS	20	1.33
NN	7	1.07
Mean	43	1.48

(56%) was shown by Nei's diversity index. Within the average population, the enzyme IDH detected the greatest and MDH-2 the least diversity (Tables 6.7 and 6.8), while across the species as a whole the enzyme revealing the greatest diversity was ME and the least diverse was G6PD.

The distribution of diversity between and within populations was found to vary between enzyme loci. Shannon's diversity index revealed that seven of the thirteen polymorphic loci apportioned more variability between populations than within populations (Table 6.8), namely MDH-2, ME, PGM, 6PGD, GOT-1, GSR and FUM. Nei's diversity index revealed a similar result with the exception that GPI was also shown to apportion more variation between than within populations (Table 6.7).

Table 6.5. Estimates of Nei's gene diversity (D) within populations of *G. graminis*.

Locus	CV	DY	WN	JM	MD	WI	MN	BT	HM	WP	GM	TM	TW	BB	HS	NN
ACON	0.000	0.058	0.320	0.000	0.498	0.490	0.498	0.180	0.428	0.226	0.466	0.000	0.282	0.282	0.000	0.000
FUM	0.000	0.354	0.130	0.130	0.000	0.000	0.466	0.000	0.466	0.000	0.000	0.180	0.282	0.130	0.000	0.000
GOT-1	0.000	0.000	0.282	0.000	0.000	0.282	0.320	0.420	0.000	0.000	0.000	0.000	0.494	0.180	0.000	0.000
GOT-2	0.282	0.466	0.282	0.000	0.000	0.482	0.000	0.000	0.320	0.000	0.000	0.282	0.000	0.180	0.000	0.282
G6PD	0.000	0.058	0.130	0.000	0.000	0.130	0.320	0.000	0.180	0.000	0.130	0.000	0.282	0.000	0.000	0.000
GSR	0.130	0.320	0.282	0.000	0.130	0.282	0.494	0.130	0.638	0.000	0.130	0.354	0.226	0.180	0.000	0.000
HK-2	0.000	0.000	0.000	0.000	0.180	0.354	0.320	0.414	0.000	0.000	0.000	0.000	0.130	0.000	0.000	0.000
IDH	0.000	0.320	0.000	0.180	0.480	0.320	0.226	0.320	0.180	0.394	0.180	0.000	0.494	0.354	0.488	0.000
GPI	0.000	0.000	0.000	0.000	0.354	0.466	0.320	0.282	0.534	0.422	0.282	0.000	0.394	0.000	0.296	0.000
MDH-2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.480	0.354	0.180	0.000	0.000	0.000	0.000	0.000	0.000
ME	0.130	0.130	0.282	0.000	0.000	0.490	0.180	0.420	0.180	0.000	0.000	0.000	0.354	0.130	0.320	0.000
6PGD	0.000	0.000	0.000	0.000	0.000	0.354	0.320	0.000	0.584	0.000	0.180	0.000	0.226	0.130	0.000	0.000
PGM	0.000	0.420	0.320	0.000	0.000	0.130	0.282	0.130	0.180	0.000	0.000	0.000	0.498	0.180	0.000	0.000
Mean	0.042	0.164	0.156	0.024	0.126	0.291	0.288	0.214	0.311	0.094	0.105	0.063	0.282	0.134	0.085	0.022

Table 6.6. Estimates of genetic diversity (H_0) within populations of *G. graminis*.

Locus	CV	DY	WN	JM	MD	WI	MN	BT	HM	WP	GM	TM	TW	BB	HS	NN
ACON	0.000	0.194	0.722	0.000	0.997	0.986	0.997	0.469	1.098	0.557	0.951	0.000	0.658	0.658	0.000	0.000
FUM	0.000	0.778	0.366	0.366	0.000	0.000	0.951	0.000	0.951	0.000	0.000	0.469	0.658	0.366	0.000	0.000
GOT-1	0.000	0.000	0.658	0.000	0.000	0.658	0.722	0.881	0.000	0.000	0.000	0.000	1.234	0.469	0.000	0.000
GOT-2	0.658	0.951	0.658	0.000	0.000	1.207	0.000	0.000	0.722	0.000	0.000	0.658	0.000	0.469	0.000	0.658
G6PD	0.000	0.194	0.366	0.000	0.000	0.366	0.722	0.000	0.469	0.000	0.366	0.000	0.658	0.000	0.000	0.000
GSR	0.000	0.722	0.658	0.000	0.366	0.658	1.234	0.366	1.519	0.000	0.366	0.778	0.557	0.469	0.000	0.000
HK-2	0.366	0.000	0.658	0.000	0.469	0.778	0.722	0.951	0.000	0.000	0.000	0.000	0.366	0.000	0.000	0.000
IDH	0.000	0.722	0.000	0.469	0.971	0.722	0.557	0.722	0.469	0.842	0.469	0.000	1.234	0.778	1.207	0.000
GPI	0.000	0.000	0.000	0.000	0.778	0.951	0.722	0.658	1.319	1.116	0.658	0.000	0.842	0.000	0.824	0.000
MDH-2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.971	0.778	0.469	0.000	0.000	0.000	0.000	0.000	0.000
ME	0.366	0.366	0.557	0.000	0.000	0.986	0.469	0.881	0.469	0.000	0.000	0.000	0.778	0.366	0.722	0.000
6PGD	0.000	0.000	0.000	0.000	0.000	0.778	0.722	0.000	1.368	0.000	0.469	0.000	0.557	0.366	0.000	0.000
PGM	0.000	0.881	0.722	0.000	0.000	0.366	0.658	0.366	0.469	0.000	0.000	0.000	0.997	0.469	0.000	0.000
Mean	0.107	0.370	0.413	0.064	0.276	0.650	0.652	0.482	0.741	0.230	0.252	0.147	0.657	0.339	0.212	0.051

Table 6.7. Partitioning of Nei's gene diversity (D) within and between populations of *G. graminis* for 13 polymorphic enzyme loci.

Locus	Average within populations	Total within species	Proportion within populations	Proportion between populations
	Dpop	Dsp	$\frac{Dpop}{Dsp}$	$\frac{Dsp-Dpop}{Dsp}$
ACON	0.233	0.378	0.617	0.383
FUM	0.134	0.410	0.326	0.674
GOT-1	0.124	0.491	0.252	0.748
GOT-2	0.161	0.261	0.618	0.382
G6PD	0.077	0.085	0.910	0.090
GSR	0.206	0.659	0.313	0.687
HK-2	0.087	0.114	0.770	0.230
IDH	0.246	0.330	0.746	0.254
GPI	0.211	0.629	0.336	0.664
MDH-2	0.063	0.358	0.177	0.823
ME	0.164	0.714	0.229	0.771
6PGD	0.112	0.492	0.228	0.772
PGM	0.114	0.571	0.199	0.801
Mean	0.148	0.422	0.440	0.560

6.3.2 Application of isozymes as possible taxonomic markers within *G. graminis*

Experiments to assess isozyme diversity within *G. graminis* indicated that allelic variants of GOT-1, GOT-2, GSR and 6PGD may prove useful in taxonomic identification of isolates (Chapter 3). The number of alleles resolved with these loci differed between the earlier experiments and the population analyses, thereby making taxonomic identification difficult. For example, the GOT-2 allele 0.96, found previously to be exclusive to *Gga* isolates, was not detected in the population analysis whereas GSR 0.82 and 6PGD 0.96 were new alleles. Furthermore, the frequencies of the markers were not consistent within each of the populations, indicating that they were not conserved within a particular taxonomic group of isolates. For example,

Table 6.8. Partitioning of genetic diversity (H_0) within and between populations of *G. graminis* for 13 polymorphic enzyme loci.

Locus	Average within populations	Total within species	Proportion within populations	Proportion between populations
	Hpop	Hsp	$\frac{Hpop}{Hsp}$	$\frac{Hsp-Hpop}{Hsp}$
ACON	0.518	0.854	0.607	0.393
FUM	0.307	0.950	0.323	0.677
GOT-1	0.289	1.061	0.272	0.728
GOT-2	0.374	0.732	0.512	0.488
G6PD	0.196	0.301	0.652	0.348
GSR	0.504	1.667	0.302	0.698
HK-2	0.205	0.329	0.624	0.376
IDH	0.573	1.019	0.562	0.438
GPI	0.492	0.938	0.525	0.475
MDH-2	0.139	0.784	0.177	0.823
ME	0.373	1.871	0.199	0.801
6PGD	0.266	1.095	0.243	0.757
PGM	0.308	1.411	0.218	0.782
Mean	0.349	1.001	0.401	0.599

within the WI population, GOT-1 documented 17% of the population as *Gga* and 83% as *Ggt* (alleles 0.94 and 0.87, respectively) whereas GSR indicated that 83% was *Gga* and the remaining 17% could not be identified (alleles 0.86 and 0.82, respectively) (Table 6.3).

Some populations, however, showed consistency of the allelic markers. For example, the NN (oat) population was monomorphic at GSR 0.82, 6PGD 1.04 and GOT-1 0.94, the three alleles most likely to be useful in the identification of *Gga* since this is the only variety which is capable of parasitising oats. Other populations in which these *Gga*-type alleles were present in high frequency were WN, JM, DY and GM.

Consistency between markers was also observed within the HB and WP populations, which were monomorphic for all four of the *Ggt* markers (Table 6.3).

6.3.3 Genetic identities of *G. graminis* populations

The genetic identities of populations ranged from 0.378 between the JM and WI populations to 0.996 between the Western Australian populations NN and JM (Table 6.9). The average, overall genetic identity between all 16 populations was approximately 0.60.

Cluster analysis of the identity values separated the populations into two primary groups, one containing seven populations and the other nine (Figure 6.2). The group of seven had an average genetic identity of approximately 0.80 and included the two populations with the highest identity together with two other West Australian

Figure 6.2. Dendrogram of *G. graminis* populations generated by group average cluster analysis of the genetic identities among populations.

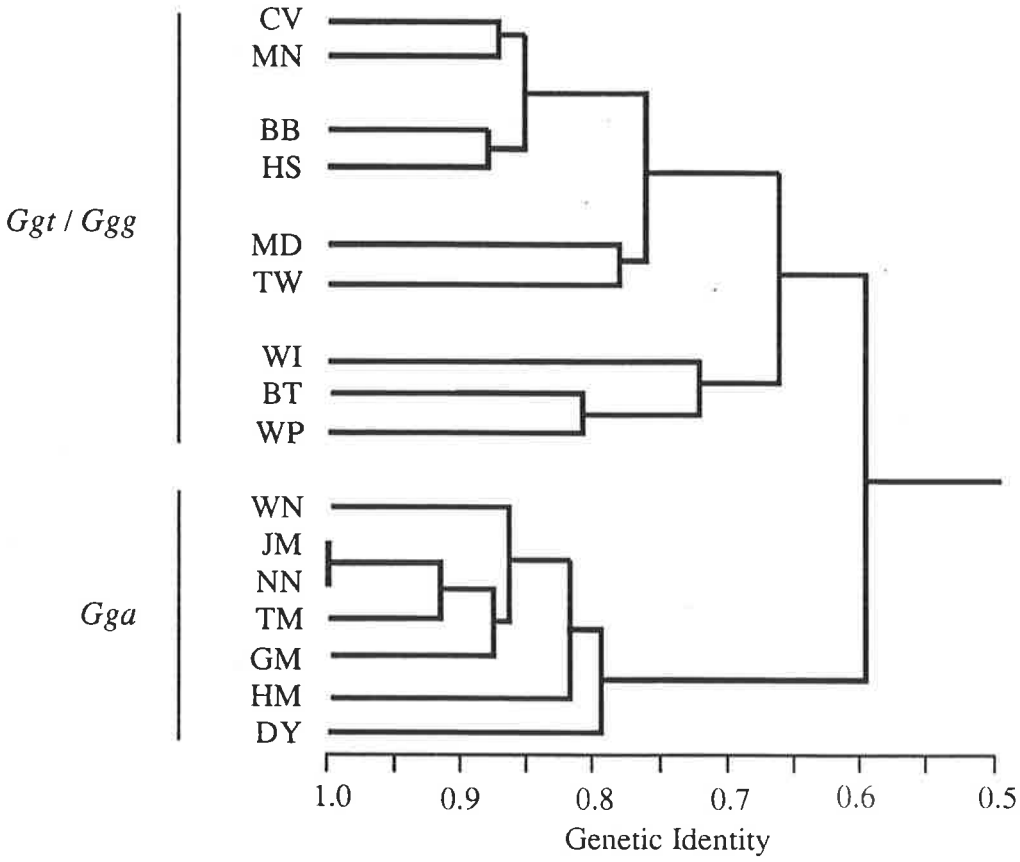


Table 6.9. Nei's genetic identity (I) among isolates of *G. graminis* from 16 populations and three host species based on frequencies of 42 alleles at 13 polymorphic enzyme loci.

WN	0.553							
DY	0.552	0.753						
JM	0.535	0.901	0.821					
MD	0.770	0.540	0.516	0.583				
WI	0.627	0.540	0.500	0.378	0.646			
MN	0.870	0.568	0.608	0.601	0.865	0.679		
BT	0.608	0.498	0.445	0.451	0.669	0.723	0.767	
HM	0.704	0.803	0.802	0.838	0.604	0.570	0.712	0.655
WP	0.613	0.616	0.540	0.518	0.627	0.718	0.787	0.807
GM	0.658	0.839	0.765	0.903	0.689	0.468	0.696	0.441
TM	0.623	0.814	0.772	0.912	0.651	0.457	0.681	0.543
TW	0.720	0.545	0.734	0.580	0.778	0.676	0.803	0.656
BB	0.859	0.663	0.730	0.700	0.684	0.564	0.829	0.642
HS	0.869	0.541	0.583	0.567	0.776	0.562	0.840	0.672
NN	0.539	0.904	0.835	0.997	0.579	0.383	0.596	0.442
	CV	WN	DY	JM	MD	WI	MN	BT

WP	0.689						
GM	0.754	0.591					
TM	0.847	0.604	0.818				
TW	0.659	0.607	0.620	0.695			
BB	0.861	0.751	0.763	0.782	0.735		
HS	0.701	0.671	0.635	0.647	0.734	0.878	
NN	0.836	0.510	0.900	0.915	0.604	0.699	0.560
	HM	WP	GM	TM	TW	BB	HS

populations, WN and DY. These four geographically related populations do not form a discrete, highly related cluster since the eastern Australian populations, TM, GM and HM, are observed to have higher genetic identities to the NN and JM populations

relative to those shown by the DY population. DY had the lowest identity to the other populations within this group.

The group of nine also covered all of the geographical regions and was clearly subdivided into three groups, each of which also included diverse host species and regions. The two populations with the highest genetic identity (0.877) were BB (Western Australia) and HS (Victoria) which were both isolated from barley crops. Additionally, they have genetic identities of 0.840 and 0.803 respectively to the MN population, whose previous host was also barley. The remaining populations were isolated from wheat crops and fell into two separate groups.

6.4 Discussion

Previous experiments to assess diversity within *G. graminis* indicated that four isozyme loci may provide useful markers for taxonomic identification of isolates (Chapter 3). The number of alleles resolved with these putative marker loci differed between the earlier experiments and the population analyses. This implied that isozymes could not be applied as definitive variety-specific markers, as was indicated from the inconsistencies in the frequency distributions of the markers within populations. Some populations, however, did show consistency between the variety-specific markers (e.g. *Gga* - NN, JM, GM, DY and WN; *Ggt* - HB and WP) implying that isozymes have at least provided a partial estimate of the taxonomic composition of pathogen populations.

Isozyme analysis revealed high levels of genetic diversity within and between populations of *G. graminis*. The total gene diversity (Dsp) within all 480 isolates, taken as the average across all polymorphic loci, was 0.422. These figures indicate that *G. graminis* is highly variable in comparison to the plant pathogens *Magnaporthe grisea* (Dsp 0.030) (Leung and Williams 1986), *Atkinsonella hypoxylon* (Dsp 0.229) (Leuchtmann and Clay, 1989) and *Leptographium wageneri* (Dsp 0.227) (Zambino and Harrington, 1989). Genetic identity values showed no correlations between the geographical and genetic identities of populations, implying that geographically

restricted pathotypes have not evolved within *G. graminis*. Factors responsible for the distribution of genetic diversity and the resulting relationships between populations are discussed below.

6.4.1 Partitioning of genetic diversity within and between populations of *G. graminis*

In general, the four mechanisms by which variation could be generated and maintained within populations of *G. graminis* include mutation, somatic hybridisation, migration of genetically diverse isolates from other populations and sexual recombination. As has been described previously (Chapter 2) migration of individuals between populations may occur via wind dispersal of ascospores or fine particles of infected plant materials, but the frequencies of successful establishment of isolates in new locations is likely to be low.

Somatic hybridisation has been induced in the laboratory (Asher, 1981), but only closely related isolates are likely to anastomose. Mitotic recombination in these heterokaryotic isolates is apparently rare (Blanch *et al.*, 1981) so it seems unlikely to be a major source of genetic variation within populations.

G. graminis is homothallic and the primary mode of reproduction is thought to involve self-fertilisation. Since the pathogen is haploid, self-fertilisation results in progeny which are genetically identical to the parental strains and does not contribute to genetic diversity within populations (Asher, 1981). Crossing between genetically diverse isolates requires hyphal anastomosis within the developing perithecia for the exchange of nuclei and the generation of hybrid progeny. Given the low frequency of somatic hybridisation within *G. graminis* it is unlikely that sexual recombination between different individuals occurs frequently enough to generate high levels of diversity within populations. This implies that long periods of time would be required for outcrossing to lead to significantly increased levels of intra-population diversity. Therefore, mutation may account for a large proportion of the variation within populations.

