

Genetics of pathogenicity in *Pyrenophora* leaf diseases of barley

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Declaration

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Summary

Net blotch of barley, caused by *Pyrenophora teres*, is one of the most important diseases of this cereal in the south Western Cape Province of South Africa. This fungus exists as two different types (forms), namely a net-type and a spot-type that are distinguished by differential symptom expression on barley leaves. Based on this specific plant pathological difference a series of studies of agricultural importance were executed to investigate the effects of sexual recombination between these two types. In addition, studies were done to determine the difference between local net- and spot-type populations with regards to population structure and fungicide sensitivity. This dissertation therefore, consists of a collection of separate publications and as a result a certain degree of redundancy has been unavoidable.

Recombination is one of the most important evolutionary forces involved with sexual reproduction. In plant-fungal agricultural ecosystems this may result in pathogenic fungal populations adapting more rapidly to control programs such as fungicide applications. The first section of the review in part 1 of this dissertation covers different aspects of sexual reproduction in ascomycetes, specifically focussing on mating-type genes, vegetative incompatibility and recombination. The major part of the review is then dedicated to various plant pathological aspects of *P.teres*, specifically addressing the differences between the two types, and in various cases highlighting the significance of sexual recombination within and between the net- and spot-type.

Using morphological criteria for identification purposes there have been many conflicting reports concerning the identity of leaf spot isolates in the Western Cape Province of South Africa. In part 2, the correct identity was eventually achieved employing mating studies and molecular markers. This was accomplished after single ascospores were obtained from pseudothecia after *in vitro* mating had occurred between a verified *P. teres* net-blotch isolate from Denmark and a representative *Pyrenophora* leaf spot isolate from South Africa. Using amplified fragment length polymorphism (AFLP) and RAPD markers, recombination was demonstrated in the progeny that had DNA

banding patterns different from the two parental isolates. Pathogenicity trials also confirmed that recombination had taken place during mating. Inoculations were conducted on the differential cultivars susceptible to the net-blotch and leaf spot forms. The two parents induced typical net-blotch or leaf spot symptoms whereas the progeny mostly induced a jagged spot symptom on each cultivar. Fungicide sensitivity tests using the ergosterol biosynthesis inhibitors showed that, due to recombination, some progeny could have increased resistance to these fungicides. Due to mating and subsequent recombination between a net blotch isolate of *P. teres* and a representative leaf spot isolate, it was concluded that the latter was *P. teres* f. *maculata*.

Fifteen of the net-spot hybrid progeny (F_1) produced from the mating study in Part 2 were screened in Part 3 to assess their viability and genetic stability. Hybrid progeny (F_1) inoculated onto barley seedlings consisting of the cultivars Stirling (differentially susceptible to net-type isolates), B87/14 and Clipper (both differentially susceptible to spot-type isolates) produced intermediate symptoms on all cultivars. Axenic cultures (F_{1-1}) isolated from foliar lesions, followed by repeated inoculation and isolation (F_{1-2}) onto a healthy set of seedlings produced similar intermediate symptoms. RAPDs conducted with two 10-mer primers on all isolates of F_{1-1} and F_{1-2} progeny revealed profiles similar to those obtained for F_1 isolates. RAPD molecular data, therefore, indicated that hybrid progeny of this net x spot mating were genetically stable after having been subjected to two repetitive inoculation and reisolation cycles. Phylogenetic analysis of DNA sequences of the internal transcribed spacers (ITS1 and ITS2) flanking the 5.8S nuclear ribosomal RNA gene and the 5' end partial histone-3 gene confirmed the genetic stability of the hybrid progeny. These results also indicated that the hybrid progeny produced consistent symptoms throughout the series of experiments, and maintained their virulence to the differential cultivars screened.

Both types of *P. teres* are prevalent in the south Western Cape Province of South Africa, found on susceptible cultivars often grown within close proximity of each other. In Part 4, a net- and spot-type population were characterised in terms of their population structure using RAPD markers. Samples were collected from infected barley leaves from two separate

quadrants in each field, the two quadrants positioned in corners of the fields, diagonal to one another. A total of 65 loci were produced of which 54 were polymorphic. Total gene diversities determined for all loci resulted in mean indices of 0.063 and 0.082 being obtained respectively for the net- and spot-type populations. A coefficient of genetic differentiation (G_S) of 0.0149 was obtained between sites within populations while a coefficient (G_T) of 0.63 was obtained between the two populations. Genotypic variation revealed 13 distinct multilocus genotypes (haplotypes) in the net-type population while there were 12 in the spot-type population. UPGMA cluster analysis done on the two populations together with six progeny from the mating between a net- and spot-type isolate resulted in three main clusters being produced, one for each population and one for the progeny. One isolate collected from the net-type population also contained a unique spot-type RAPD fragment. This suggested that sexual recombination may be taking place between isolates of the net- and spot-type under field conditions.