Most of the genetic diversity detected at isozyme loci (60%) was apportioned between populations of *G. graminis*. This is consistent with the results accumulated by Loveless and Hamrick (1984) where isozyme studies of natural plant populations have shown that self-fertilisation results in increased genetic variability between gene pools. The greater proportion of variation between populations also implies low levels of inter-population gene flow. If gene flow is considerably restricted, then much of the differentiation between populations may be attributed to genetic drift (Aquadro, 1992).

Populations in those areas of Western Australia which may be considered marginal for cereal production (e.g. low rainfall, nutrient deficient soils) showed lower levels of genetic variability compared with populations originating from areas which are more conducive to cereal cropping. The populations with the lowest levels of genetic diversity included NN, JM and CV and all originated from these marginal regions. These populations also represent the extremes of the species range within Western Australia (Yeats *et al.*, 1986). There are a number of possible factors which may account for lower levels of genetic variation in more isolated populations including (i) recent colonisation of these habitats by a restricted number of genetically similar individuals (founder effect), (ii) high levels of inbreeding, (iii) reductions in population size (population bottlenecks) and (iv) lower diversities of host species (Ayala, 1982; Hartl and Clark, 1989).

Take-all has been reported in these regions of Western Australia for over 100 years and has been reported to occur in crops in their first year of production (Cotterill and Sivasithamparam, 1989; B. MacCleod, personal communication, 1990). Thus, *G. graminis* is unlikely to be a recent introduction to these areas and appears to be a common component of the soil biota throughout the cereal producing regions of southern Australia (Garrett, 1981).

The lower levels of genetic diversity within these Western Australian populations may reflect the evolution of pathogen genotypes which are better adapted to surviving in semi-arid conditions in soils of low nutrient status. Recent studies of native tree species within Australia have indicated that populations at the extremes of the species

range in semi-arid and arid environments are genetically less diverse than those from more temperate environments (Coates and Sokolowski, 1989; Moore and Moran, 1989). These authors have suggested that genetic differentiation may depend on the time of geographical separation of the populations in relation to increasing aridity. Therefore, environmental selection, combined with the probable low levels of gene flow between isolated populations, may be factors contributing to the reduction of genetic diversity within some *G. graminis* populations. This hypothesis is supported by the higher levels of genetic diversity within populations existing in more temperate areas. The contrasts between pathogen variability within these two types of environment is clearly observed within Western Australia. Populations from the south-west region (e.g. WN, BB) revealed three- to four-fold increases in diversity over the populations from the agriculturally marginal regions to the north and east of the state (Tables 6.5 and 6.6).

The apparent correlation between low genetic diversity and increasing aridity is not consistent across all populations. For example, the GM and TM populations exhibited relatively low isozyme diversity and yet were isolated from regions of temperate climate known to be conducive to cereal production. The low levels of diversity within these populations may indicate that they were recently established by a few founder isolates which were relatively homogeneous at isozyme loci. This is unlikely, however, due to the apparently infrequent establishment of *G. graminis* by dispersal of wind-borne inoculum (Chapter 2).

An alternative explanation is that there may have been a reduction in the effective sizes of the populations as the result of host-mediated selection. Since phytopathogenic fungi are largely dependent upon their host plants, the genetic identities of host species may be particularly important in maintaining variation within populations of *G. graminis*. Selective pressures applied by host genotypes have been implicated in affecting isozyme diversity within the plant pathogens *Puccinia graminis* (Burdon and Roelfs, 1985), *Magnaporthe grisea* (Leung and Williams, 1986) and *Atkinsonella hypoxylon* (Leuchtmann and Clay, 1989). Intense host-mediated selection can force

populations through evolutionary bottlenecks, resulting in dramatic reductions in the size and genetic diversity of pathogen populations (Ayala, 1982; Hartl and Clark, 1989).

Host-mediated selection may be responsible for the relatively low levels of genetic variation within the GM population. The previous host of GM was oat, which is resistant to *Ggt* and *Ggg*. These varieties, in the absence of alternative hosts (e.g. grassy weeds), would have had to survive saprophytically upon host debris already present within the soil. In general, *G. graminis* is reported to be a relatively poor saprophyte (Shipton, 1981) and is known to be sensitive to general and specific microbial antagonism during this phase of the life cycle (Rovira and Wildermuth, 1981). Under these conditions the inoculum levels of *Ggt* and *Ggg* would be expected to decrease in frequency. Therefore, *Gga* is expected to have a competitive advantage over the other varieties and should respond faster to the appearance of a susceptible cereal host in the following season. The low genetic diversity within the GM population may result from the selection of genetically homogeneous *Gga* isolates due to the cultivation of oats in the previous season. Further information concerning the recent cropping history of the TM location may reveal if selection of host-specific pathotypes can account for the genetic homogeneity within this population.

Host-mediated selection has probably contributed to the loss of genetic diversity within the NN population, which was expected to be composed exclusively of *Gga* isolates, since this is the only variety which is capable of parasitising oats. NN exhibited the lowest isozyme diversity of any *G. graminis* population examined in this study, which may result from an interaction between environmental and host-mediated selection pressures.

Populations showing the highest levels of diversity were all isolated from wheat. Theoretically, all three varieties of *G. graminis* could be present within these populations since this host is susceptible to each type. Given the high levels of variation observed within *Ggt* and *Ggg* and their differentiation from *Gga* at some of the enzyme loci (Chapter 3), it is possible that these variable populations are composed of a diverse

collection of isolates representing each of the three varieties. The presence of a susceptible host should result in low host-mediated selection and maintain the genetic diversity already present within pathogen populations. It is notable that the populations with the highest levels of diversity (HM and WI) were isolated from fields which were composed of grassy pasture in the previous year. This would have provided a broad diversity of hosts for the pathogen and a means of increasing inoculum levels within the soil. Both of these populations are from locations within the central region of the species range and have evolved in areas which are expected to be environmentally favourable for the pathogen. Consequently, these populations may have evolved under low environmental selection (see above) and over the past few generations have not been exposed to strong host-mediated selection. These factors may have acted to maintain high levels of genetic diversity within these populations.

Most of the populations examined in this study were isolated from cereal hosts within fields which were grown to highly susceptible cereals or pasture grasses in the previous year. Thus, pathogen populations would have been under low host-mediated selection and the differences in the levels of genetic diversity may reflect environmental selection of genotypes adapted to differing climatic and edaphic conditions.

6.4.2 Genetic identities of *G. graminis* populations

Cluster analysis revealed the absence of a relationship between the geographical and genetic identities of populations. An exception is that four of the six Western Australian populations formed a group within one of the primary clusters but within this cluster, three populations originating from eastern Australia were also present (Figure 6.2). This implies that geographically restricted pathotypes have not evolved within *G. graminis*.

It was previously shown that isozyme analysis separated *Gga* from *Ggt* and *Ggg* and that these varieties had an overall similarity of 62% (Chapter 3). Similarly, the populations fell into two groups with an overall genetic identity of 60% and one of these groups contained the expected *Gga* populations NN and GM. It is tempting to

conclude that the other populations within this group may also contain high frequencies of *Gga*. Indeed, three *Gga*-specific alleles (GSR 0.82, 6PGD 1.04 and GOT-1 0.94) showed that five populations within this group contained high frequencies of *Gga*. Inconsistencies between the frequencies of the respective allelic markers within HM and TM indicated that these populations could not be clearly identified as *Gga*.

As shown previously, the overall genetic similarity between *Gga* isolates was approximately 80% (Chapter 3), which is the same value as the average overall genetic identities between the populations in the *Gga* group. The combination of low isozyme diversity and high genetic identities between these populations supports the hypothesis that *Gga* is genetically homogeneous relative to *Ggt* and *Ggg*, as outlined in Chapter 3.

Most of the populations absent from the group dominated by *Gga* appeared to contain some *Gga* isolates but at a much lower frequency relative to the other populations. The WI and MD populations, however, may have contained a greater proportion of this variety, since two of the *Gga*-type markers were present in high frequencies. According to these markers the only populations which may lack *Gga* were HS and WP.

The second primary cluster (Figure 6.2) grouped all nine populations proposed to contain only low frequencies of *Gga*, implying that they were dominated by *Ggt* and *Ggg*. It was not possible to distinguish between the latter two varieties on the basis of isozyme variation, as the frequency of the *Ggg*-specific alleles varied between the respective marker loci within each population. The average genetic similarity between *Ggt* and *Ggg* at the same enzyme loci was approximately 60% (Chapter 3) which is comparable with the overall genetic identities of the nine populations in this group (65%). The similarity between these identity values supports the premise that these populations contain high frequencies of *Ggt* and *Ggg*.

None of these nine populations was isolated from oats or exposed to this host in the previous season. Therefore, they were not expected to have been subjected to intense host-mediated selection during these periods. *Gga* isolates would not have been actively selected within these populations and were expected to be present in low frequencies.

There was, however, some evidence of weak host-mediated selection within the HS, BB and MN populations. These populations were isolated from barley and showed high genetic identities despite their geographical separation. There is some evidence that barley shows a greater tolerance to infection by *G. graminis* compared with wheat (Chambers, 1970; Scott, 1981), although both species are regarded as being highly susceptible. The greater tolerance of barley may apply weak selective pressures upon pathogen populations, resulting in adaptation of pathogen genotypes to this host species.

The results of this study suggest that *G. graminis* exhibits considerable genetic diversity both within and between populations throughout its range in southern Australia. Levels of genetic diversity within populations varied depending upon the host species and possibly the climatic and edaphic conditions. Isolates representing both *Ggt* and *Gga* appeared to be common throughout cereal producing regions of southern Australia. The relative frequencies of these varieties within populations may be determined by host-mediated selection. The distribution and frequency of *Ggg* is unknown. There was some evidence of differentiation within *Ggt* for pathotypes which were adapted to parasitising wheat and barley, although these observations are far from conclusive.

Given the lack of a correlation between geographical origin and genetic identity of pathogen populations, the cluster analysis probably reflects similarities of taxonomic composition of the populations, in particular the presence or absence of high frequencies of *Gga*. This result exemplifies the extent of genetic differentiation between the varieties of *G. graminis*.

The lack of consistency between the frequencies of the respective allelic markers implied that isozymes could not be used to predict the taxonomic composition of *G. graminis* populations. A more accurate determination of their composition would be achieved by examining the populations with variety-specific low-copy and repeat-sequence DNA probes. Furthermore, this should aid in identifying the factors affecting changes in the genetic structure of pathogen populations.

Chapter 7 Molecular variation within and between populations of *G. graminis*

7.1 Introduction

Electrophoretic studies at several isozyme loci have resolved high levels of genetic diversity both within and between populations of *G. graminis* (Chapter 6). The distribution of diversity has implicated several factors for maintaining genetic variability within pathogen populations. These are primarily associated with the reproductive mechanisms of *G. graminis*, the frequency of migration between populations and the relative intensities of environmental and host-mediated selection pressures.

Some populations were found to exhibit relatively low levels of genetic diversity at isozyme loci and required further analyses with potentially highly polymorphic genetic markers to clarify the extent of their variation. It has already been shown that RFLPs detected higher levels of diversity between isolates of *G. graminis* compared with those resolved by isozymes (Chapter 4). Therefore, this technique should provide more definitive estimates of intra-population diversity.

The application of highly variable repeat-sequence probes may be particularly useful in determining levels of intra-population diversity. A number of different repeat sequences have been identified in *G. graminis* and hybridisation experiments have resolved RFLP phenotypes which were unique to specific isolates (Chapter 4). Data derived from such high resolution methods should provide information on the genetic structure of populations. For example, it has been proposed (Chapter 2) that self-fertilisation and asexual reproduction are likely to be the primary modes of reproduction within *G. graminis*. This implies that populations may be composed primarily of clonally propagated lineages. The use of highly discriminatory repeat-sequence probes should provide a means of defining population structure, which may

indicate the predominant mode of pathogen reproduction in the field (Levy *et al.*, 1991).

Phenetic analysis of isozyme diversity within *G. graminis* provided no evidence for the evolution of geographically restricted pathotypes within Australia and it was proposed that inter-population relationships may be based upon similarities in their taxonomic composition (Chapter 6). Taxonomic identification with isozymes was, however, inconclusive due to discrepancies between frequencies of a few putative variety-specific alleles. Therefore, evaluations of the taxonomic composition of pathogen populations will require analyses with markers, such as low-copy DNA probes, which are more definitive (Chapter 4).

A variety of low copy number DNA probes resolved RFLP phenotypes capable of differentiating between varieties of *G. graminis* (Chapter 4) and should prove useful in clarifying the distribution of varieties of the pathogen. Families of repetitive sequences may also provide a means of discriminating between varieties of this species, as has been shown by O'Dell *et al.* (1992). Screening populations isolated from different host species with the variety-specific markers should confirm the importance of host-mediated selection in determining the taxonomic composition of *G. graminis* populations.

The purpose of this study was to estimate levels of genetic diversity at RFLP loci within and between populations of *G. graminis*. The use of low copy and repeat-sequence probes makes it possible to compare their relative abilities to resolve variation within the species and to differentiate between taxonomic varieties of the pathogen. This research should provide additional information on the fine scale genetic structure of pathogen populations, which may be of value in understanding the mechanisms responsible for the generation of diversity and the factors which have shaped the recent evolution of the species.

7.2 Materials and methods

7.2.1 Source of isolates, DNA extraction and DNA hybridisation experiments

Twenty isolates randomly selected from the 30 individuals representing each of the 16 *G. graminis* populations (Table 6.1) were analysed for RFLPs with 7 different probe/enzyme combinations.

Isolates were grown in shaking liquid culture by the methods described in Chapter 3. Methods for isolation of DNA and documentation of RFLPs were as described previously (Chapter 4). DNA was found to be of sufficient quality for restriction enzyme digestion and subsequent analyses without the requirement for purification on caesium chloride (CsCl) density gradients.

Individual samples of DNA (1.5-2.0 μg), from each of the isolates, were digested separately with the restriction enzymes BamH I and Hind III and transferred to nylon membranes. The Hind III digests were probed separately with *G. graminis* clones 4, 26, 30 and 202. BamH I digests were probed with clones 5, 23 and 26. These probe/enzyme combinations were chosen to document variation within and between populations of *G. graminis* because: (i) they had previously been shown to discriminate between taxonomic varieties of *G. graminis* and (ii) were capable of detecting variation within varieties (Chapter 4).

Each membrane also contained DNA from isolates representing each variety and since the RFLP phenotypes of these isolates had been determined previously (Chapter 4), they acted as standards for inter-population comparisons and taxonomic identification.

7.2.2 Data analysis

Frequencies of the RFLP phenotypes determined with each of the probe/enzyme combinations were calculated for each of the 16 populations and were used to estimate levels of genetic diversity within and between populations of *G. graminis* using Shannon's diversity index (Chapter 3).

Genetic identity (Nei, 1972) was calculated by pairwise comparisons of populations using the RFLP phenotype frequencies from probe/enzyme combinations 5B, 26B, 26H and 202H. The combination 30H was excluded from the analysis as it was monomorphic across all 16 populations. Identity values were used to construct a between-population identity matrix. Hierarchical cluster analysis of the identity matrix was used to generate a dendrogram illustrating the relationships between *G. graminis* populations as described in Chapter 6.

Probes/enzyme combinations 4H and 23B resolved multiple bands in each isolate and were designated as repeat-sequence probes (Goodwin *et al.*, 1992). Some bands were excluded from the analyses due to poor resolution of the lower molecular weight fragments. In the absence of crossing experiments and segregation analysis, individual bands of the hybridisation profiles could not be scored as loci or allelic forms of loci. The different banding profiles of isolates were therefore defined as separate RFLP phenotypes.

An estimate of the similarity between these individual phenotypes was made by using the similarity coefficient (Lynch, 1990, Chapter 3). Pairwise comparisons were made between probe-specific phenotypes and the results presented in similarity matrices. Cluster analyses of the similarity values were performed using the group average method and the relationships between phenotypes shown in a dendrogram (Chapter 3).

7.3 Results

7.3.1 Phenotypes identified with low-copy probes 5, 26, 30 and 202

The sizes of the hybridisation fragments diagnostic for each RFLP phenotype, their frequencies within each population and their overall frequencies averaged across all populations are shown in Table 7.1.

Each of the 320 *G. graminis* isolates used in this study exhibited clear banding patterns with each of the seven probe/enzyme combinations. All combinations were

Table 7.1. RFLP phenotype frequencies at 5 probe/enzyme combinations within 16 populations of *G. graminis*.