Fungicide application is the most important method used in the control of net blotch in South Africa. In Part 5 the fungicide sensitivities (IC_{50} values) of 89 monoconidial isolates (46 net-type and 43 spot-type) of *P. teres* to sterol demethylation inhibiting fungicides were determined, based on the inhibitory effect on radial mycelial growth. The fungicides evaluated were triadimenol, bromuconazole, flusilazole, propiconazole and tebuconazole. Both net- and spot-type isolates revealed strong resistance to triadimenol while flusilazole was shown to be the strongest inhibitor of fungal growth. Spot-type isolates showed a higher resistance than net-type isolates to all five fungicides screened. The IC_{50} values indicated significant differences between four of the fungicides (triadimenol, tebuconazole, flusilazole and propiconazole). The IC_{50} values between propiconazole and bromuconazole were not significant. This study suggested that spot-type isolates showed a higher degree of resistance to commercially used fungicides than net-type isolates.

The overall conclusion of this study is that the spot-type of *P. teres* is the pathogen associated with leaf spots of barley in the south western Cape province of South Africa and not *P. japonica* as earlier reported. Together with the net-type, both types exist as genetically variable populations in this barley production region. Mating between the two types results in sexual

progeny that are genetically stable. This implies that barley fields adjacent to one another in which either net- or spot-type susceptible cultivars are being cultivated may lead to sexual progeny being produced. This in turn may lead to an increased rate at which fungal populations may become resistant to commercially used fungicides. It is furthermore suggested that an alternative fungicide seed treatment is used instead of triadimenol due to high resistance of *P. teres* to this fungicide.

Opsomming

Netvlek op gars is een van die belangrikste siektes van hierdie graansoort in die suidelike deel van die Westelike Kaaprovincie. Dié siekte word veroorsaak deur die swam *Pyrenophora teres*. Hierdie swam kom voor as twee verskillende tipes, naamlik 'n net-tipe en 'n kol-tipe wat onderskei word op grond van die voorkoms van hulle simptome op garsblare. Hierdie plantpatologiese verskil in ag genome, is 'n reeks studies van landboukundige waarde uitgevoer om die effek van geslagtelike rekombinasie tussen die twee tipes te ondersoek. Daarbenewens is ook studies uitgevoer om om die verskil te bepaal tussen plaaslike net- en kol-tipe populasies ten opsigte van populasiestruktuur en fungisiedsensitiwiteit. Hierdie verhandeling bestaan dus uit 'n versameling afsonderlike publikasies en as gevolg daarvan is daar onvermydelik'n mate van oorvleueling.

Rekombinasie is een van die belangrikste evolusionêre kragte betrokke by geslagtelike voortplanting. In plant-swam landboukundige ekostelsels kan dit veroorsaak dat patogene swampopulasiess vinniger aanpas by beheerprogramme soos fungisiedtoediening. Die eerste gedeelte in deel 1 van hierdie verhandeling dek die verskillende aspekte van geslagtelike voortplanting van ascomycetes, met spesifieke verwysing na paringstipe gene, vegetatiewe onverenigbaarheid en rekombinasie. Die grootste gedeelte van die oorsig word gewy aan verskeie plantpatologiese aspekte van *P. teres*, en wys veral op die verskille tussen die twee tipes. In verskeie gevalle word die betekenis van geslagsrekombinasie binne en tussen die net- en kol-tipe uitgelig.

Deur morfologiese kenmerke vir identifikasiedoeleindes te gebruik, is daar baie teenstrydige verslae rakende die identifikasie van blaarvlekisolate in die Westlike Kaaprovincie van Suid-Afrika. In deel 2 is die korrekte identifikasie eventueel verkry deur gebruik te maak van paringstudies en molekulêre merkers. Dit is bereik nadat enkel ascospore verkry is uit pseudothecia gevorm na *in vitro* paring plaasgevind het tussen 'n bevestigde *P. teres* netvlek isolaat uit Denemarken en 'n verteenwoordigende *Pyrenophora* blaarvlekisolaat van Suid-Afrika. Deur gebruik te maak van versterkte fragmentlengte polimorfisme [AFLP]

en RAPD merkers, is rekombinasie gedemonstreer in die nasate wat DNA bandpatrone gehad het wat verskil het van dié van die "ouer" isolate. Patogenisiteitstoetse het ook bevestig dat rekombinasie tydens paring plaasgevind het. Inokulasies is uitgevoer op die verskillende cultivars wat vatbaar is vir die netvlek en blaarvlek vorme. Die twee ouers het tipiese netvlek of blaarvlek simptome veroorsaak, terwyl die nasate hoekige vlekke veroorsaak het op elke cultivar. Toetse vir fungisiedsensitiwiteit deur gebruik van die ergosterol biosintese inhibeerders het gewys dat a.g.v. rekombinasie sekere nasate verhoogde weerstand teen hierdie fungisiedes het. As gevolg van paring en daaropvolgende rekombinasie tussen 'n netvlek isolaat van *P. teres* en 'n verteenwoordigende blaarvlek isolaat is afgelei dat laasgenoemde *P. teres* f. *maculata* is. Vyftien van die netvlek hibried nakomelinge (F_1) verkry van die paringstudie in deel 2 is ondersoek in deel 3 om hul lewensvatbaarheid en genetiese stabiliteit te bepaal. Hibried nasate (F_1) geïnokuleer op garssaaillinge bestaande uit die volgende cultivars: Stirling (soms vatbaar vir net-tipe isolate), B87/14 en Clipper (albei soms vatbaar vir kol-tipe isolate) het intermediêre simptome op al die cultivars veroorsaak. Akseniese kulture (F_{1-1}) geïsoleer uit blaarletsels gevolg deur herhaalde inokulasie en isolasie (F_{1-2}) op 'n gesonde stel saaillinge het dieselfde intermediêre simptome veroorsaak. RAPDs uitgevoer met twee 10-mer inleiers op al die isolate van F_{1-1} en F_{1-2} nasate het profiele opgelewer soortgelyk aan dié wat vir F_1 isolate verkry is. RAPD molekulêre data het dus gewys dat die hibried nasate van hierdie net x kol paring geneties stabiel was nadat dit onderwerp is aan twee inokulasie en reïsolasie sikkusse. Genetiese stabiliteit van die hibried nageslag is bevestig deur filogenetiese analise van die DNA volgorde van die interne getranskribeerde spasieerders (ITS1 en ITS2) reg langs die 5.8S nukluêre ribosomale RNA geen en die 5' end gedeeltelike histoon-3 geen. Hierdie resultate het ook gewys dat die hibried nasate konstante simptome getoon het tydens die hele reeks eksperimente en hulle virulensie behou het vir die kultivars wat getoets is.

Beide tipes van *P. teres* kom algemeen voor in die suidelike deel van die Westelike Kaapprovincie en word gevind op vatbare cultivars wat dikwels nabysteende

mekaar groei. In deel 4 is 'n net- en kol-tipe populasie gekarakteriseer in terme van hulle populasiestruktuur deur gebruik van RAPD merkers. Monsters is versamel van geïnfekteerde garsblare van twee aparte kwadrante in elke saailand. Die twee kwadrante is geplaas in die hoeke van die saailand, diagonaal tot mekaar. 'n Totaal van 65 lokusse is gevorm, waarvan 54 polimorfies was. Die algehele genetiese verskeidenheid bepaal vir alle lokusse, het geleid tot gemiddelde indekse van 0.063 en 0.082 soos gevind vir die net- en kol-tipe populasies. 'n Koëffisiënt van genetiese differensiasie (G_S) van 0.0149 is gevind tussen gebiede tussen populasies, terwyl 'n koëffisiënt (G_T) van 0.63 gevind is tussen die twee populasies. Genotipiese variasie het 13 duidelike multilokus genotypes (haplotypes) getoon in die net-tipe populasie, terwyl daar twaalf was in die kol-tipe populasie. UPGMA groeperingsanalises wat gedoen is op die twee populasies tesame met ses nasate van die paring van 'n net- en kol-tipe isolaat het tot gevolg gehad dat drie hoof groepe gevorm is, een vir elke populasie en een vir die nasate. Een isolaat wat versamel is, van die net-tipe populasie het 'n unieke kol-tipe RAPD fragment bevat. Dit wys daarop dat geslagtelike rekombinasie in veldomstandighede mag voorkom tussen isolate van die net- en kol-tipe.

Fungisiedtoediening is die belangrikste metode wat gebruik word om netvlek in Suid-Afrika te beheer. In deel 5 is die fungisiedsensitiwiteit (IC_{50} waardes) van 89 enkelkonidiale isolate (46 net-tipe en 43 kol-tipe) van *P. teres* teen sterol demetielasie inhiberende fungisiedes bepaal, op die basis van die onderdrukkende effek op die radiale groei van die miselium. Die volgende fungisiedes is geëvalueer: triadimenol, bromuconazole, flusilazole, propiconazole en tebuconazole. Beide net- en kol-tipe isolate het 'n sterk weerstand teen triadimenol openbaar, terwyl flusilazole gevind is as die sterkste onderdrukker van swamgroei. Kol-tipe isolate het 'n hoër weerstand as die net-tipe isolate teen al vyf fungisiedes wat getoets is, gehad. Die IC_{50} waardes het aangedui dat daar beduidende verskille tussen vier van die fungisiedes is (triadimenol, tebuconazole, flusilazole en propiconazole). Die IC_{50} waardes tussen propiconazole en bromuconazole was nie beduidend nie. Die gevolg trekking van

hierdie studie is dus dat die kol-tipe isolate 'n hoër graad van weerstand teen kommersiëel gebruikte fungisiedes as die net-tipe isolate gehad het.