Combination	RFLP	Fragment sizes (kbp)	variety	CV	WN	DY	JM	MD	WI	MN	BT	HM	WP	GM	TM	TW	BB	HS	NN	Sum
5/BamH I	1	6.31 + 2.51	*tritici*	0.75	0.05			1.00	0.70	0.75	1.00	0.05	0.65		0.05	0.85	0.90	1.00		0.48
	2	15.85 + 2.51	*tritici*	0.25					0.20	0.25			0.25				0.05			0.06
	3	12.96 + 2.51	*avenae*		0.95	1.00	1.00		0.10			0.95	0.10	1.00	0.95	0.15	0.05		1.00	0.46
26/BamH I	1	9.12	*tritici*	0.05				0.40	0.20	0.80			0.15		0.10	0.30	0.75	1.00		0.23
	2	7.49	*avenae*		0.95	1.00	1.00		0.10			0.95		1.00	0.90	0.20	0.05		1.00	0.45
	3	4.47	*tritici*	0.95	0.05			0.60	0.70	0.20	1.00	0.05	0.85			0.50	0.20			0.32
26/Hind III	1	10.80	*tritici*	1.00				1.00	1.00	1.00	1.00	0.05	1.00		0.10	1.00	1.00	1.00		0.57
	2	6.03	*avenae*		1.00	1.00	1.00					0.95		1.00	0.90				1.00	0.43
30/Hind III	1	6.68		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
202/Hind III	1	6.60 + 3.63 + 3.31	*tritici*	0.85	0.15			0.20	0.30	1.00	0.05		0.80			0.25	0.75	0.05		0.28
	2	6.60 + 3.31	*tritici*	0.15				0.80	0.60		0.95	0.10	0.20		0.10	0.60	0.20	0.95		0.29
	3	10.50	*avenae*		0.85	1.00	1.00		0.10			0.90		1.00	0.90	0.15	0.05		1.00	0.43

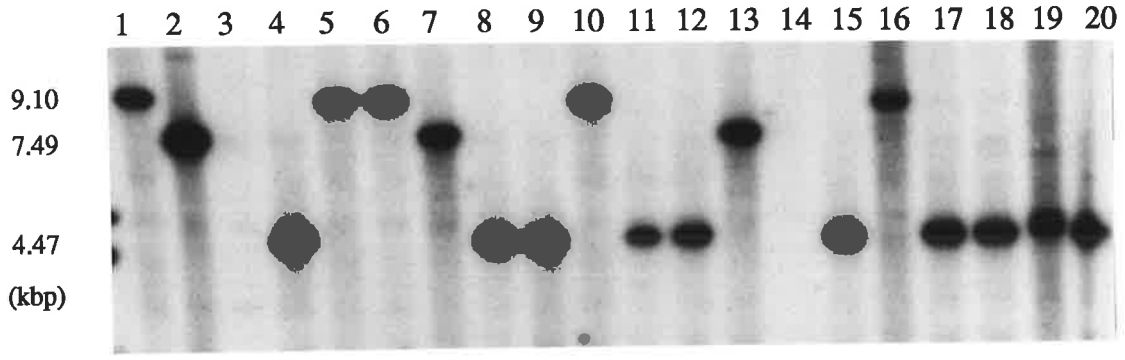
polymorphic with the exception of 30H, which was invariant for a 6.68 kilobase pair (kbp) fragment across all 16 populations.

Combination 5B resolved three RFLP phenotypes between the 16 populations. This was much lower than the nine phenotypes observed in the original screening of *G. graminis* isolates (Chapter 4). Phenotype 1, characterised by two fragments of 6.31 and 2.51 kbp, was observed in 12 of the populations and was previously shown to be common to *Ggt* isolates. Phenotype 2 had the lowest overall frequency across the populations (0.06) and was detected in only five populations. Previously this phenotype was shown to be unique to *Ggt* isolate EBI and was characterised by hybridisation fragments of 15.85 and 2.51 kbp (Chapter 4). Both phenotypes 1 and 2 were assumed to identify isolates as *Ggt*. Phenotype 3 (12.96+2.51 kbp fragments) was assumed to identify isolates as *Gga* as it was previously observed to be specific for *Gga* isolate 35793.

Combination 26B resolved three distinct phenotypes in the population analysis, each isolate showing a single band of hybridisation. Phenotype 1 was present in nine populations and was characterised by a 9.12 kbp fragment, previously shown to be specific for *Ggt*. The 7.49 kbp fragment (phenotype 2), shown to be characteristic of *Gga*, was detected in ten populations. Phenotype 3 (4.47 kbp) was detected in ten populations and was common to *Ggt* and *Ggg* (Figure 7.1). With combination 26H, only two RFLP phenotypes were detected amongst the population samples. Phenotype 1 (10.80 kbp) was detected in 11 populations and was fixed in nine (frequency = 1.0). This phenotype was shown to be characteristic of *Ggt*, whereas phenotype 2 (6.03 kbp) was characteristic of *Gga* (Chapter 4).

Combination 202H detected three RFLP phenotypes among the populations, the number of bands varying from 1-3 in each isolate. Phenotype 1 had hybridisation fragments of 6.60, 3.63 and 3.31 kbp and was detected in ten populations. This phenotype was not resolved in previous experiments (Chapter 4) but differed from the *Ggt* marker (phenotype 2) by the inclusion of the 3.36 kbp band. Phenotype 2 (characterised by the 6.60 and 3.31 kbp bands) was detected in ten of the populations

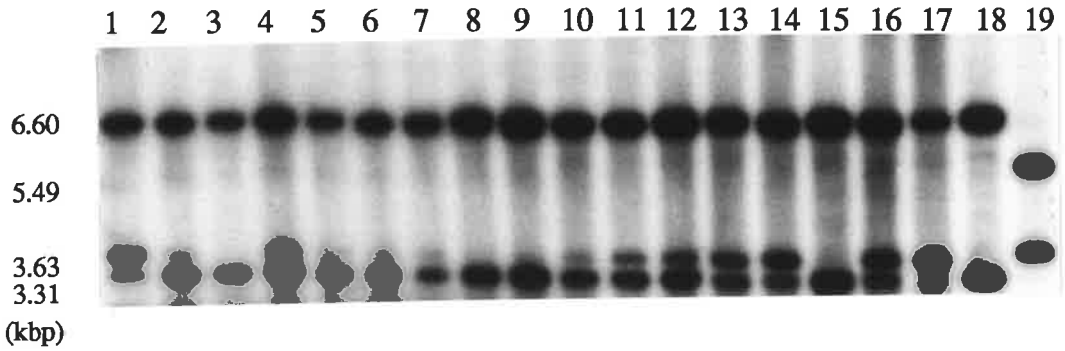
Figure 7.1. Hybridisation of labelled clone 26 with DNA isolated from 17 *G. graminis* isolates within the WI population. DNA was digested with *Bam*H I (ie. combination 26B). Lanes: 1, *Ggt*- 800; 2, *Gga*- 192M; 3-20, WI 2 (degraded DNA), WI 1, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 (degraded DNA), 14, 16, 17, 18, 19, 20. Sizes of fragments are in kilobase pairs (kbp).



(Figure 7.2). A single fragment of 10.50 kbp characterised phenotype 3, which in the original screening of isolates, was present in *Gga* isolate 35793 and *Ggt* isolate 500 (weakly pathogenic upon oats, and reclassified as *Gga* from RFLP and PCR variety-specific markers). This RFLP phenotype identified isolates as *Gga* and was detected in ten of the populations.

The 5B, 30H and 202H RFLP phenotypes of *Gga* isolates from *Agrostis* spp. were not detected in any of the populations. Similarly, the 5B and 202H phenotypes detected in *Ggg* isolates in earlier experiments were also absent from the populations (Chapter 4).

Figure 7.2. Hybridisation of labelled clone 202 with DNA isolated from 17 *G. graminis* isolates within the WP population. DNA was digested with *Hind* III (ie. combination 202H). Lanes: 18, *Ggt*- 17916; 19, *Ggg*- ADI; 1-17, WP 21, 22, 26, 27, 28, 29, 30, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20. Sizes of fragments are in kilobase pairs (kbp).



7.3.2 Phenotypes identified with repeat-sequence probes 4 and 23

Combinations 4H and 23B revealed many polymorphisms between isolates of *G. graminis*. Complex banding patterns were observed for each isolate, with 4H resolving 7-12 scorable bands, ranging in size from 1.20 kbp to 11.00 kbp. Combination 23B resolved 5-9 bands per isolate which varied in size from 0.50 kbp to 14.60 kbp.

The banding profiles were used to assign a DNA phenotype to each isolate. Twenty 23B and 34 4H phenotypes were detected amongst the 320 isolates used in this study, with 2-4 phenotypes found within any one population, except JM and NN which were monomorphic with 4H and 23B, respectively (Table 7.2). Of the thirty four phenotypes detected with 4H, twenty nine were unique to specific populations and only five were observed to occur in more than one population (Table 7.3). Similarly, the majority of phenotypes detected with 23B were population-specific, with only five present in more than one population (Table 7.4).

Table 7.2. Numbers of DNA repeat sequence phenotypes detected within 16 populations of *G. graminis* with probe/enzyme combinations 4H and 23B.

Population	4H		23B	
	Total	unique*	Total	unique*
CV	3	2	3	1
WN	2	2	2	2
DY	2	2	2	1
JM	1	1	3	1
MD	3	2	2	0
WI	4	3	2	1
MN	2	2	4	2
BT	3	2	3	0
HM	2	1	2	1
WP	2	2	3	0
GM	2	2	2	0
TM	2	1	3	3
TW	3	1	3	2
BB	4	4	4	2
HS	2	1	2	0
NN	2	2	1	0
	34	30	20	16

Unique* Refers to the number of repeat-sequence phenotypes unique to a particular pathogen population.

7.3.3 Partitioning of genetic diversity within and between populations of *G. graminis*

The average level of polymorphism across all populations and markers was 58%, with the average number of phenotypes per combination varying between populations (Table 7.5). The percentage of polymorphic combinations ranged from 14% in the JM and NN populations to 86% in the HM and TM populations.

Table 7.3. Distribution and frequency of the 34 repeat-sequence phenotypes detected with 4H within and between populations of *G. graminis*.

RFLP phenotype	Population	*tritici* isolates*	*avenae* isolates*	overall	RFLP phenotype	Population	*tritici* isolates*	*avenae* isolates*	overall
1	MD	0.70		0.70	18	WI	0.20		0.20
	CV	0.55		0.55		TW	0.55	0.05	0.60
2	CV	0.35		0.35	19	WI	0.15		0.15
3	CV	0.10		0.10	20	WI	0.10		0.10
4	NN		0.80	0.80	21	MN	0.75		0.75
5	NN		0.20	0.20	22	MN	0.25		0.25
6	BB	0.65		0.65	23	BT	0.70		0.70
	TW	0.25	0.05	0.30	24	BT	0.20		0.20
7	BB	0.25		0.25	25	HB	0.65		0.65
8	BB		0.05	0.05		BT	0.10		0.10
9	BB	0.05		0.05	26	HM		0.95	0.95
10	WN	0.05	0.85	0.90		TM		0.90	0.90
11	WN		0.10	0.10	27	HM	0.05		0.05
12	DY		0.75	0.75	28	HS	0.35		0.35
13	DY		0.25	0.25	29	WP	0.85		0.85
14	JM		1.00	1.00	30	WP	0.15		0.15
15	MD	0.20		0.20	31	GM		1.00	0.85
16	MD	0.10		0.10	32	GM		1.00	0.15
17	WI	0.45	0.10	0.55	33	TM	0.10		0.10
					34	TW		0.10	0.10

Table 7.4. Distribution and frequency of the 20 repeat-sequence phenotypes detected with 23B within and between populations of *G. graminis*.

RFLP phenotype	Population	*tritici* isolates*	*avenae* isolates*	overall	RFLP phenotype	Population	*tritici* isolates*	*avenae* isolates*	overall
1	MD	0.85		0.85	4	NN		1.00	1.00
	CV	0.60		0.60		JM		0.10	0.10
	MN	0.45		0.45	5	BB	0.50		0.50
	WP	0.35		0.35	6	BT	0.75		0.75
	HS	0.25		0.25		BB	0.10		0.10
	BB	0.15		0.15		WP	0.10		0.10
	BT	0.05		0.05	7	WN	0.05	0.85	0.90
	GM		0.05	0.05	8	WN		0.10	0.10
2	CV	0.25		0.25	9	DY		0.80	0.80
3	GM		0.95	0.95	10	JM		0.80	0.80
	HS	0.75		0.75	11	HM		0.95	0.95
	WI	0.75		0.75		JM		0.10	0.10
	TW	0.60	0.05	0.65	12	WI	0.15	0.10	0.25
	WP	0.55		0.55	13	MN	0.15		0.15
	BB	0.20	0.05	0.25	14	MN	0.15		0.15
	MN	0.25		0.25	15	HM	0.05		0.05
	BT	0.20		0.20	16	TM		0.80	0.80
	DY		0.20	0.20	17	TM		0.10	0.10
	CV	0.15		0.15	18	TM	0.10		0.10
	MD	0.15		0.15	19	TW	0.20		0.20
					20	TW		0.15	0.15

Table 7.5. Descriptive statistics of RFLP diversity with 7 probe/enzyme combinations, within the 16 populations of *G. graminis* .

Population	% Polymorphic RFLP markers	Average No. phenotypes/RFLP marker
CV	71	2.00
WN	71	1.71
DY	29	1.29
JM	14	1.29
MD	57	1.71
WI	71	2.43
MN	57	1.86
BT	43	1.71
HM	86	1.86
WP	71	2.00
GM	71	1.71
TM	86	1.86
TW	71	2.29
BB	71	2.57
HS	43	1.43
NN	14	1.14
Mean	58	1.80

Genetic diversity within each of the populations (Table 7.6) was estimated from the phenotypic frequencies. Populations with the low levels of genetic diversity were NN, GM, JM and DY, all of which were monomorphic with each of the combinations except one or both of the repeat-sequence probes. Populations exhibiting particularly high levels of diversity were WI, TW and BB, each of which was polymorphic with all combinations except 26H and 30H. Only the HM and TM populations were polymorphic with 26H.

Genetic diversity was partitioned into within- and between-population components (Table 7.7). The repeat-sequence probes accounted for more of the diversity than the low copy probes, both within populations (Hpop) and across the species (Hsp). All of

Table 7.6. Estimates of genetic diversity (H_0) within populations of *G. graminis* at each of the probe/enzyme combinations used in this study.

Probe	CV	DY	WN	JM	MD	WI	MN	BT	HM	WP	GM	TM	TW	BB	HS	NN
5B	0.811	0.000	0.286	0.000	0.000	1.157	0.811	0.000	0.286	1.236	0.000	0.286	0.610	0.569	0.000	0.000
26B	0.286	0.000	0.286	0.000	0.971	1.157	0.722	0.000	0.286	0.610	0.000	0.469	1.486	0.992	0.000	0.000
26H	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.286	0.000	0.000	0.469	0.000	0.000	0.000	0.000
30H	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
202H	0.610	0.000	0.610	0.000	0.722	1.296	0.000	0.286	0.469	0.722	0.000	0.469	1.353	0.992	0.286	0.000
23B	1.353	0.722	0.469	0.922	0.610	0.811	1.840	0.992	0.286	1.381	0.286	0.922	1.279	1.743	0.811	0.000
4H	1.337	0.811	0.469	0.000	1.157	1.682	0.811	1.157	0.286	0.610	0.610	0.469	1.296	1.336	0.934	0.722
Mean	0.628	0.219	0.303	0.132	0.494	0.872	0.598	0.348	0.272	0.651	0.128	0.441	0.860	0.805	0.290	0.103

Table 7.7. Partitioning of the genetic diversity between and within populations of *G. graminis* as determined with seven probe/enzyme combinations.

Combination	Average within populations	Total within species	Proportion within populations	Proportion between populations
	Hpop	Hsp	$\frac{Hpop}{Hsp}$	$\frac{Hsp-Hpop}{Hsp}$
5B	0.378	1.274	0.297	0.703
26B	0.454	1.536	0.296	0.704
26H	0.047	0.986	0.048	0.952
202H	0.488	1.553	0.314	0.686
23B	0.902	3.380	0.267	0.733
4H	0.855	4.543	0.188	0.812
Mean	0.521	2.212	0.235	0.765

the combinations resolved more diversity between, rather than within, populations, with 76% of the overall diversity apportioned between populations.

7.3.4 Application of low-copy RFLP phenotypes as taxonomic markers within *G. graminis*

The NN population, which was isolated from oats, showed only *Gga*-type markers. The correspondence between pathogenicity and all four RFLP phenotypes implied that the markers may be used to determine the taxonomic composition of pathogen populations. The NN, GM, JM and DY populations showed identical frequencies of these four phenotypes implying that each population was entirely composed of *Gga*. Combinations 26B, 26H and 202H indicated that 90% of the TM isolates were also classified as *Gga*, whereas 5B indicated the proportion to be 95%. Three of the four markers suggest that 95% of the HM population was *Gga*, whereas 202H indicated only 90% of the population to be of this type. Combinations 5B and 26B classified 95% of the isolates within the WN population to be *Gga*. Discrepancies occurred at the other

two combinations where 202H classified 85% and 26H 100% of these isolates to be *Gga*.

Populations BB, TW, WP and WI also contained isolates with the *Gga* RFLP phenotypes. The frequency of these phenotypes at three of the four markers within BB was 0.05, suggesting that one isolate within this population was *Gga*, but 26H did not distinguish this isolate from *Ggt*. A similar discrepancy between the markers was observed within the WI population, where 10% of the isolates were identified as *Gga*. Within the TW and WP populations the proportion of *Gga* isolates detected by the different markers varied, ranging between 0-15% and 0-20% respectively (Table 7.1).

Within the entire collection of isolates, combinations 5B and 26B identified 45% and 26H and 202H 43% of the isolates as *Gga*, the rest being classified as *Ggt*.

7.3.5 Genetic identity of populations revealed with probes 5, 26 and 202

Nei's genetic identity was used to estimate the similarities between populations based on the RFLP phenotype frequencies listed in Table 7.1. The average, overall genetic identity between all 16 populations was approximately 0.15 and identities ranged between 0.00 and 1.00 (Table 7.8). Four populations (NN, GM, JM and DY) were phenotypically identical and had no phenotypes in common with CV, MD, MN, BT and HS.

Cluster analysis of the identity matrix separated the populations into two primary groups (Figure 7.3). Both of these groups contained populations of diverse geographical origin, with the average, overall, genetic identity between populations within each group observed to be approximately 0.75. Within one group the HS and BB populations (isolated from barley) showed a genetic identity of 0.837, despite having originated from Victoria and Western Australia respectively. These two populations had genetic identities of 0.973 and 0.724 respectively to the MN population, whose previous host was also barley. The MN and BB populations formed a discrete group. CV and WP showed the greatest genetic identity within this primary cluster (0.994), despite having originated thousands of kilometres apart. These populations were both isolated from

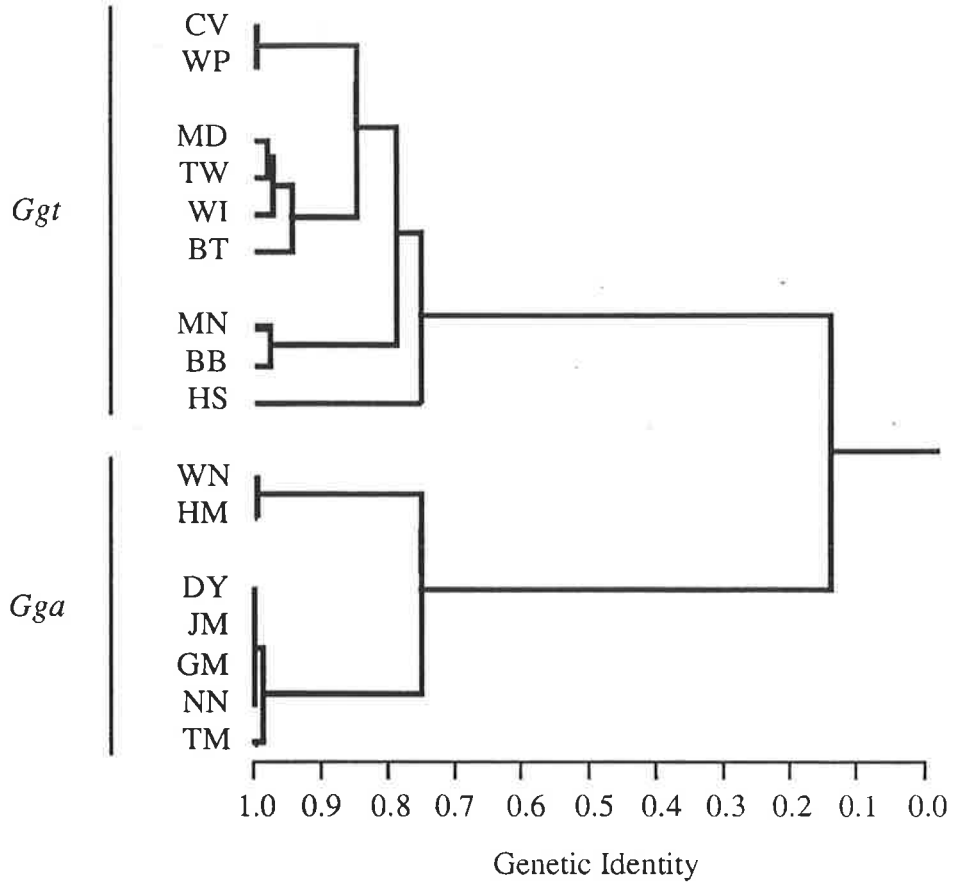
wheat and showed a closer relationship to other populations from the same host, compared to those isolated from barley.

Table 7.8. Nei's genetic identity (I) among the 16 populations of *G. graminis* based on frequencies of 11 RFLP phenotypes from 4 probe/enzyme combinations.

WN	0.355							
DY	0.000	0.729						
JM	0.000	0.729	1.000					
MD	0.812	0.329	0.000	0.000				
WI	0.900	0.462	0.094	0.094	0.961			
MN	0.822	0.349	0.000	0.000	0.735	0.751		
BT	0.807	0.297	0.000	0.000	0.953	0.948	0.557	
HM	0.309	0.994	0.758	0.758	0.330	0.454	0.292	0.308
WP	0.994	0.400	0.029	0.029	0.831	0.918	0.850	0.809
GM	0.000	0.729	1.000	1.000	0.000	0.094	0.000	0.000
TM	0.047	0.759	0.995	0.995	0.082	0.168	0.065	0.068
TW	0.838	0.518	0.156	0.156	0.976	0.974	0.764	0.926
BB	0.820	0.398	0.043	0.043	0.877	0.826	0.973	0.677
HS	0.555	0.284	0.000	0.000	0.897	0.789	0.724	0.744
NN	0.000	0.729	1.000	1.000	0.000	0.094	0.000	0.000
	CV	WN	DY	JM	MD	WI	MN	BT

HM							
WP	0.355						
GM	0.758	0.029					
TM	0.789	0.084	0.995				
TW	0.513	0.861	0.156	0.235			
BB	0.357	0.850	0.043	0.118	0.869		
HS	0.295	0.601	0.000	0.095	0.862	0.837	
NN	0.758	0.029	1.000	0.995	0.156	0.043	0.000
	HM	WP	GM	TM	TW	BB	HS

Figure 7.3. Dendrogram of *G. graminis* populations generated by group average cluster analysis of the genetic identities among populations.



7.3.6 Genetic similarities of populations resolved by probes 4 and 23

Combination 4H

The average overall similarity between the 34 phenotypes detected with combination 4H was approximately 40%. The greatest similarity values of 94.1% were observed in comparisons between eight phenotypes (Table 7.9), representing the majority of isolates from the CV, MD, WI, TW, BT and HS populations. Repeat-sequence phenotypes which showed the least similarity of 23.5% were in the following comparisons; (i) BB9 with WI18, 19, TW18 and MN22, (ii) BT23 with BB8, TW34 and GM31 and (iii) BT24 with GM31.

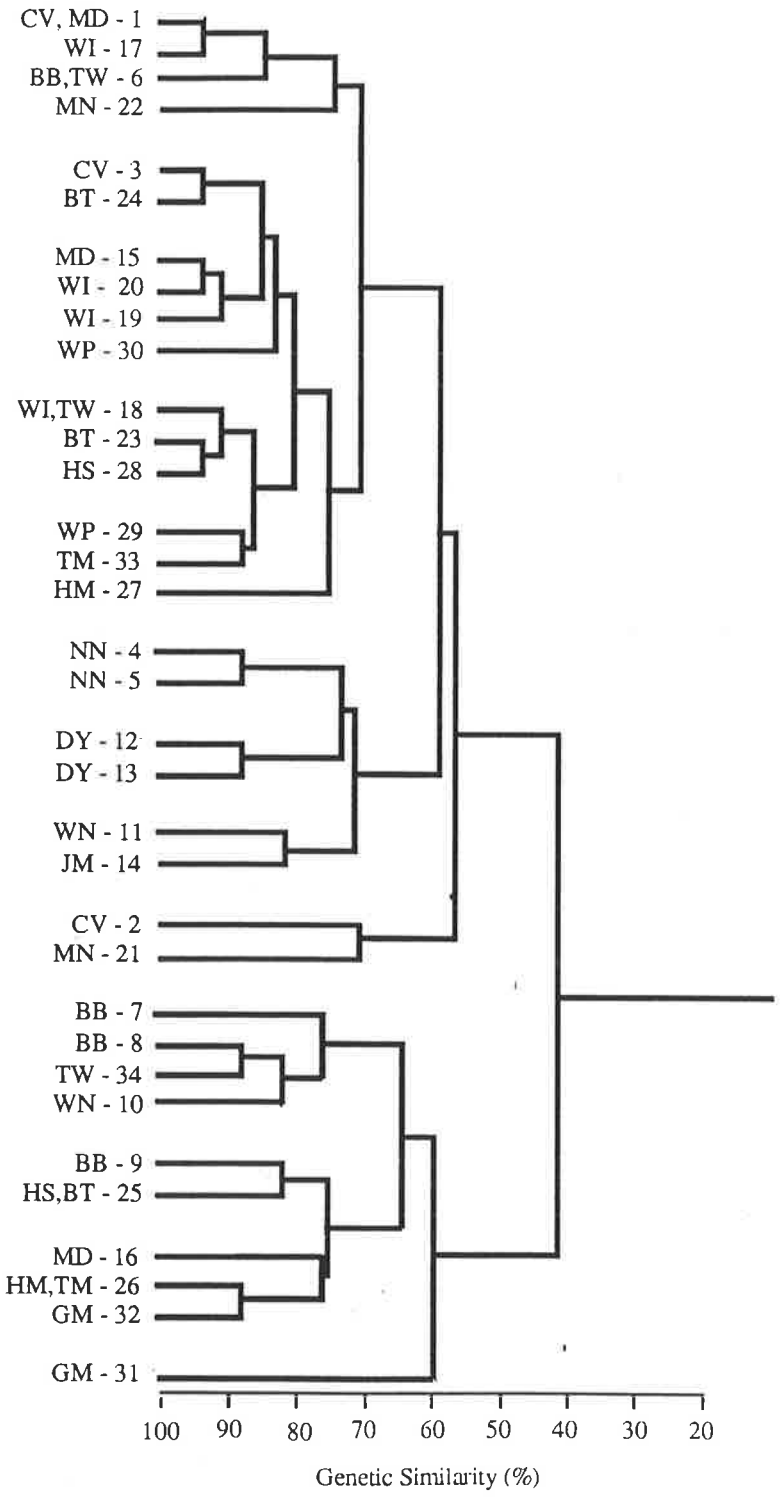
Table 7.9. Genetic similarities (%) between banding profiles of the 34 repeat sequence phenotypes detected with probe/enzyme combination 4H.

2	70.6															
3	76.5	58.8														
4	64.7	47.1	76.5													
5	52.9	58.8	64.7	88.2												
6	82.4	52.9	70.6	47.1	35.3											
7	76.5	70.6	52.9	41.2	29.4	70.6										
8	64.7	58.8	41.2	41.2	41.2	58.8	76.5									
9	41.2	47.1	29.4	41.2	52.9	35.3	52.9	64.7								
10	64.7	58.8	41.2	52.9	52.9	58.8	76.5	88.2	64.7							
11	58.8	52.9	58.8	70.6	70.6	52.9	58.8	52.9	58.8	82.4						
12	76.5	58.8	64.7	76.5	76.5	58.8	52.9	52.9	41.2	64.7	70.6					
13	64.7	58.8	52.9	64.7	76.5	58.8	52.9	52.9	52.9	64.7	70.6	88.2				
14	52.9	47.1	52.9	76.5	76.5	35.3	41.2	64.7	52.9	64.7	82.4	76.5	64.7			
15	76.5	70.6	88.2	64.7	64.7	70.6	52.9	41.2	29.4	41.2	58.8	64.7	64.7	52.9		
16	70.6	52.9	47.1	58.8	47.1	52.9	58.8	58.8	70.6	58.8	52.9	58.8	47.1	58.8	47.1	
17	94.1	64.7	70.6	58.8	47.1	88.2	70.6	58.8	35.3	58.8	52.9	70.6	58.8	47.1	70.6	64.7
18	70.6	64.7	82.4	58.8	58.8	76.5	47.1	35.3	23.5	35.3	52.9	58.8	58.8	47.1	94.1	41.2
19	82.4	64.7	94.1	70.6	58.8	76.5	58.8	47.1	23.5	47.1	64.7	70.6	58.8	58.8	94.1	52.9
20	70.6	64.7	82.4	58.8	58.8	76.5	58.8	47.1	35.3	47.1	64.7	58.8	70.6	47.1	94.1	41.2
21	64.7	70.6	52.9	41.2	52.9	47.1	64.7	64.7	41.2	64.7	58.8	64.7	64.7	52.9	64.7	35.3
22	70.6	41.2	70.6	47.1	35.3	76.5	58.8	47.1	23.5	47.1	52.9	58.8	47.1	47.1	70.6	41.2
23	58.8	52.9	82.4	70.6	70.6	64.7	35.3	23.5	35.3	35.3	52.9	58.8	58.8	47.1	82.4	41.2
24	70.6	52.9	94.1	82.4	70.6	64.7	47.1	35.3	35.3	47.1	64.7	70.6	58.8	58.8	82.4	52.9
25	58.8	64.7	47.1	58.8	70.6	41.2	47.1	58.8	82.4	58.8	52.9	58.8	58.8	58.8	47.1	76.5
26	64.7	58.8	41.2	52.9	52.9	47.1	64.7	76.5	76.5	76.5	70.6	64.7	52.9	76.5	41.2	82.4
27	64.7	58.8	76.5	64.7	52.9	58.8	64.7	41.2	41.2	52.9	70.6	64.7	52.9	64.7	76.5	58.8
28	64.7	58.8	88.2	64.7	64.7	70.6	41.2	29.4	29.4	29.4	47.1	52.9	52.9	41.2	88.2	35.3
29	70.6	52.9	70.6	58.8	58.8	76.5	47.1	35.3	35.3	47.1	52.9	70.6	70.6	47.1	82.4	52.9
30	82.4	52.9	82.4	70.6	58.8	76.5	58.8	47.1	35.3	58.8	64.7	82.4	70.6	58.8	82.4	64.7
31	41.2	35.3	29.4	41.2	41.2	47.1	52.9	64.7	52.9	52.9	58.8	52.9	52.9	64.7	29.4	47.1
32	52.9	47.1	29.4	41.2	41.2	58.8	64.7	76.5	76.5	76.5	70.6	52.9	52.9	64.7	29.4	70.6
33	58.8	52.9	70.6	58.8	58.8	64.7	47.1	35.3	35.3	47.1	64.7	58.8	58.8	58.8	82.4	41.2
34	52.9	58.8	41.2	41.2	41.2	47.1	76.5	88.2	64.7	76.5	70.6	52.9	52.9	64.7	41.2	58.8
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**

18	76.5																
19	76.5	88.2															
20	64.7	88.2	88.2														
21	58.8	58.8	58.8	58.8													
22	76.5	76.5	76.5	64.7	70.6												
23	64.7	88.2	76.5	76.5	47.1	64.7											
24	64.7	76.5	88.2	76.5	47.1	64.7	88.2										
25	52.9	41.2	41.2	41.2	47.1	29.4	52.9	52.9									
26	58.8	35.3	47.1	35.3	52.9	47.1	35.3	47.1	82.4								
27	58.8	70.6	82.4	70.6	52.9	70.6	70.6	82.4	47.1	64.7							
28	70.6	94.1	82.4	82.4	52.9	70.6	94.1	82.4	47.1	29.4	64.7						
29	76.5	88.2	76.5	76.5	41.2	76.5	88.2	76.5	52.9	47.1	70.6	82.4					
30	76.5	76.5	88.2	76.5	58.8	76.5	76.5	88.2	52.9	58.8	82.4	70.6	88.2				
31	47.1	35.3	35.3	35.3	52.9	58.8	23.5	23.5	47.1	64.7	41.2	29.4	35.3	35.3			
32	58.8	35.3	35.3	35.3	41.2	47.1	35.3	35.3	70.6	88.2	52.9	29.4	47.1	47.1	76.5		
33	64.7	88.2	76.5	76.5	58.8	76.5	88.2	76.5	41.2	47.1	82.4	82.4	88.2	76.5	35.3	47.1	
34	47.1	35.3	47.1	47.1	64.7	47.1	23.5	35.3	58.8	76.5	52.9	29.4	35.3	47.1	76.5	76.5	35.3
	17	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**

Cluster analysis placed the phenotypes into two primary groups, each of which had an average similarity of approximately 60% (Figure 7.4). Eight populations (WN, MD,

Figure 7.4. Dendrogram showing relationships between 4H repeat sequence phenotypes detected in populations of *G. graminis*.



BT, HM, TM, TW, BB and HS) contained phenotypes which fell into both primary groups. Phenotypes showed a wide range of inter-population similarities; low (e.g. BB6 and BB9, 35.3%), moderate (eg, WI17 and WI20, 64.7%) and high (DN12 and DN13, 88.2%). In general, it was rare for phenotypes from the same population to have very high similarities and in some cases there were greater similarities between, rather than within, populations (e.g. CV1 and WI17, 94.1% compared with CV1 and CV2, 70.6%).

Comparisons of the 4H phenotypes with the taxonomic classifications of isolates from low-copy RFLP loci (Table 7.3) indicated that all NN, DY, JM and WN11 *Gga* isolates formed a discrete group within the largest of the primary clusters. The JM and WN isolates showed a closer similarity to each other than they did to *Gga* isolates from the NN and DY populations. The average similarity between these 6 phenotypes was approximately 70% and they were clearly differentiated from *Ggt* isolates within this primary cluster.

The NN, DY, JM and WN11 *Gga* isolates were, however, more closely related to *Ggt* than they were to the BB8, WN10, TW34, TM26, HM26 and GM31, 32 *Gga* isolates, which formed two groups within the second primary cluster (Table 7.3, Figure 7.4). The WN10, TW34 and BB8 isolates formed a discrete cluster with an average similarity of 80%. It is notable that BB8 characterised a single *Gga* isolate, which showed a closer similarity to *Gga* isolates in geographically disjunct populations than it did to the *Ggt* isolates within its own population. The HM26, TM26 and GM32 *Gga* isolates showed an average similarity of approximately 90%, but were very similar to the MD16 *Ggt* isolates (82%). GM31 *Gga* isolates were generally dissimilar to all other isolates within this primary cluster. *Gga* isolates with 4H phenotypes 17 and 18 (i.e. WI and TW) were identical to *Ggt* isolates within their respective populations.

Combination 23B

The average overall similarity between all 23B phenotypes was approximately 68%. The greatest similarity values (95.2%) were in comparisons between 10 phenotypes (Table 7.10), which included isolates from most of the populations.

Table 7.10. Genetic similarities (%) between banding profiles of the 20 repeat sequence phenotypes detected with probe/enzyme combination 23B.