Die algehele gevolgtrekking van hierdie studie is dat die kol-tipe van *P. teres*, die patogeen is wat geassosieer word met blaarvlekke op gars in die suidwestelike Kaapprovincie van Suid-Afrika, en nie *P. japonica* soos voorheen gerapporteer nie. Tesame met die net-tipe, kom altwee tipes voor as geneties veranderlike populasies in hierdie gars verbouingstreek. Paring tussen die twee tipes lei tot geslagtelike nasate wat geneties stabiel is. Dit impliseer dat aangrensende garsvelde waarop net- of kol-tipe vatbare kultivars verbou word, mag lei tot die produksie van geslagtelike nasate. Dit kan weer lei tot 'n verhoogde tempo waarteen swampopulasiës weerstandbiedend teenoor kommersiële fungisiedes raak. Daar word verder ook voorgestel dat alternatiewe fungisied saadbehandelings gebruik word in plaas van triadimenol as gevolg van verhoogde weerstand van *P. teres* teenoor laasgenoemde.

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1. Aspects of ascomycete sexual reproduction, and implications thereof in the barley net blotch pathogen *Pyrenophora teres*

INTRODUCTION

Sexual reproduction in plant/fungal ecosystems is important as it can increase levels of genetic diversity and as a result fungal populations may adapt more rapidly to fungicide applications. Mating-type genes and vegetative incompatibility are two fundamental components involved in sexual reproduction. These aspects are increasingly enjoying more attention in research projects specifically aimed at the elucidation of pathogenicity in plant pathogenic fungi, with the aim of developing strategies to exploit resistance against phytopathogens in agricultural systems.

This review outlines the basic processes involved in sexual reproduction as well as mating-type gene structure and various aspects of vegetative incompatibility. Recombination occurring during the sexual cycle, which may directly lead to increased levels of resistant genotypes, is reviewed as well, specifically focussing on its detection in plant/fungal systems. The major section of this review is devoted to the barley net blotch pathogen, *Pyrenophora teres*, in which various plant pathological aspects that may be affected by sexual recombination are discussed.

Based on specific plant pathological aspects of the *P. teres* pathosystem in the Western Cape province of South Africa, four different studies are proposed for further investigation (parts 2-5).

FUNGAL SEXUAL REPRODUCTION AND ITS CONSEQUENCES

What is the purpose of mating? Some organisms mate regularly as part of their life cycles while others propagate themselves by vegetative growth (Metzenberg & Glass, 1990). As both forms of reproduction exist, each must have its own advantages. Only the advantages of sexual reproduction will, however, be discussed. In fungi the most important consequence of sexual reproduction is the production of offspring with new favourable combinations of alleles from both parents via genetic recombination (Hawker, 1966). A second advantage of sexual reproduction is that it may allow the possibility of each partner repairing random epigenetic or conventional genetic damage (Bernstein *et al.*, 1985). Genetic damages are physical alterations in the structural framework of DNA such as breaks, thymine dimers, depurinations and depyrimidations. As a result these damages interfere with DNA replication and transcription. Conventional genetic damages constitute mutations which arise from substitution, addition, deletion or rearrangements of base pairs. Bernstein *et al.* (1985) argued that the two principal features of sexual reproduction, namely recombination and outcrossing are maintained respectively for repairing DNA damage and masking mutations. Furthermore, genetic variation is produced as a by-product of sexual reproduction. A further advantage of sexual reproduction is that the process may allow the survival of a species through the formation of dormant fruit bodies and sexual spores, resistant to adverse environmental conditions such as killing by heat, desiccation and ultraviolet light (Perkins & Turner, 1988). At the end of a dormant period, when conditions will probably be different from those at the start, sufficient variation will have been generated to increase the likelihood that some of the offspring will succeed in the changed conditions, with a repair mechanism being in place at the same time to eliminate gross abnormalities. There are also disadvantages to sexual reproduction. These include the metabolic costs of producing pheromones and construction costs of structures related to sex, failure to find a compatible partner and the possibility that recombination may break up successful combinations of alleles (Metzenberg & Glass, 1990).

Life cycle of a two-allele heterothallic fungus

Two-allele physiological heterothallism is common among various ascomycetes (Yoder *et al.*, 1986; Metzenberg & Glass, 1990; Debuchy & Coppin, 1992) in which compatibility is determined by genetic factors which behave like two alleles of a single locus and confer different mating-types which may be referred to as *A* or *a* (Metzenberg & Glass, 1990), *MAT1-1* and *MAT1-2* (Yoder *et al.*, 1986) or *mat+* and *mat-* (Debuchy & Coppin, 1992). The sexual phase of a life cycle is initiated by the fusion of haploid cells that are morphologically indistinguishable (Bölker & Kahmann, 1993) (Fig. 1). However, these cells can only fuse if they are of opposing mating-types. The term mating-type defines all specific activities of a cell that are required to develop from a haploid to a diploid stage and to continue on to meiosis. The mating-type is determined by the genetic information that is encoded at the mating-type locus.