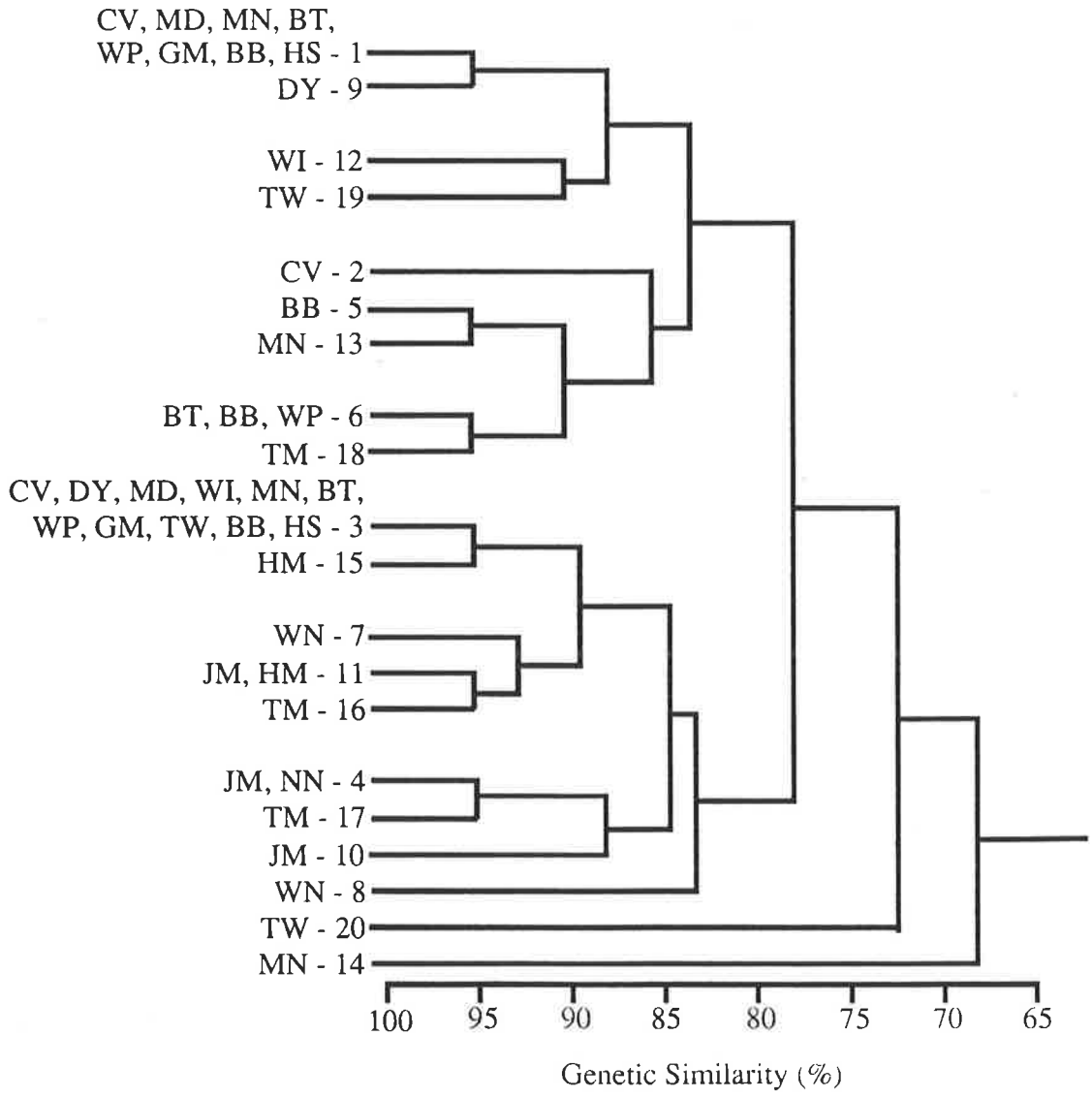
2	90.5									
3	90.5	90.5								
4	81.0	71.4	81.0							
5	90.5	81.0	90.5	81.0						
6	90.5	90.5	90.5	71.4	90.5					
7	76.2	76.2	85.7	85.7	76.2	76.2				
8	85.7	76.2	76.2	85.7	76.2	76.2	90.5			
9	95.2	85.7	85.7	85.7	85.7	85.7	71.4	81.0		
10	81.0	71.4	81.0	90.5	81.0	71.4	85.7	85.7	76.2	
11	81.0	81.0	90.5	90.5	81.0	81.0	95.2	85.7	76.2	90.5
12	90.5	81.0	90.5	81.0	90.5	81.0	76.2	76.2	85.7	81.0
13	85.7	85.7	95.2	76.2	95.2	95.2	81.0	71.4	81.0	76.2
14	71.4	71.4	71.4	71.4	61.9	71.4	66.7	66.7	76.2	71.4
15	85.7	85.7	95.2	85.7	85.7	85.7	90.5	81.0	81.0	85.7
16	76.2	76.2	85.7	85.7	76.2	76.2	90.5	81.0	71.4	85.7
17	76.2	66.7	76.2	95.2	76.2	66.7	81.0	81.0	81.0	85.7
18	85.7	85.7	85.7	66.7	85.7	95.2	71.4	71.4	81.0	66.7
19	90.5	81.0	81.0	71.4	81.0	81.0	66.7	76.2	85.7	71.4
20	66.7	66.7	76.2	76.2	66.7	66.7	81.0	71.4	61.9	76.2
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**

12	81.0								
13	85.7	85.7							
14	71.4	61.9	66.7						
15	95.2	85.7	90.5	66.7					
16	95.2	76.2	81.0	66.7	90.5				
17	85.7	76.2	71.4	66.7	81.0	90.5			
18	76.2	76.2	90.5	66.7	81.0	81.0	71.4		
19	71.4	90.5	76.2	71.4	76.2	66.7	66.7	76.2	
20	85.7	76.2	71.4	57.1	81.0	81.0	71.4	61.9	66.7
	11	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**

Phenotypes which exhibited the least similarity (57.1%) were MN14 and TW20. Phenotypes 1 and 3 were present in 8 and 12 of the populations respectively, and represented all of the MD, HS and GM isolates. The only populations not containing these phenotypes were HM, NN, TM, JM and WN.

Cluster analysis formed two primary groups of related phenotypes and two dissimilar individuals, namely MN14 and TW20 (Figure 7.5). When these two were

Figure 7.5. Dendrogram showing relationships between 23B repeat-sequence phenotypes detected in populations of *G. graminis*.



excluded from the analysis the average similarity between phenotypes was 78%, indicating very little differentiation of these families of repeat-sequences between populations. The two primary groups both contained nine phenotypes with an average within-group similarity of approximately 85%. All of the populations were represented on the primary cluster containing phenotype 3, whereas populations NN, HM, JM and WN were absent from the second primary cluster containing phenotype 1. There was no case where phenotypes from the same population formed highly related groups, which implied greater differentiation between families of repeat-sequences within, rather than between, some populations.

Relationships between the taxonomic identities of isolates and their respective 23B phenotypes are documented in Table 7.4 and Figure 7.5. The *Gga* isolates with phenotypes 4 (NN and JM), 11 (JM and HM), 16, 17 (TM), 7, 8 (WN) and 3 (DY and GM) all grouped into one of the primary clusters showing an overall genetic similarity of approximately 85%. These isolates then formed discrete clusters in which TM16, WN7, JM11 and HM11 were closely related to each other (93%). However, these *Gga* isolates showed greater similarities to *Ggt* than they did to *Gga* isolates NN4, JM4, 10, TM17 and WN8. The GM1, GM3, TW3, BB3, DY3, DY9 and WI12 *Gga* isolates were identical to the majority of *Ggt* isolates with these repetitive sequences.

7.4 Discussion

Low-copy RFLP markers provided few ambiguities in the identification of the taxonomic status of isolates and indicated that individual populations were dominated by either *Ggt* or *Gga*. Repeat-sequence phenotypes, were generally not conserved within individual varieties and were too variable to be useful in taxonomic identification within the species as a whole.

Genetic identities between populations at low-copy markers revealed no correlations between the geographical and genetic identities of populations, implying that the relationships between populations were based on similarities in their respective taxonomic compositions. Genetic similarities between repeat-sequence phenotypes

indicated a random distribution of variation between populations which generally did not agree with the relationships shown by low-copy variety-specific markers. The distinctions between these results are discussed below.

The frequencies of low copy RFLP phenotypes within populations varied between probe/enzyme combinations, implying that each probe targeted different genomic sequences. This was best illustrated in the CV, WP and TW populations (Table 7.1). If the probes were annealing to the same restriction fragments then the phenotypic frequencies would not differ. This distribution implied that the three different probes acted as independent markers in assigning taxonomic status to the population isolates.

Probes 4 and 23 also appeared to be hybridising to different families of repeat-sequences as may be inferred from the differences in diversity within populations: e.g. only 23B revealed variation among the twenty isolates within the JM population, whereas only 4H resolved variation within NN. (Table 7.6).

It has previously been shown that low-copy RFLP markers could be used for taxonomic classification of isolates (Chapter 4). The present results showed no populations with *Ggg*-specific markers, implying a low incidence of this variety under agricultural conditions. Consequently, isolates within pathogen populations have been identified as being either *Ggt* or *Gga*.

Of the seven probe/enzyme combinations used in this study only 30H was monomorphic across all 16 populations. The 6.68 kbp fragment resolved by this combination was detected in all three varieties of *G. graminis* in previous experiments, but was not detected in *Gga* strains isolated from *Agrostis* species (Chapter 4). Furthermore, combinations 5B and 202H did not resolve any phenotypes characteristic of the *Agrostis* isolates. This shows that these *Gga*-type isolates are absent from areas of cereal cultivation and implies that *Gga* may be differentiated into cereal and turf-grass pathotypes.

7.4.1 Partitioning of genetic diversity within and between populations of *G. graminis*

The total genetic diversity (Hsp) averaged across all polymorphic probe/enzyme combinations was 2.212, which indicated that *G. graminis* shows much greater levels of RFLP diversity than the outcrossing plant species *Rudbeckia missouriensis* (Hsp 0.202) (King and Schaal, 1989). Most of the diversity (76%) was apportioned between populations of *G. graminis*, implying that most of the populations are composed of high frequencies of a particular variety and do not contain them in equal proportions.

The conservation of low-copy RFLP phenotypes within varieties of the pathogen indicated that these markers are unlikely to detect much variation within populations but, if populations are composed of different frequencies of the respective varieties, considerable variation may be resolved. This was observed to be the case within the TW and WI populations which showed high levels of diversity with these markers.

Repeat-sequence probes have often been reported to reveal high levels of genetic variation between closely related organisms that could not be differentiated by other means (Gilbert *et al.*, 1990; Nybom *et al.*, 1990). This observation was supported by probes 4 and 23 used in this study. On average these probes revealed the highest levels of diversity within and between populations of *G. graminis*, indicating that most of the diversity can be attributed to variation in families of repetitive sequences.

Genomic rearrangements, such as transposition, gene conversion and unequal crossing over, are thought to be responsible for the generation of considerable diversity in repeat-sequence DNA and are believed to result in differentiation between isolated, inbreeding populations by the process of molecular drive (Dover, 1982, Flavell, 1986). Given the considerable diversity of repetitive sequences and the presence of unique phenotypes within populations, this process may be particularly relevant in accounting for genetic diversity within *G. graminis*.

Other factors which are known to affect levels of genetic diversity are associated with the evolutionary histories of the respective populations. For example, populations which have passed through an evolutionary bottleneck, causing a reduction in the

effective population size, are also expected to show low variability (Hartl and Clark, 1989). Reductions in population size may occur frequently within *G. graminis* due to intense host-mediated selection, resulting from cultivation of resistant hosts such as oats or rye. Populations NN and GM were recently exposed to intense selection via the cultivation of oats and had the lowest levels of genetic diversity. These populations are proposed to be composed entirely of *Gga* isolates, which is supported by the pathogenicity of NN isolates upon oats and fixation of RFLP phenotypes specific for this variety within both populations.

In general, populations with high frequencies of *Gga* also revealed low levels of genetic diversity. However, the HS and BT populations were dominated by *Ggt* and were genetically less diverse than the *Gga* populations TM and WN. This is probably due to the fixation of *Ggt* markers within the former populations whereas the latter two contained a few *Ggt* isolates (5% -10%) and therefore exhibited greater overall diversity.

Populations with the highest levels of diversity were isolated from wheat and barley and theoretically could contain all three varieties of *G. graminis*, since these hosts are susceptible to infection by either variety. These hosts would thus be expected to exert only low host-mediated selection and hence to support a genetically variable pathogen population. The three most variable populations were WI, TW and BB, each of which contained isolates representing both *Ggt* and *Gga*. The greater levels of variation within taxonomically diverse populations reflects the extent of genetic differentiation between *Ggt* and *Gga*.

The distribution of diversity between populations of *G. graminis* may provide clues to help determine the factors which have shaped the evolution of the species. Studies of genetic variation in some plant pathogenic fungi have indicated a division of pathogen populations into host-based groups, presumably resulting from host-mediated selection and infrequent sexual recombination between pathotypes (O'Dell *et al.*, 1989; Levy *et al.*, 1991). Host-mediated selection is assumed to be responsible for the high frequencies of *Gga* isolates within the NN and GM populations, but cannot be used to

explain the prevalence of this variety within the other populations dominated by *Gga*. It is possible that wild oats may have provided some selection pressure to these populations. At least two species are known to occur within the cereal belt of southern Australia (*Avena fatua* L. and *A. barbata* Brot.) and both were introduced into Australia from the Mediterranean region (Gardner, 1952). These species frequently occur as a component of self-sown pastures and also as weeds within cereal crops. *A. fatua* is known to be susceptible to *Gga* but is resistant to *Ggt* (Yeats *et al.*, 1986). Exposure of pathogen populations to these hosts may account for the high frequencies of *Gga* within populations not recently exposed to cultivated oats.

7.4.2 Genetic identities of *G. graminis* populations revealed by probes 5, 26, and 202

Cluster analysis of genetic identities between populations indicated that these probes identified isolates as either *Ggt* or *Gga*, with few ambiguities. The populations clustered primarily on the basis of their taxonomic composition, not upon similarities in geographical origin. The distribution of these RFLP phenotypes and the topography of the dendrogram implied that both varieties were widespread throughout the cereal growing regions of southern Australia.

Populations of *G. graminis* were divided into two principal clusters, one dominated by *Gga* and the other dominated by *Ggt*. The high genetic identities between populations within each of the principal clusters indicated an overall lack of genetic differentiation within each variety and confirmed that the constituent populations were dominated by only one of the respective taxonomic varieties. The presence of isolates within populations which were clearly identified as different varieties, supports the hypothesis that inter-variety hybridisation between *Gga* and *Ggt* is rare or non-existent (Chapter 2).

7.4.3 Genetic similarities between populations estimated from repetitive sequences

Genetic identities estimated from repetitive sequences showed different relationships between the populations to those observed with the variety-specific RFLP

probes (compare Figure 7.3 with Figures 7.4 and 7.5). There appeared to be no simple correlation between the low-copy and repeat-sequence RFLP markers in discriminating between varieties of *G. graminis*. Some isolates, identified from the low-copy probes as different varieties, resolved identical repeat-sequence phenotypes (e.g. 4H phenotypes BB6 and WI17). Furthermore, some phenotypes were present in populations which were dominated by different varieties of *G. graminis*, e.g. phenotype 3 (23B) was present in 12 populations representing both *Ggt* and *Gga*.

Combination 4H did not reveal a simple marker (i.e. one particular phenotype) for distinguishing *Gga* from *Ggt*. It did, however, reveal closer relationships between most *Gga* isolates than those observed with 23B and in general, differentiated between varieties existing within individual populations. Intra-population similarities of 4H *Gga* phenotypes were quite high and were generally greater than those observed between *Ggt* isolates within populations. Conversely, inter-population comparisons between *Gga* phenotypes revealed considerable genetic differentiation between some populations.

Cluster analysis of genetic similarities between repeat-sequence phenotypes generally indicated that populations did not group either according to their taxonomic status or the geographic similarities between populations (Figures 7.4 and 7.5). Isolates identified as *Gga* often showed closer genetic similarities to *Ggt* isolates than to other *Gga* isolates. This was evident in the high genetic similarities between populations composed of different varieties of *G. graminis* and the separation of isolates which were monomorphic at other RFLP markers, into discrete highly differentiated repeat-sequence phenotypes. These repeat-sequence probes did not provide any markers which could be used to differentiate between the different varieties of *G. graminis*.

The inability of 4H and 23B repeat-sequence phenotypes to identify varieties of *G. graminis* contrasts with the study by O'Dell *et al.* (1992) where ribosomal DNA repeat-sequences were useful in the discrimination of *Ggt* and *Gga*. Given these results and the numerous other reports of ribosomal probes differentiating closely related fungal taxa

(Vilgalys and Gonzalez, 1990; Priestley *et al.*, 1992), it is unlikely that the repeat-sequence probes used in this study represent ribosomal DNA repeats.

Genetic similarities between repeat-sequence phenotypes tend to deny the genetic distinctness and apparent reproductive isolation of the varieties and imply that *G. graminis* may represent a single panmictic population (Prodohl *et al.*, 1992). This is most apparent with phenotypes 1 and 3 resolved by combination 23B. These two phenotypes were present in 8 and 12 of the populations respectively, representing the geographic range of the species within Australia. This implied that there may be substantial migration of isolates between geographically isolated populations. In contrast, the absence of these phenotypes from other populations, which originated from the same geographical region, denies the likelihood of frequent inter-population gene flow.

The lack of correspondence between the genetic similarities and geographical origins of many populations indicated that variation in repeat sequences was randomly distributed between populations. This implied that populations may be evolving in relative isolation and was supported by the evolution of large numbers of population-specific phenotypes. Repeat sequences are often heterochromatic regions of DNA and they may accumulate mutations and exhibit variation which has little effect on the overall phenotype of the organism (Doolittle and Sapienza, 1980). Consequently, repeat sequences are generally not subject to strong selection, implying that variation may be maintained within populations and result in divergence between populations.

Variation in families of repeat sequences is generated by mechanisms of genome rearrangement and can occur independently within different individuals. Fixation of these variants can occur relatively rapidly in evolutionary time, but requires a large number of generations within interbreeding populations (Dover, 1982). However, in haploid organisms, where the primary mode of reproduction is asexual or occurs via self-fertilisation, repeat-sequence variants may accumulate rapidly, resulting in the predominance of individual clonal lineages within populations (Hartl and Clark, 1989; Tibayrenc *et al.*, 1991). Since *G. graminis* is haploid and self-compatible, the random

generation of variation by genomic rearrangements may account for the presence of a diversity of repeat-sequence phenotypes within and between populations. Therefore, it is possible that the identical phenotypes observed between populations may have arisen independently and may not have resulted from inter-population migration. Independent origins of these phenotypes may also account for the identical repeat-sequence phenotypes observed between *Ggt* and *Gga* isolates.

The results of RFLP analyses indicate that *G. graminis* is highly variable both within and between populations. The extent and distribution of genetic diversity between populations supports the hypotheses that (i) host-mediated selection is important in determining the taxonomic composition of populations, (ii) the predominant mode of pathogen reproduction is via asexual reproduction or self-fertilisation and (iii) inter-population gene flow is infrequent (Chapter 2). This implies that populations of *G. graminis* may be composed of a series of clonal lineages in which variation is predominantly generated by simple mutation and genomic rearrangements. Furthermore, the lack of gene flow suggests that the populations may be evolving in relative isolation.

The abilities of repeat sequences to differentiate between taxonomic varieties of *G. graminis* appeared to be population-dependent and indicated that they cannot be applied as taxonomic markers within the species as a whole. Low-copy RFLP phenotypes, however, provided an assessment of the taxonomic composition of pathogen populations and indicated that both *Ggt* and *Gga* were widespread throughout the cereal belt of southern Australia. The absence of phenotypes common to *Gga* isolates originating from *Agrostis* species implied that this variety may be differentiated into two pathotypes which are adapted to turf grasses and cereals, respectively. *Ggg* may be absent from pathogen populations and probably represents only a minor component of the total species diversity in regions under intensive cereal production. Previous experiments have indicated that PCR provided a rapid method for distinguishing between varieties of *G. graminis* (Chapter 5) and should confirm the relative frequencies of each variety within pathogen populations.

Chapter 8 Use of the polymerase chain reaction to determine the extent and distribution of genetic diversity between populations of *G. graminis*

8.1 Introduction

Previous experiments have indicated that sequences amplified by the intron splice junction primer R1 provided markers to discriminate between the varieties of *G. graminis* (Chapter 5). This technique provides a rapid and relatively inexpensive method to determine the taxonomic composition of *G. graminis* populations. Additionally, PCR revealed considerable diversity between isolates which was not detected with other DNA markers and therefore should provide a more definitive assessment of diversity within pathogen populations. In this respect primer R1 has the advantage over DNA repeat-sequence phenotypes in that it can detect variation between closely related isolates whilst still being capable of determining the taxonomic status of isolates.

The primary aim of this study was to assess the applicability of PCR with primer R1 to determine the taxonomic composition of *G. graminis* populations. Comparing these results with those from the RFLP analyses should provide an accurate description of distribution of the different varieties within and between populations. A secondary aim was to assess the ability of the technique to detect variation within populations which were generally invariant with other genetic markers.

To meet these aims, four populations were selected for PCR analysis on the basis of their proposed taxonomic composition and levels of genetic variability at RFLP loci. The JM and GM populations were observed to exhibit generally low variation at isozyme and RFLP loci and all isolates within these populations were classified as *Gga*. This was shown by the fixation of RFLP variety-specific markers within these populations. The HS population also exhibited extremely low within-population diversity and was fixed for the presence of the *Ggt*-specific RFLP markers. The WI

population was observed to be highly variable for the RFLP analyses and was included to provide a comparison of the relative abilities of the different techniques to document variation between isolates. RFLP variety-specific markers indicated that 10% of the population (i.e. 2 isolates) was classified as *Gga*. PCR was used to clarify the identity of these isolates and to provide further evidence of a correlation between the variety-specific DNA markers.

8.2 Materials and methods

8.2.1 Source of isolates, DNA extraction and PCR analysis

Details of the geographical origins of the populations and the respective host species are listed in Table 6.1. *G. graminis* isolates used in these experiments were grown in liquid culture by the methods described in Chapter 3. Methods for DNA extraction and PCR were as outlined in Chapters 4 and 5 respectively.

PCR products of isolates previously identified as *Ggt* and *Gga* were included on each gel and acted as positive controls to identify the presence of the variety-specific amplification products within population samples (Chapter 5). In addition, one isolate randomly selected from each of the 16 populations, was subjected to PCR. The results were used to confirm the taxonomic classification of these isolates within their respective populations.

8.2.2 Data analysis

Amplification profiles were compared between all isolates to determine the number of R1 PCR phenotypes and their distributions among populations. Frequencies of these phenotypes were calculated and used to determine genetic diversity within and between *G. graminis* populations using Shannon's diversity index (Chapter 3).

An estimate of the genetic similarity between R1 phenotypes of every pair of isolates within a population was made using the similarity coefficient (Chapter 3). The similarity matrices from the individual populations were then combined to calculate a

between-population similarity matrix. This analysis is analogous to Nei's genetic identity between populations (Chapter 6). A cluster analysis was performed to illustrate the genetic similarities between populations and the results presented in a dendrogram. Methods of analysis are as described in Chapter 3.

8.3 Results

8.3.1 Application of PCR amplification products as taxonomic markers

Primer R1 generated amplification products of varying sizes which detected polymorphisms both within and between populations of *G. graminis* (Figures 8.1 and 8.2). The JM population (Figure 8.1) was monomorphic for the 1.30 kbp band characteristic of *Gga* together with the 500 and 550 bp bands characteristic of *Ggt*. All isolates within the GM population were also characterised by the amplification of the 1.30 kbp and 500 bp products and all but two (GM 21 and 29) by the 550 bp bands (data not shown).

The WI population (Figure 8.2) contained three isolates (numbers 5, 11 and 12) which showed the 1.30 kbp band. Isolates 5 and 11 also showed a band of approximately 700 bp. Most of the WI isolates showed the 500 and 550 bp products. The high molecular weight bands (i.e. greater than 1.70 kbp) were removed from the analysis as they were not amplified consistently in repeated experiments. All isolates within the HS population showed amplification of the 500 and 550 bp products in the absence of the 1.30 kbp band.

Individual isolates were selected at random from each of the remaining 16 populations. The 1.30 kbp product was amplified in isolates NN 17, WN 22, DN 11, JM 22, TM 27 and GAN 1 but not in any of the isolates from the other populations. Every individual exhibited one or both of the 500 and 550 bp products.

Figure 8.1. PCR products generated with intron splice junction (ISJ) primer R1 from 20 *G. graminis* isolates within the JM population. Lanes: M, λ dVI *Hae* III molecular size standards; 2-21, JM 1-20. Sizes of products are in base pairs (bp).

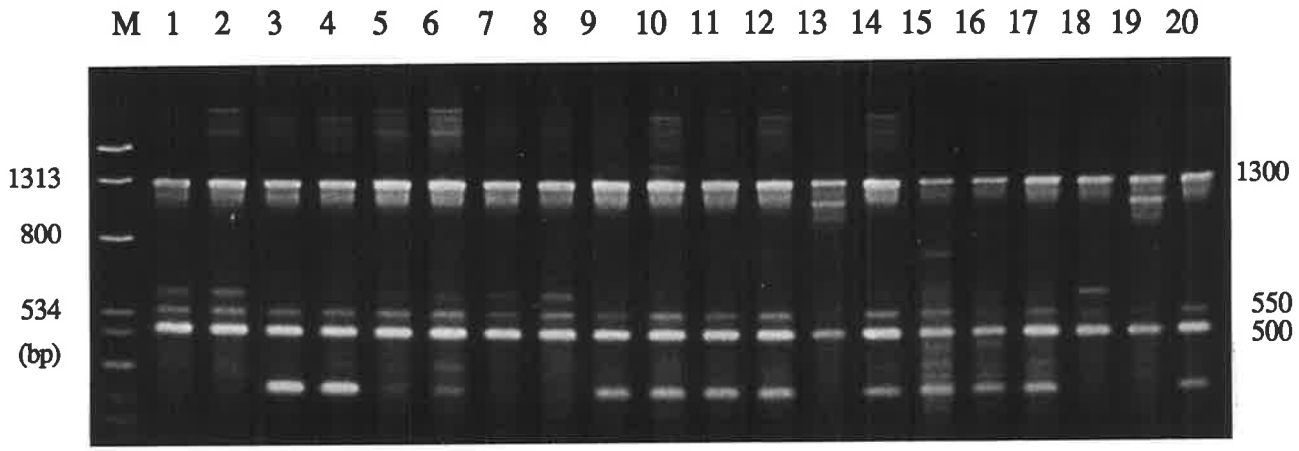
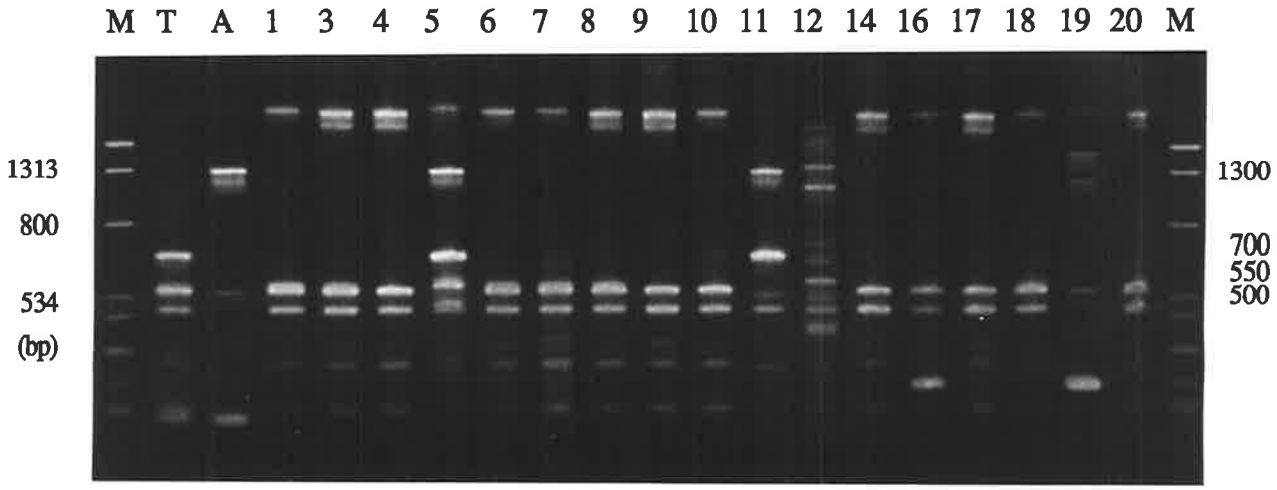


Figure 8.2. PCR products generated with intron splice junction (ISJ) primer R1 from 17 *G. graminis* isolates within the WI population. Lanes: M, λ dVI *Hae* III molecular size standards; T, *Ggt*- 800; A, *Gga*- 192M; WI 1, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 16, 17, 18, 19, 20. Sizes of products are in base pairs (bp).



8.3.2 Partitioning genetic diversity within and between populations of *G. graminis*

The number of the nine R1 phenotypes found within each of the four populations ranged between two (HS) and four (WI) (Table 8.1). Six phenotypes were found to be unique to a particular population e.g. phenotypes 1 and 8 were unique to the JM and GM populations respectively. The frequencies of the nine phenotypes were calculated within the entire collection of 71 isolates (representing the four populations) with phenotype 2 being the most common (32%) and phenotype 7 the least (1.4%). No phenotype was found in more than two populations, although many of the individual amplification products were.

Twelve different PCR phenotypes were detected amongst the 16 isolates randomly sampled from each population. The number of bands per isolate ranged from 1 (550 bp product in BT 9) to 7 (HM 22 and TW 28). Isolates WI 4, MN 15, HB 31 and WP 23 were characterised by phenotype 4 from the population analysis (Figure 8.2, Table 8.1). HM 22 and TW 28 were identical in their amplification profiles but this phenotype was not detected in the population analysis.

Table 8.1. Frequencies of DNA amplification phenotypes within 4 populations of *G. graminis*, generated by PCR with the intron splice junction primer R1.

Phenotype	Population				Sum
	JM (20 isolates)	WI (17)	HS (17)	GM (17)	(71)
1	0.350				0.100
2	0.550			0.706	0.324
3	0.100			0.176	0.070
4		0.705	0.176		0.211
5		0.118			0.028
6		0.118			0.028
7		0.059			0.014
8				0.118	0.028
9			0.824		0.197

The WI and JM populations showed the greatest within-population diversity and the HS population the least (Table 8.2). Genetic diversity detected within the GM population was nearly equivalent to that observed in the JM and WI populations. The overall diversity detected within the entire group of 71 isolates (Hsp) was more than double the average level of diversity observed within populations (Hpop) (Table 8.2). Fifty five percent of the genetic diversity was apportioned between these four populations.

Phenetic analysis revealed that the average similarity between these four populations was approximately 63%. The greatest similarity was observed between the HS and WI populations (80%) and the least between JM and WI (55%) (Table 8.3). Hierarchical clustering of these similarity values showed that the GM and JM populations were more closely related to each other than either was to the HS or WI populations (Figure 8.3).

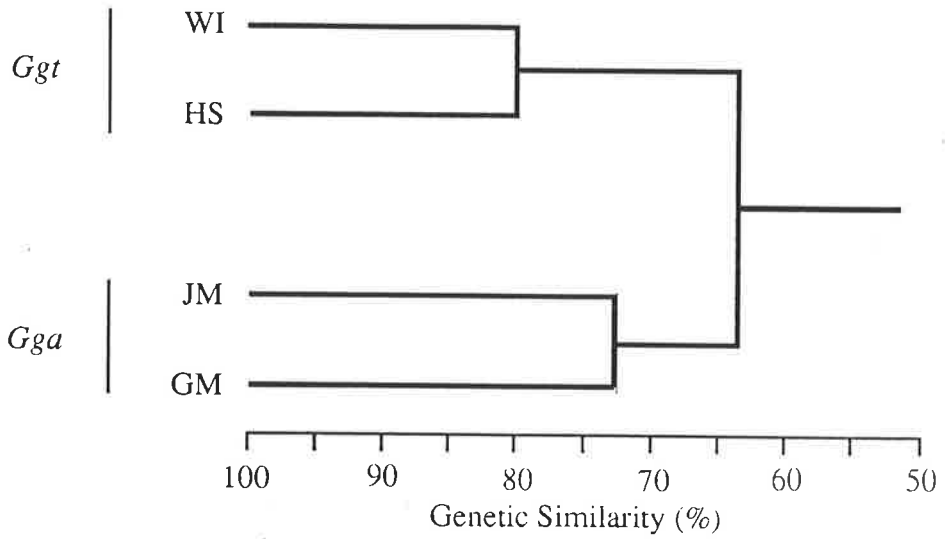
Table 8.2. Estimates of genetic diversity (H_0) within and the partitioning of the diversity between populations of *G. graminis*.

Population				Average within populations	Total within species	Proportion within populations	Proportion between populations
JM	WI	HS	GM	Hpop	Hsp	$\frac{Hpop}{Hsp}$	$\frac{Hsp-Hpop}{Hsp}$
1.337	1.338	0.680	1.173	1.132	2.539	0.446	0.554

Table 8.3. Genetic similarity (%) between populations of *G. graminis* based on the total amplification products resolved with primer R1.

HS	74.0		
JM	72.5	55.4	
WI	70.0	79.9	54.7
	GM	HS	JM

Figure 8.3. Dendrogram of genetic similarities between populations of *G. graminis* derived from the total amplification products of intron splice junction (ISJ) primer R1.



8.4 Discussion

8.4.1 Application of PCR amplification products as taxonomic markers

These results indicated that PCR confirmed the taxonomic compositions of *G. graminis* populations. The JM and GM populations both consistently presented the 1.30 kbp product characteristic of *Gga* together with the 500 and 550 bp products observed in the majority of *Ggt* isolates. The combination of these bands produced phenotypes

which were most similar to *Gga* isolate 35793 (Chapter 5) which was originally isolated from oats. The hypothesis that the GM and JM populations were entirely composed of *Gga* was supported by the results of the 3 isozyme and 4 RFLP taxonomic markers (Chapters 6 and 7). The HS population was monomorphic for the *Ggt* PCR markers which confirmed the results of the RFLP and isozyme analyses.

The WI population contained three isolates in which the 1.30 kbp *Gga* product was detected. The remaining isolates all showed PCR phenotypes characteristic of *Ggt*. RFLP phenotypes detected with probe/enzyme combination 26H identified all WI isolates as *Ggt* whereas the other RFLP markers classified isolates 5 and 11 as *Gga*. Isolates 5 and 11 also showed amplification of a 700 bp product, shown to be specific to *Ggt* isolate 800 (Chapter 5). The lack of a complete correlation between the different molecular taxonomic markers indicates that the distinction between varieties within this population is not complete. It is possible that sexual recombination between varieties within the WI population may account for the inconsistencies between the RFLP and PCR markers, but no isolates were observed to contain the 700 bp *Ggt* product without the 1.30 kbp *Gga* product. Therefore, from this sample of isolates it is impossible to identify the parental genotypes which may have produced these putative hybrid strains. Further detailed sampling at this location may determine if the parental phenotypes exist in low frequency within the population. Given the apparently low frequency of outcrossing within *G. graminis* it is likely that similar polymorphisms may have arisen independently within each variety or that the *Gga* isolates may have been recent introductions into the population from another location.