Neurospora crassa is a haploid heterothallic fungus with the mating-types *A* and *a* (Perkins & Barry, 1977; Johnson, 1978; Perkins & Turner, 1988; Metzenberg & Glass, 1990). Only two strains of opposite mating-type, including those isogenic at all other loci, are competent to mate (Metzenberg & Glass, 1990). Under suitable environmental conditions i.e., nitrogen starvation (Perkins & Barry, 1977; Glass & Lorimer, 1991) and not too high a temperature can the sexual cycle be initiated. Either the *A* or a mating-type produces a nearly spherical pre-fruiting body composed of sterile specialized hyphae (Fig. 1). This structure, termed a protoperithecium is the female element. Specialized hyphae, called trichogynes, now grow directionally from the protoperithecium. Mating occurs between these trichogynes and a target cell of the opposite mating-type which functions as the male cell. This target cell may be a conidium, a microconidium or a vegetative hypha (Glass & Lorimer, 1991). The directional growth of trichogynes as well as the specific cell recognition between cells of the opposite mating-type is mediated by pheromones (Bistis 1981, 1983; Bölker & Kahmann, 1993) that are secreted by cells of each mating-type. After cytoplasmic fusion of the trichogyne and its target cell, the nucleus from the male cell is conducted through the trichogyne into the protoperithecium (Metzenberg & Glass, 1990), which

becomes by definition a perithecium. The A and a haploid nuclei now proliferate to form fertile ascogenous hyphae. In the final few nuclear divisions nuclei of opposite mating-type are paired after which fusion (karyogamy) occurs to produce diploid cells. Nuclear fusion is immediately followed by the two meiotic divisions to produce four haploid nuclei which subsequently undergo a mitotic division to give eight nuclei around which ascospores are soon delineated.

MATING-TYPE GENES

Many species of ascomycetes, including *Pyrenophora* have bipolar mating systems i.e., they are self sterile and require for successful mating, partners with alternate genes at the single, regulatory mating-type (*MAT*) locus (Kües & Casselton, 1992; Turgeon *et al.*, 1995). Mating-type genes have been cloned from *Neurospora crassa* (Glass *et al.*, 1988, 1990; Staben & Yanofsky, 1990), *Podospora anserina* (Picard *et al.*, 1991; Debuchy & Coppin, 1992), as well as from various plant pathogens including the rice blast fungus *Magnaporthe grisea* (Kang *et al.*, 1994) and *Cochliobolus heterostrophus* (Turgeon *et al.*, 1993), the causal agent of southern corn leaf blight. The most striking discovery in the cloning of these genes (alleles) was the fact that the two genes showed a very high degree of dissimilarity (Glass *et al.*, 1988) (Fig. 2). The two alleles at the mating-type locus were subsequently referred to as idiomorphs (Metzenberg & Glass, 1990). Further evidence for dissimilarity has also been shown in the DNA regions adjacent to the mating-type genes. Randall and Metzenberg (1995) showed that in several heterothallic *Neurospora* species the DNA regions adjacent to the mating-type genes could have as little as 20% similarity between the A and a mating-types of *N. discreta* and 21% in *N. intermedia*, while the similarity between *N. crassa* mating-types was 66%.

Transcripts of these mating-type genes contain regions coding for high mobility group (HMG) motifs that are found in all DNA-binding proteins (Kelly *et al.*, 1988; Debuchy & Coppin, 1992; Griess *et al.*, 1993). Further investigations (Philley & Staben, 1994) have indicated that the HMG boxes of

the mating-type polypeptides also bind to specific DNA sequences in a similar way to other closely related DNA-binding proteins (Sinclair *et al.*, 1990; Sugimoto *et al.*, 1991; Alexander-Bridges *et al.*, 1992; Hartley *et al.*, 1992; Hartmann *et al.*, 1996). As a result of the high degree of conservation of certain amino acids in the HMG DNA-binding motif, PCR methods have been developed for cloning *MAT* genes across and within genus lines of both sexual and asexual fungi (Arie *et al.*, 1997; Christiansen *et al.*, 1998). Some of the mating-type genes isolated have been those of severe cereal pathogens, namely *P. teres* (net blotch of barley), *P. tritici-repentis* (tan spot of wheat) and *Gaeumannomyces graminis* (take-all of wheat) (Arie *et al.*, 1997).

It has been proposed that these mating-type polypeptides may function in a similar fashion as pheromones in the yeast *Saccharomyces cerevisiae* (Herskowitz, 1989). Furthermore, other regions within the *MAT* locus play a role in fertilisation (Arnaise *et al.*, 1993), postfertilisation functions, vegetative incompatibility, ascospore production (Ferreira *et al.*, 1996, 1998) and fruiting body development (Debuchy *et al.*, 1993; Turgeon *et al.*, 1995).

VEGETATIVE INCOMPATIBILITY

All fungi possess a system for regulating heterokaryon formation (Glass & Kulda, 1992). If two opposite mating-types are vegetatively incompatible then sexual reproduction cannot take place. Vegetative incompatibility also known as heterokaryon incompatibility results in failure to form heterokaryotic strains via hyphal fusion between different strains. This is due to the coexpression of incompatible genes in the heterokaryotic cells and results in a cell death reaction (Bourges *et al.*, 1998).