Further evidence to support the PCR products of primer R1 as variety-specific markers is shown in Table 8.4. HM 22 was the only one of these isolates which had been previously identified as *Gga* (by the variety-specific RFLP markers) to lack the 1.30 kbp PCR band. Isolates NN 17, WN 22, DY 11, JM 22 and GM 1 showed a complete correlation between the respective variety-specific markers. Isolates from the remaining populations showed amplification of one or both of the 500 and 550 bp products, which in the absence of the 1.30 kbp product, was diagnostic for *Ggt*. The

Table 8.4. Classification of isolates of *G. graminis* based upon variety-specific PCR products and RFLP phenotypes.

Isolate	Host	PCR	Probe/enzyme combination			
		Primer R1	5/BamH I	26/BamH I	26/Hind III	202/Hind III
CV 27	wheat	T	T	T	T	T
NN 17	oats	A	A	A	A	A
BB 26	barley	T	T	T	T	T
WN 22	wheat	A	A	A	A	A
DY 11	wheat	A	A	A	A	A
JM 22	wheat	A	A	A	A	A
MD 28	wheat	T	T	T	T	T
WI 4	wheat	T	T	T	T	T
MN 15	wheat	T	T	T	T	T
BT 9	wheat	T	T	T	T	T
HM 22	wheat	T	A	A	A	A
HB 31	barley	T	T	T	T	T
WP 23	wheat	T	T	T	T	T
TW 28	wheat	T	T	T	T	T
TM 27	wheat	A	A	A	A	A
GM 1	wheat	A	A	A	A	A

T: *Ggt*, A: *Gga*.

identity of these isolates as *Ggt* is supported unequivocally by the variety-specific RFLP phenotypes (Table 8.4).

As discussed previously (Chapter 7), RFLP phenotypes detected with combinations 5B and 202H implied that *Ggg* isolates were absent from the population samples. This was confirmed in this study, where every isolate examined showed amplification of PCR products specific for either *Ggt* or *Gga*.

The taxonomic identities of isolates determined with PCR agreed with their classifications at RFLP markers. Only two isolates showed anomalies in this otherwise

perfect correlation between the different markers, WI 12 and HM 22. WI 12 was classified as *Ggt* by the RFLP markers and showed a band slightly larger than the 1.30 kbp product observed in other *Gga* isolates. The larger band may represent a different product, which could be determined by isolating the 1.30 kbp band from an unambiguously identified *Gga* isolate and using it as a probe to check for homology with the band resolved by isolate WI 12. Amplification of this product was weaker compared to all other *Gga* isolates. Increasing the stringency of the PCR by raising the primer annealing temperature or lowering magnesium ion concentrations (Henson, 1992) may result in the elimination of this aberrant band, and assist in confirming the taxonomic identity of this isolate.

The majority of isolates (95%) within the HM population, including HM 22, were identified as *Gga* by RFLP markers (Chapter 7), but this isolate, the only one tested from this population, lacked the PCR product characteristic of *Gga*. Therefore, HM requires more detailed RFLP and PCR analysis before its taxonomic composition can be determined with confidence.

It may be possible to increase the sensitivity and accuracy of taxonomic identification within *G. graminis* by sequencing the relevant PCR products. This may allow the design of specific primers for these loci rather than relying on the sequences of the exon regions flanking the intron splice junctions. Products derived from sequences unique to a particular variety should provide identification of isolates whose classification may have been difficult with other markers (e.g. HM 22, WI 12). The use of primer R1 in initial rounds of amplification, followed by the addition of the specific primers derived from internal sequences of the products, should produce bands which accurately identify the taxonomic status of isolates. Such an approach was used by Schesser *et al.* (1991) in attempts to differentiate between *G. graminis* and other root-infecting fungi, which showed non-specific amplification of products using primers derived from a *G. graminis*-specific mitochondrial DNA clone (Henson, 1989).

8.4.2 Partitioning genetic diversity within and between populations of *G. graminis*

Most of the diversity (55%) was apportioned between populations, which probably reflects the number of population-specific phenotypes resolved in the analysis. Other phenotypes were conserved between populations dominated by a particular pathogenic variety of *G. graminis*. For example, both the WI and HS populations contain high frequencies of *Ggt*, most of which are characterised by R1 phenotype 4 in the former but are less frequent in the latter population. The distinctions between the two populations may reflect host-mediated selection of pathogen genotypes better adapted to parasitising wheat (WI) relative to barley (HS) as was indicated in the isozyme and RFLP analyses (Chapters 6 and 7). Determination of R1 phenotypes within the Boyup Brook (BB) and Monarto (MN) populations, both thought to have undergone some host-mediated selection resulting from growth on barley, may indicate whether there is a conservation of R1 phenotypes between these populations.

Cluster analysis indicated that inter-population relationships were based upon the taxonomic composition of the populations rather than the geographical identities of the locations (Figure 8.3). The overall genetic similarity between the four populations was approximately 65%, which was almost identical to that observed between isolates of *Ggt* and *Gga* in previous experiments (Chapter 5). This similarity provides further support for the separation of the populations into two groups, based upon the taxonomic identities of their respective pathogen genotypes.

PCR products were derived from exon regions near the intron splice junction, some of which may have been derived from sequences which are highly conserved within varieties of *G. graminis*. The taxon-specific amplification products may be examples of these conserved sequences whereas the variable products may be derived from intron sequences not under strong selection. Many of the R1 phenotypes within populations were observed to differ by the presence or absence of a few non-specific bands, indicating that duplications and/or deletions of intron sequences may be responsible for most of the variation between isolates within a variety. If this variation is non-deleterious, variant isolates may increase in frequency within populations due to the

production of large numbers of progeny resulting from self-fertilisation or asexual reproduction (Chapter 2). Once established within the population these variants may fluctuate in frequency depending upon their relative capacities for saprophytic survival and competitive abilities for a diversity of host resources.

The results of PCR experiments complemented the isozyme and RFLP analyses in describing the taxonomic composition of *G. graminis* populations. In doing so PCR provided a rapid and efficient method for the classification of isolates to their taxonomic status. PCR detected high levels of genetic diversity within populations and was capable of discriminating between closely related isolates which were invarient at other markers. Consequently, PCR may be used to determine the fine scale genetic structure of pathogen populations and to follow the dispersal and survival of individuals within and between locations.

Chapter 9 General Discussion

9.1 Molecular markers for the identification of taxonomic varieties within *G. graminis*

The results of this research clearly showed that molecular techniques can be used to discriminate between the taxonomic varieties of the take-all fungus *G. graminis*. Early experiments to identify variety-specific markers indicated that isozyme, RFLP and PCR analyses were capable of discriminating *Gga* from *Ggt* and *Ggg*. Isozyme markers however, could not clearly differentiate between *Ggt* and *Ggg* isolates.

The taxonomic composition of pathogen populations was determined on the basis of RFLP phenotypes resolved with low-copy genomic probes and also by PCR-directed amplification of sequences derived from intron-exon splice junctions. Isozyme and DNA repeat-sequence phenotypes were found to be of only limited value in the taxonomic identification of pathogen isolates, since both methods proved to be highly variable and resolved markers which were not detected in earlier screening experiments. Nevertheless, a limited number of isozyme alleles provided putative identifications of isolates, with the classifications generally confirmed by low-copy RFLP and PCR analyses.

Population analyses indicated that both *Ggt* and *Gga* were widespread throughout the cereal producing regions of southern Australia. RFLP markers used to identify *Gga* were detected in a strain originally isolated from oats, whilst those found in *Gga* parasitising turf grass (*Agrostis* spp.) were not detected in populations isolated from cereals. This implied that there may be differentiation within *Gga* into pathotypes which are better adapted to parasitising these respective host species. RFLP and PCR analyses indicated that *Ggg* was absent from the population samples and may only occur in low frequency in areas under intensive cereal production.

The absence of *Ggg* from populations isolated from wheat and barley was unexpected, given that this variety is capable of infecting these hosts even though it is weakly pathogenic in comparison to *Ggt* and *Gga* (Walker, 1981). The greater pathogenicity of *Ggt* and *Gga* implies a greater degree of host specialisation than may be expected to have evolved within *Ggg*, so the competitive abilities of *Ggg* for cereal host resources may be less than those of *Ggt* and *Gga*. Intensive cereal cropping may cause the frequency of *Ggg* to decrease over time, leading to a prevalence of *Ggt* and *Gga* within populations. Analysis of populations isolated from non-agricultural regions (e.g. undisturbed grasslands) may indicate the importance of host susceptibility in determining the taxonomic composition of *G. graminis* populations.

The frequencies of *Ggt* and *Gga*, averaged across all isolates, were equivalent. Populations which were isolated from or had a recent exposure to oats contained high frequencies of *Gga*, whereas populations exposed to wheat were dominated by either *Gga* or *Ggt*. Those isolated from barley were composed almost exclusively of *Ggt*. Many of the populations had a mixed taxonomic composition, consisting of both varieties, but they were not found to coexist in equivalent frequencies within the one location, one variety always being dominant over the other.

This distribution of pathotypes implies that if host genotype can exert intense selection upon the pathogen population, rapid changes in the taxonomic composition of pathogen populations may occur. For example, cultivation of oat appeared to select *Gga* types and suppress those of *Ggt*, due to the relative susceptibilities of this host to these two varieties. Under these conditions the frequency of *Gga* would be expected to increase relative to that of *Ggt*. Cultivation of wheat in following seasons would then provide a susceptible host and provide for increases in the inoculum levels of both varieties. Therefore, taxonomic composition of pathogen populations appears to be strongly influenced by host-mediated selection pressures.

The ability to discriminate between taxonomic varieties of the pathogen existing within the one location implies that sexual hybridisation between the respective varieties must be a rare event. Further evidence to support the relative isolation of each

variety was the high genetic similarity observed between *Ggt* isolates from Australia and Eire. Cluster analyses with isozyme, RFLP and PCR markers consistently grouped EBI (Eire) with Australian *Ggt* isolates and EBI showed RFLP and PCR phenotypes which were identical to many of the Australian isolates. Similarly, *Gga* isolates from Britain and New Zealand showed a high genetic similarity to each other (O'Dell *et al.* 1992). The similarities between these geographically disjunct isolates representing one variety were greater than those between varieties from the same region.

It is unlikely that these genetic similarities reflect limited divergence due to a recent introduction of *G. graminis* into Australia at the time of European settlement. The pan-continental distribution of the pathogen and the reports of severe take-all in wheat crops in the first season of production following the clearing of natural vegetation, imply that the species is endemic to the Australian region (Garrett, 1981; Cotterill and Sivasithamparam, 1989). The ability to discriminate between varieties of the pathogen from diverse locations, implies that these techniques could have applications in epidemiological studies of *G. graminis* internationally.

9.2 Partitioning genetic diversity within and between populations of *G. graminis*

Molecular analyses revealed high levels of genetic variation within and between populations of *G. graminis* isolated from three cereal host species. The total gene diversity (D_{sp}) within the 480 isolates (assessed from variation at isozyme loci) was 0.422 which is higher than values obtained for the plant pathogens *Magnaporthe grisea* (0.030) (Leung and Williams 1986), *Atkinsonella hypoxylon* (0.229) (Leuchtmann and Clay, 1989) and *Leptographium wageneri* (0.227) (Zambino and Harrington, 1989), but lower that observed within the nitrogen-fixing bacterium *Rhizobium leguminosarum* (D_{sp} 0.50) (Harrison *et al.*, 1989).

The total genetic diversity (H_{sp}) averaged across all polymorphic probe/enzyme combinations in the RFLP analysis was 2.212. This value is approximately double that revealed with isozyme loci (1.001) even though significantly fewer isolates were

examined in the RFLP analysis. This probably reflects the relatively slow evolutionary rates of the protein-coding sequences relative to some of the sequences detected by RFLP analyses, in particular the DNA repeat sequences (Brown and Weir, 1983).

The total genetic diversity (H_{sp}) detected with PCR was 2.539, a high value considering that only one primer was used. A comparable level of variation (Hsp 2.976) was detected in an analysis of the outbreeding tree species *Gliricidia sepium*, using nine random PCR primers (Chalmers *et al.*, 1992). PCR revealed much greater levels of diversity (H_{pop} 1.132) compared with those shown by isozyme (H_{pop} 0.126) and RFLP (H_{pop} 0.355) analyses. The differences between the respective techniques probably reflects the ability of primer R1 to target multiple intron regions within the genome, which are known to be highly variable both in sequence and length. This would lead to the simultaneous identification of multiple polymorphisms between isolates (Hawkins, 1988; Weining and Langridge, 1991).

Isozyme and RFLP analyses produced similar rankings of populations with respect to their overall levels of genetic diversity. In general, populations which had a mixed taxonomic composition but were dominated by *Ggt* were more variable (e.g. WI, TW) whereas those populations which contained high frequencies of *Gga* were less diverse (e.g. NN, GM). Associations between pathogen populations and a resistant host (oat) would be expected to apply intense host-mediated selection, which has resulted in the selection of a few homogeneous *Gga* pathogen genotypes.

There was, however, an outstanding contradiction between the two techniques. HM was the most diverse population at isozyme loci (H_0 0.741) and yet was generally invariant at RFLP markers (H_0 0.272). The lower RFLP diversity was consistent with the majority of the population (95%) being classified as *Gga*, with the variation at each marker being attributed to the presence of a single *Ggt* isolate within the population. The taxonomic composition of this population was unexpected, since HM had a long-term association with a range of grass hosts and was assumed to have evolved in the absence of intense host-mediated selection. The cropping history and extensive isozyme diversity of this population may indicate that populations of *Gga*, which have not been

exposed to intense selection, may remain genetically diverse. This could be checked by assessing levels of genetic diversity within *Gga*-type populations from native grasslands, exposing them to selection by a variety of cereal hosts and following changes in the genetic composition of the populations over time.

Differentiation of pathogen populations into host-based groups has been postulated to have occurred within a number of plant pathogens including *Erysiphe graminis* (O'Dell *et al.*, 1989), *Magnaporthe grisea* (Levy *et al.*, 1991) and *Pseudocercosporella herpotrichoides* (Priestley *et al.*, 1992) and is likely to be a common evolutionary consequence of associations between pathogens and their hosts. Host-mediated selection and reproductive isolation probably accounts for the degree of differentiation observed between varieties of *G. graminis* and accordingly for the populations being dominated by the respective varieties. Consequently, the genetic identities and distributions of host species are important factors determining the taxonomic composition and genetic diversity of pathogen populations.

Studies of natural plant populations have shown that self-fertilisation results in increased genetic variability between populations (Loveless and Hamrick, 1984). Isozyme, RFLP and PCR analyses indicated that most of the diversity (60%, 76% and 55%, respectively) was apportioned between populations of *G. graminis*. This distribution of variation supports the assumption that self-fertilisation may be the primary mode of reproduction, implying that *G. graminis* does not represent a panmictic population within southern Australia. Instead, populations appear to be evolving in relative isolation in the absence of high levels of inter-population gene flow. If gene flow is considerably restricted, then much of the differentiation between populations may be attributed to genetic drift (Aquadro, 1992).

RFLP analysis revealed that most of the genetic diversity between populations was attributed to variation in repetitive sequences. These sequences are a common feature of many eukaryotic genomes and are thought to evolve more rapidly than single-copy sequences by processes of genome rearrangement (Dover, 1982; Flavell 1986; Chapter 2). Given the nature of these processes, repeat-sequence variation may frequently arise

independently and if variants are not subject to strong selection they may spread through interbreeding pathogen demes as a result of molecular drive (Dover, 1982). Thus, groups of individuals which belong to separate populations due to reproductive or geographical isolation accumulate different variants of repeat sequences. These processes may account for the high levels of repeat-sequence diversity resolved within *G. graminis*. The evolution of repeat-sequence phenotypes which were unique to specific populations may be a consequence of the lack of gene flow between populations.

Most of the RFLP variation within populations was apportioned as a limited number of repeat-sequence variants, implying that significant outcrossing and recombination of the loci is probably rare (Dover, 1982). A number of independent variants could accumulate within populations through isolates producing large numbers of progeny (ascospores and conidia). This implies that populations may be composed of a series of clonal lineages of isolates, which may fluctuate in frequency due to the effects of host-mediated selection. Pathogenic potential and the ability to survive saprophytically in the absence of a compatible host, appear to be important factors determining the evolutionary dynamics of *G. graminis* populations.

Analysis of repeat-sequence variation allows for the detection of diversity at a fine scale and may provide the opportunity to determine changes in pathogen populations in response to a variety of selective pressures. For example, exposure of pathogen populations to different host species and monitoring their genetic composition over consecutive years may determine the rates with which host-adapted lineages develop. Such studies are crucial in developing an understanding of the epidemiology of take-all and should indicate the evolutionary capacity of the pathogen to respond to control methods.

9.3 Phenetic relationships within *G. graminis*

Determination of the genetic similarities between varieties of *G. graminis* indicated that isolates of *Ggt* and *Gga* formed their own discrete groups. In contrast, *Ggg* was

subdivided into two groups of isolates, one showing a greater similarity to *Ggt* than to *Gga* and another group clearly distinct from these two varieties, implying that *Ggt* and *Gga* may have been derived from *Ggg* if the species had a monophyletic origin. This has also been suggested by Walker (1981), on the basis of morphological and pathogenic similarities between *Ggt* and *Gga* which are not shared with *Ggg*. Furthermore, it is possible that *Gga* may represent a subgroup of genotypes which were derived from the genetically variable progenitor populations of *Ggt*. Associations between the pathogen and a resistant host (oat) would be expected to apply strong host-mediated selection, which may have resulted in the selection of a genetically restricted pathogen genotype, namely *Gga*. The low diversity within this variety would be maintained through the predominance of self-fertilisation (Asher, 1981, Blanch *et al.*, 1981), thereby preventing inter-varietal hybridisation and the generation of diversity.

The isozyme and low-copy RFLP dendrograms (Figures 6.2 and 7.3) were nearly identical in their topology and revealed two distinct evolutionary lineages within *G. graminis*. The populations known to contain high frequencies of *Gga* or *Ggt* formed discrete variety-specific groups with both isozymes and RFLPs, which provided further support for their genetic isolation. PCR analysis also indicated that inter-population relationships were based upon similarities in their respective taxonomic compositions (Figure 8.3). The lack of a correlation between genetic and geographical identities of populations implied that geographically restricted pathotypes have not evolved within Australia.

High genetic identities between populations isolated from, or recently exposed to, the same host species supports the hypothesis that host-mediated selection is a major factor affecting the distribution and relative frequencies of the different varieties, within and between populations. Populations isolated from, or recently exposed to, barley also showed high genetic identities at RFLP and isozyme loci, implying that selection may have acted within *Ggt*, resulting in differentiation between genotypes better adapted to parasitising barley relative to wheat. The lack of specific markers for these putative

pathotypes implied that differentiation between them is limited and therefore the selection pressures are relatively weak.

Isozyme analysis indicated that the overall average genetic identity between pathogen populations was approximately 0.60, a relatively low value for an intra-specific analysis (Ayala, 1975). Reviews of isozyme studies on a variety of taxonomic groups indicated that genetic identities between subspecies usually range between 0.70 and 0.90 whereas those between different species range from 0.30 to 0.80 (Ayala, 1982; Thorpe, 1983). Identity values observed between taxonomic varieties of the root pathogen *Leptographium wageneri* (0.57-0.74) (Zambino and Harrington, 1989) and between host-specific populations of *Atkinsonella hypoxylon* (0.21-0.88) (Leuchtmann and Clay 1989) were low enough for these authors to suggest a separation of taxa into discrete species. The genetic identities between *G. graminis* populations were low enough, in relation to these figures, to imply that *Ggt* and *Gga* represent different species.

Isozyme and RFLP analyses between host-specific and geographically disjunct populations of the nitrogen-fixing bacterium, *Rhizobium meliloti*, also revealed two distinct evolutionary lineages within the species (Eardly *et al.*, 1990). The genetic identity between the two lineages was approximately 20%, with the authors suggesting that these two highly differentiated groups warranted specific status. The average genetic identity between *G. graminis* populations with low-copy RFLP probes was only 15%, which provides further support for the reclassification of *Ggt* and *Gga* as separate species.

Crossing experiments between isolates of different varieties within populations and between isolates of the same variety from widely separated locations should determine if sexual incompatibility is common within the species as a whole, regardless of taxonomic status. If out-crossing occurs only within a variety of *G. graminis*, regardless of geographical origin, separation of the taxonomic varieties into distinct biological species may be warranted.

Analyses of genetic similarities estimated from repetitive sequences, indicated a random distribution of phenotypes between populations, which did not correlate with the genetic identities of populations estimated from isozyme or low-copy RFLP loci. This distinction between the two types of RFLP analyses was also been reported by McDonald and Martinez (1991) in their studies of the phytopathogenic fungus *Septoria tritici*. The low repeat-sequence similarities between isolates which were identical at other markers, supports the hypothesis that repeat-sequence variants may arise independently and frequently by mechanisms of genome rearrangement. Therefore, these processes may constitute genetic mechanisms for the origin of evolutionary novelties which do not reflect evolutionary relationships within the species. The families of repetitive sequences analysed in this study appear to fall into this category and exhibited too much variation to allow accurate determination of phylogenetic relationships within *G. graminis*.

9.4 Further research.

Genetic analysis of *G. graminis*

The establishment of a suite of genetic markers opens the possibility of constructing a comprehensive genetic map of the *G. graminis* genome, for example by hybridising the genomic clones to chromosomes after separation by pulse-field gel electrophoresis (Schwartz and Cantor, 1984). This technique has many applications in the genetic analysis of fungi and has been used to determine the chromosomal locations of repetitive sequences in *Magnaporthe grisea* (Hamer *et al.*, 1989) and *Cladosporium fulvum* (Talbot *et al.*, 1991), to analyse the extent of chromosomal polymorphisms in *Saccharomyces cerevisiae* (Bidenne *et al.*, 1992) and to map the cellulase and xylanase genes of the fungus *Trichoderma reesei* (Carter *et al.*, 1992). DNA hybridisation experiments with the cloned sequences used in this study would provide an assessment of the copy number and distribution of these sequences throughout the genome. By these methods the low-copy clones, with chromosomal locations determined, could be selected to produce a genetic linkage map.

Intra-variety crosses between isolates which were polymorphic at numerous RFLP markers would provide a test-cross population to assess the number of individual loci detected with each of the probe/enzyme combinations. Segregation ratios at each of the individual loci could then provide an assessment of the patterns of inheritance within the pathogen and linkage analysis could be performed to generate a tentative chromosome map (Hulbert *et al.*, 1988; Christiansen and Giese, 1990; Anderson *et al.*, 1992). Since *G. graminis* has a haploid vegetative stage, this should allow the unequivocal identification of recombinant and parental genotypes, providing accurate estimates of recombination frequencies from the segregating RFLP markers.

The use of RFLP markers to determine both the chromosomal locations of genes and the genetic linkage between them allows for a comparison between physical and genetic maps of the *G. graminis* genome. This should aid in locating genes of interest, such as those controlling pathogenicity and virulence. It is likely that cDNA clones will be more appropriate for this purpose, since they are derived from actively transcribed genes and will avoid targeting dispersed heterochromatic regions of DNA, which are abundant in the genome of this species.

PCR products generated from intron-exon splice junctions should also be useful markers for mapping the *G. graminis* genome as they probably represent sequences derived from non-heterochromatic regions of DNA. Additionally, since introns are present in many fungal genes (Scazzacchio, 1989) it is possible to target a multitude of different genes and rapidly identify many genetic markers. This method has been used previously to identify and map polymorphisms in cereals (Weining and Langridge, 1991).

The physical locations of clones with homology to the PCR products could be determined by DNA hybridisation to electrophoretically separated chromosomes. Chromosomes which hybridise with the probes could be excised from the gel, partially digested with enzymes known to produce large restriction fragments and the DNA electroeluted from the agarose matrix. Purification of the DNA and subsequent cloning into cosmid vectors would produce chromosome-specific libraries to facilitate

molecular analysis of the genes from which the PCR products were originally derived. A similar approach has been used to isolate and map mutant genes from the fungus *Coprinus cinereus* (Zolan *et al.*, 1992).

It may be possible to develop laboratory strains of *G. graminis* which are capable of inter-variety sexual hybridization, as has been achieved with *Magnaporthe grisea* (Valent *et al.*, 1991). Inter-variety crosses between pathogenic and non-pathogenic isolates of *G. graminis* could be used to follow the inheritance of gene(s) associated with pathogenicity. Hybrid progeny could be backcrossed to both parental genotypes for a number of generations and their pathogenicity assessed on a range of cereal hosts. Any correlations between the acquisition or loss of pathogenic potential associated with the presence or absence of these marker loci in the progeny, may help to develop an understanding the genetics of pathogenicity within *G. graminis*.

In the absence of successful crosses it may be possible to transform non-pathogenic isolates with sequences thought to be associated with pathogenicity. *G. graminis* has been shown to be amenable to genetic transformation (Henson *et al.*, 1988). This approach offers an alternative method to study the molecular mechanisms underlying pathogenicity within the species.

9.5 Implications for disease control

The development of epidemics of take-all takes several years and furthermore depends both on the range of hosts to which the pathogen has been exposed and the effects of microbial antagonism. The role of specific soil micro-flora in the biological control of *G. graminis* is well documented (for a review see Rovira and Wildermuth, 1981). As the fertility of the soil is improved the severity of the disease frequently diminishes, a process which is associated with the build up of antagonistic soil bacteria under intensive cereal cropping. This suppression of fungal infectivity is known as take-all decline (T.A.D.).

T.A.D. is thought to be prevalent in temperate climates in soils which are richer in organic matter and support relatively high populations of soil micro-biota (Yarham,

1981). The bacteria responsible for the disease suppression can, however, be destroyed by non-gramineous hosts or by high temperatures in soils of low water potential (Rovira and Wildermuth, 1981). In the southern Australian cereal belt the combination of high summer temperatures, low rainfall and generally nutrient-poor soils results in lower numbers of antagonistic soil microorganisms and a perpetuation of *G. graminis* inoculum. Consequently, the development and persistence of take-all suppressive soils, either by continuous cereal cropping or introduction of T.A.D. bacteria as seed dressings, is considered unlikely within most of the cereal belt of southern Australia.

Cross-protection of crops, using avirulent fungi, has also been proposed as a means of preventing severe take-all. Precolonisation of wheat roots by *Phialophora graminicola* (the conidial state of *Gaeumannomyces cylindrosporus*) (Scott, 1970) and *Ggg* (Wong, 1981) have been shown to protect against infection by *Gga* and *Ggt*. *Ggg* and *Phialophora* spp. are only mildly pathogenic upon grasses and cultivated cereals and colonisation is believed to elicit a host response leading to increased lignification and suberisation of endodermal tissues, which restricts stelar infection by *Ggt* and *Gga* (Wong, 1981).

Avirulent fungi within the *Gaeumannomyces-Phialophora* complex are abundant in British grasslands and in cereal crops following grass pastures and have been successful in short-term disease control, primarily because they occupy the same ecological niche as the pathogen and appear to displace it by their prior occupation (Deacon, 1973). Control by this method requires persistence of the fungal antagonists and inoculum levels can be increased by using grass leys between cereal crops (Deacon, 1973). This approach, however, is not practical within Australia since this study has shown that *Ggg* is absent from regions under intensive cereal cropping and grass leys will only exacerbate the disease problem by allowing the persistence of large populations of *Ggt* and *Gga*.

In the absence of wheat and barley cultivars with resistance to *G. graminis*, the only effective method for short term disease control involves attempts to reduce inoculum levels by the use of non-susceptible hosts in regular rotation with cereals (Cotterill and

Sivasithamparam, 1988). For this procedure to be effective it is essential that these break crops and pastures should be free of alternative grass hosts. Disease forecasting schemes are necessary to advise farmers on the implementation and timing of these control measures to prevent the sowing of highly susceptible cereal crops in potential risk situations.

The RFLP and PCR variety-specific markers developed in this research can be used to determine the pathotype composition of *G. graminis* populations and provide a means of advising control practices aimed at limiting high incidences of take-all. For example, oats are often grown in rotation with wheat to reduce inoculum levels of *G. graminis* and prevent high incidence of take-all in the successive wheat crops. This practice is successful if the pathogen populations are dominated by *Ggt*. Conversely, if populations contain high frequencies of *Gga*, cropping oats will act to select those isolates which are equally well adapted to parasitising wheat in the following crops. Therefore, a wheat-oats-wheat rotation could result in the perpetuation of take-all disease, as has been reported to occur within the Western Australian grain belt (Yeats *et al.*, 1986). If the frequency of *Gga* isolates is high, a non-cereal host (e.g. grain legume, clover pasture) should be planted to prevent increases in take-all inoculum and improve soil fertility (Cotterill and Sivasithamparam, 1988).

In situations where fields contain large amounts of *Ggt* it may be advisable to expose the pathogen population to oats for one or more seasons before sowing a non-cereal break crop. This would select *Gga* and suppress *Ggt*, resulting in significant reductions in the density of inoculum. Furthermore, since *Gga* has been shown to be genetically homogeneous in comparison to *Ggt*, the overall levels of genetic diversity within the pathogen population would be significantly reduced by growing oats. Restricting the genetic base of the population may make the pathogen more amenable to control by factors such as limited non-specific microbiological antagonism, when surviving saprophytically in the presence of non-susceptible break crops.

These methods can be hoped to provide only short-term control of take-all. Efficient long-term disease control may come about only by developing a greater

understanding of the genetic determinants of pathogenicity and the genetic control of host defence mechanisms in response to infection.

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Appendix A Composition of solutions and media

A.1 Isozyme analysis

Isozyme extraction buffer: (per 100 ml distilled water) 0.4 g Tris, 0.01 mg NADP, 0.1 ml 2-mercaptoethanol, 0.1 ml Triton x-100, pH 7.0.

NDY culture medium: (per litre of distilled water) 2 g $NaNO_3$, 1 g KH_2PO_4, 0.5 g $MgSO_4$. $7H_2O$, 0.5 g KCl, 10 ml 0.1 % solution $FeSO_4$, 0.5 g yeast extract, 30 g sucrose.

SM-GGT3 (*G. graminis* selective isolation medium): (per litre of distilled water) 39 g Difco PDA, 10 mg dicloran, 10 mg metalaxyl, 25 mg 1-(3,5-dichlorophenyl)-3-methoxymethylpyrrolidin-2,4-dion (Hoe 00703), 100 mg streptomycin sulphate, 500 mg L-β-3,4-dihydroxyphenylalanine (L-DOPA).

A.2 RFLP analysis

(2 x)pUC19 specific oligolabelling buffer: 40 µM d(ATP, GTP, TTP), 100 mM Tris (pH 7.6), 100 mM NaCl, 20 mM $MgCl_2$, 200 µg/ml acetylated DNAse free bovine serum albumin (Fraction V; Sigma). Per 500 µl add 2 µg each pUC19 specific primers, primer 1: 5' ACAGCTATGACCATG 3', primer 2: 5' TMCCAGTMACGACGT 3'. Store as 12.5 µl aliquots at -20° C.

5 x HSB: 3 M NaCl, 100 mM PIPES, 25 mM Na_2EDTA, pH 6.8 with 4 M NaOH.

10 x CIP dephosphorylation buffer: 10 mM $ZnCl_2$, 10 mM $MgCl_2$, 100 mM Tris, pH 8.3.

10 x gel loading buffer: 100 mM Tris-HCl, 200 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol, 30% ficoll type 4000, pH 8.0.

10 x Ligation buffer: 500 mM Tris (pH 7.6), 100 mM $MgCl_2$, 100 mM dithiothreitol, 500 µg/ml bovine serum albumin (Fraction V; Sigma).

10 x Restriction endonuclease buffer B: 10 mM Tris-HCl, 5 mM $MgCl_2$, 100 mM NaCl, 1 mM 2-mercaptoethanol, pH 8.0.

10 x Restriction endonuclease buffer H: 10 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, pH 7.5.

10 x Restriction endonuclease buffer L: 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.5.

10 x Restriction endonuclease buffer M: 10 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol, pH 7.5.

10 x TAE: 400 mM Tris, 30 mM sodium acetate, 10 mM Na_2EDTA, pH 7.8

20 x SSC: 3 M NaCl, 300 mM Na_3citrate.

Denaturing solution: 1.5 M NaCl, 500 mM NaOH.

Denhardts III: 2% gelatin, 2% ficoll, 2% polyvinyl pyrollidone (PVP), 10% SDS, 5% tetrasodium pyrophosphate. Filter at 65° C.

DNA extraction buffer: 100 mM Tris, 100 mM NaCl, 1% SDS, 20 mM EDTA, 100 mM Na_2SO_3, pH 8.0.

LB medium: (per litre distilled water) 10 g bacto-tryptone, 5 g yeast extract, 10 g NaCl, pH 7.0.

LB agar: (per litre of LB medium) 15 g bacto-agar.

LB plates (for identification of recombinant *E. coli*): To 100 ml of LB agar add 100 µl of 100 mM isopropyl β-thiogalactopyranoside (IPTG), 200 µl bromo-(5)-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) [2% w/v in dimethyl formamide (DMF)], 100 µl 50 mg/ml ampicillin.

Neutralising solution: 1.5 M NaCl, 500 mM Tris-HCl, 10 mM Na_2EDTA, pH 7.0.

Phenol/chloroform/iso-amyl alcohol (25:24:1): redistilled phenol was saturated with 0.5 M Tris-HCl (pH 8.0) and mixed with chloroform and iso-amyl alcohol as indicated.

Salmon sperm DNA: add 0.5 g salmon sperm DNA to 100 ml nanopure H_2O, autoclave.

Sephadex G -100: To 300 ml TE buffer add 10 g Sephadex G -100, incubate with gentle shaking for 2 h at 65° C.

SOB: To 950 ml of deionised water, add: 20 g bacto-tryptone, 5 g bacto-yeast extract, 0.5 g NaCl, 10 ml 250 mM KCl (pH 7.0). Just before use, add 1 ml each sterile solution 10 mM $MgCl_2$, 10 mM $MgSO_4$:$7H_2O$ per 100 ml SOB.

SOC: 1 ml SOB, 10 µl 2 M $MgCl_2$, 7 µl sterile 50% glucose.

TE buffer: 10 mM Tris-HCl (pH 7.5), 1 mM Na_2EDTA.

TFB buffer: 10 mM (Morpholino)ethanesulphonic acid (MES), 45 mM $MnCl_2$:$4H_2O$, 100 mM RbCl, 10 mM $CaCl_2$:$2H_2O$, 3 mM $HACoCl_3$ (hexylamine cobalt chloride). Sterile filter and freeze.

A.3 PCR analysis

10 x PCR reaction buffer: 500 mM KCl, 200 mM Tris-HCl, 25 mM $MgCl_2$, 1 mg/ml bovine serum albumin, pH 8.4.

10 x TBE buffer: 1 M Tris, 10 mM Na_2EDTA, 860 mM boric acid, pH 8.3.