Various reasons for this phenomenon have been proposed. It has been reported that it may limit the spread of cytoplasmic genetic elements in natural populations of some fungi. It has also been suggested that it may serve to protect fungi against "genetic infection" by preventing the spread of viruses and other extrachromosomal genetic elements such as double-stranded RNA or altered mitochondrial DNA, and in this way provides a

selective advantage (Caten, 1972). It has also been proposed that the presence of these incompatibility genes would limit outbreeding and favour the evolution of isolated groups within a species, so creating a basis for evolution (Esser & Blaich, 1973). Prevention of heterokaryosis would be beneficial and incompatibility genes would have primarily been selected and have evolved to limit heterokaryon formation. Hartl *et al.* (1975) proposed that it functions to protect established, adapted mycelia from exploitative nuclei which are less well adapted to survive as homokaryons in that environment, and which might compete with the nuclei of the established mycelium if introduced by hyphal fusion. Recently Debets and Griffiths (1998) proposed that vegetative incompatibility may function in sexual crosses to protect unfertilised cultures from germ cell parasitism by conidia of other colonies.

Vegetative incompatibility can be assessed using one of three basic types of techniques (Leslie, 1993). The first involves a direct test of heterokaryon formation usually involved in the establishment of a stable prototrophic heterokaryon under conditions in which neither of the two auxotrophic components could survive. Any genetic markers whose defect can be remedied by complementation can be used to detect heterokaryon formation. Auxotrophic or pigmentation markers are usually preferred due to the ease of distinguishing the heterokaryon colony from its components. Forced markers can also be used, as in the case of nitrate nonutilising (*nit*) mutants. When a heterokaryon is forced between auxotrophic strains in the same vegetative compatibility group (VCG), then a prototrophic heterokaryon results. If the strains are in different VCGs, then no prototrophic growth occurs. Incompatibility using the barrage method generally makes use of culturing the fungal isolates on PDA amended with methionine and biotin (Anagnostakis, 1977; Meijer *et al.*, 1994; Powell, 1995; Liu & Milgroom, 1996) although oatmeal agar has also been used (Bégueret *et al.*, 1994). To make this barrage zone easier to detect, a technique utilising red food colouring dye has been employed (Kohn *et al.*, 1990, 1991; Rizwana & Powell, 1992). However, certain fungi produce acids during growth and, thereby reduce the pH of the medium resulting in the fading of the red food dye. This problem may be circumvented by the employment of bromocresol green to detect the

areas of dead or dying mycelium (Powell, 1995). In some fungi activated charcoal has been incorporated in the culture medium (Julián *et al.*, 1996).

Vegetative incompatibility can be observed on agar medium as a barrage reaction produced at the point where two incompatible strains meet. The barrage phenomenon requires hyphal fusion and in the barrage region numerous lethal hyphal fusions will occur. This zone is characterised by degenerating, dying hyphae and aborted heterokaryotic cells. The barrage may also be observed as a clear zone due to heterokaryotic cells which have undergone self-lysis as a result of the incompatibility reaction. A dark layer of pigment may also be deposited in this zone. On either side of this central region the mycelia form a higher, thicker layer of growth that in some cases may be accompanied by the formation of fruiting bodies (Andes, 1961; Anagnostakis, 1987; 1988).

The genetic determination of vegetative incompatibility is well documented with regards to *N. crassa* (Garnjobst & Wilson, 1956; Mylyk, 1975), *P. anserina* (Turcq *et al.*, 1990) and *C. heterostrophus* (Leach & Yoder, 1983; Turgeon *et al.*, 1993). Furthermore, genes involved with vegetative incompatibility in these three species have been cloned (Turcq *et al.*, 1990, 1991; Turgeon *et al.*, 1993; Saupe *et al.*, 1995, 1996). Some of these genes encode proteins associated with the binding of fungal cell walls (Saupe *et al.*, 1996), barrage formation (Turcq *et al.*, 1991) and vacuolisation of the cytoplasm during cell death (Saupe *et al.*, 1995).

RECOMBINATION

The mode of reproduction by which fungi reproduce and pass their genes on to the next generation have enormous implications with regards to the patterns of genetic variation in populations (Milgroom, 1996). Asexual reproduction results in progeny that are genetically identical to each other and the parent, the result of which is clonal population structure. Clonal populations have distinctive features such as widespread occurrence of identical genotypes, absence of recombinant genotypes and correlations between independent sets of genetic markers (Tibayrenc *et al.*, 1991).

During sexual reproduction meiosis results in an independent assortment of chromosomes and recombination within chromosomes. During sexual reproduction unlinked genes segregate independently, resulting in offspring with recombinant genotypes (Lamb, 1996). Therefore, sexual populations tend to be more genetically diverse than asexual populations of the same species (Milgroom, 1996). Independent assortment and recombination have two major effects on population structure: 1) relatively high levels of genotypic diversity (genotypic diversity is a function of the number and frequencies of combinations of alleles at multiple loci - multilocus genotype or haplotype) and 2) random association between alleles at different loci, such that genotype frequencies can be predicted from the allele frequencies at each locus.

The relevance of recombination in plant pathology is most profound in the evolution of novel pathotypes capable of overcoming resistant cultivars (Brown, 1995) and resistant to multiple fungicides. A thorough understanding of recombination in *Bremia lactucae* was required in the disease management process for lettuce in the United Kingdom (Crute, 1989). During the 1980s resistance to the fungicide metalaxyl occurred in *B. lactucae*. However, resistance to metalaxyl initially arose only in a single clone with the B2 mating-type, which was avirulent on cultivars with the *Dm11* resistance gene. In order for metalaxyl resistance genes to be recombined into pathotypes virulent on *Dm11* cultivars, resistant and sensitive isolates had to occur on the same cultivar for successful mating. Mating between resistant and sensitive isolates could only occur on cultivars that lacked *Dm11* (to allow metalaxyl-resistant isolates to colonise) and were not treated with metalaxyl (to allow metalaxyl-sensitive isolates to colonise). Therefore, to prevent recombination, growers were recommended to apply metalaxyl to cultivars lacking *Dm11* even though control against the resistant isolates would be ineffective.

Detection of recombination (sexual reproduction) in fungal populations

To assess if a fungal population has the potential for sexual reproduction either one of two lines of thought may be followed. In the first instance inferences can be made concerning random mating in populations (Milgroom,

1996), either by gametic disequilibrium which involves statistical analyses of different sets of genetic or phenotypic loci, or by more direct methods, e.g., presence of sexual structures under field conditions, mating-type ratios, and the calculation of genotypic diversity indices. Secondly, potential for sexual reproduction may be inferred directly by assaying for recombinant genotypes. This process, however, normally requires molecular genetic markers (Sujkowski *et al.*, 1994; Anderson & Kohn, 1995; Goodwin *et al.*, 1995; Burt *et al.*, 1996).

1. Random mating

Gametic disequilibrium. The null hypothesis (H_0) in tests for random mating is based on the population structure expected, given random mating. If H_0 is rejected the conclusion is made that the population is not randomly mating. For the H_0 hypothesis to be ‘rejected’ or ‘not rejected’ (Milgroom, 1996) one of two main statistical analyses can be used, namely, the two-locus gametic disequilibrium method (Hedrick, 1987; Lewontin, 1988) or the index of association (Brown *et al.*, 1980; Maynard Smith *et al.*, 1993). These two methods have been extensively reviewed by Milgroom (1996). In the two-locus gametic disequilibrium method, gametic disequilibrium is analysed between pairs of loci. Estimation of gametic disequilibrium (Table 1) is done by comparing the observed gametic frequencies to those expected as products of allele frequencies. This method has been used in various studies involving multilocus population structure using phenotypic loci (Brown & Wolfe, 1990; Welz & Leonard, 1995) or genetic loci (Geiser *et al.*, 1994; McDonald *et al.*, 1994, 1995). The other method, the index of association estimates overall nonrandom associations of alleles between loci (Maynard Smith *et al.*, 1993). This analysis is based on the variance in the number of heterozygous loci found in pairwise comparisons of all gametes (or haploid individuals).

Direct methods. The presence of sexual structures under field conditions is an indication that a species has the potential to reproduce sexually (Louw *et al.*, 1994). However, this scenario should be approached with caution as

there are documented reports in which sexual structures have been found, yet statistically the mode of reproduction was found to be clonal (Wolfe & McDermott, 1994).

Mating-type surveys are the simplest way to detect the potential for sex and recombination in some species (Milgroom, 1996). For heterothallic species, populations that have only a single mating-type cannot be mating randomly. If populations are randomly mating, the mating-type ratio is expected to be 1:1. This was found in a population of 833 isolates of *Cochliobolus carbonum* (Welz & Leonard, 1995).

Inferences of sexual reproduction can also be made on the grounds of increases in genetic diversity. Allele frequencies form the basis to measure genetic diversity (Drenth, 1998). Allele frequencies are preferred to genotypic frequencies because allele frequencies remain relatively stable over time and are independent of the mating system, whereas genotypic frequencies are randomised at each generation of mating.

Genetic diversity can be measured in a number of ways: firstly, by calculating the number of polymorphic loci; secondly, by calculating the average frequency of heterozygous individuals per locus (heterozygosity); thirdly, by calculating the gene diversity (allelic diversity index) (Table 1), which is a measure of the probability of obtaining two different alleles at a locus when two haploid individuals are sampled from a population; and fourthly, by calculating the genotypic diversity. The advantage of using the gene diversity index is that this statistic is applicable to both sexual and asexual populations. For determining the genotypic diversity index, there are again various measures that can be employed of which the most widely used are the Shannon diversity index (Goodwin *et al.*, 1993) (Table 1) and the determination of the clonal fraction (Drenth, 1998) (Table 1). The Shannon diversity index has a great advantage over the clonal fracton measure in that it is relatively stable when sample sizes differ (Peever & Milgroom ,1994).

Sexual populations have a greater diversity of genotypes than asexual populations (Groth & Roelfs, 1982; Tooley *et al.*, 1985; Welz & Kranz, 1987). This direct method can also be statistically conducted, by comparing the observed diversity to that expected under random mating (Stoddart & Taylor,

1988). This approach to ascertain random mating has been used for populations of *Pyrenophora teres* (Peever & Milgroom, 1994) and *Cryphonectria parasitica* (Milgroom *et al.*, 1992).

Another method to test for deviations from random mating is to assess correlations between genetic markers and phenotypes such as pathotypes (Burdon & Roelfs, 1985) or vegetative compatibility (Kohn *et al.*, 1991).

2. Direct observation of recombinant genotypes

Using molecular genetic techniques it is possible to assess populations over a given period of time, and in due course to identify novel recombinant genotypes (Tibayrenc *et al.*, 1991; Sujkowski *et al.*, 1994). In a population of *Phytophthora infestans*, 247 isolates were collected over a period between 1985 and 1991. Between 1985 and 1987 all isolates were found to be of the A1 mating-type, and consisted of a single clonal lineage based on allozyme and DNA fingerprint analyses. The A2 mating-type was first detected in 1988. At a later period a novel genotype was detected which was then concluded to have evolved due to sexual reproduction. Another example is provided by Goodwin *et al.* (1995), also involving a population of *P. infestans* in which novel genotypes were found and thought to have arisen due to sexual reproduction following the presence of both mating-types in the same field.

To gain a better understanding of the epidemiology of plant-fungal interactions it is sometimes necessary to determine if recombination can occur following mating between e.g., pathotypes, under laboratory conditions. Novel recombinant genotypes following mating between W- and C-pathotypes of *Tapesia yallundae* were identified using RAPD markers (Nicholson *et al.*, 1995). Other similar studies have been reported (Dyer *et al.*, 1993, 1994; Daniels *et al.*, 1995). There are also reports for the detection of recombination under laboratory conditions using phenotypic markers. Smedegård-Petersen (1971) could not morphologically distinguish the double-type symptom producing isolates of *Pyrenophora teres*, but could induce mating between the two types. He concluded that the two types were formae of the same species and, therefore, named them *P. teres* f. *teres* (net-type symptom) and *P. teres* f. *maculata* (spot-type symptom). In later studies

involving mating between the two formae of *P. teres*, Smedegård-Petersen (1976, 1977) demonstrated that ascospore progeny from a net x spot mating caused intermediate symptoms on susceptible cultivars. This indicated that recombination had taken place and, therefore, proved that the two formae were indeed of the same species.

PYRENOPHORA TERES: A PATHOGEN OF BARLEY

Pyrenophora teres (anamorph: *Drechsler teres*) the causal agent of net blotch of barley (*Hordeum vulgare*) is an economically important pathogen throughout most barley growing regions of the world (Shipton *et al.*, 1973; Jordan *et al.*, 1985; Delsereone & Cole, 1987; Steffenson *et al.*, 1991; Louw *et al.*, 1996). Yield losses of up to 77% have been reported for *P. teres* f. *teres* (Steffenson *et al.*, 1991), while losses up to 26% have been reported for *P. teres* f. *maculata* (Khan, 1989). Net blotch is a foliar pathogen, thereby having a detrimental effect on photosynthesis in the leaves, resulting in a reduction in the production of sugars required for growth (Shipton *et al.*, 1973). This in turn leads to yield losses attributed mainly to a reduction in 1000-kernel weight, and also to a reduced number of kernels per ear (Jordan, 1981).

Taxonomy of *Pyrenophora*

Nisikado originally divided the graminicolous *Helminthosporium* species into two subgenera based upon conidium morphology and germination (Sivanesan, 1987). Species with straight, cylindrical conidia (Fig. 3) that germinate by one or more germ tubes from any conidium cell were placed in the subgenus Cylindro-*Helminthosporium*. Those species with fusoid, often curved conidia and bipolar germination were grouped in the subgenus Eu-*Helminthosporium*. Ito raised the subgenus Cylindro-*Helminthosporium* to generic rank as *Drechslera*, and indicated that these species are often associated with *Pyrenophora* teleomorphs. It was suggested by Hughes (Sivanesan, 1987) that these species be correctly referred to as *Drechslera* and *Helminthosporium* subgenus Eu-*Helminthosporium*, for which a generic

name needed to be proposed at that time. However, Shoemaker (Sivanesan, 1987) recognised the species placed in the subgenus Eu-*Helminthosporium* as distinct from those in *Drechslera*, and therefore proposed the generic name *Bipolaris* for these taxa. In addition, the latter genus is characterised by fusoid, straight to curved conidia that germinate by one germ tube from each end, and is associated with *Cochliobolus* teleomorphs.

Drechslera spp. are separated on differences in their conidia and conidiophore morphology, as well as cultural criteria (Drechsler, 1923; Smith & Rattray, 1930; Pon, 1949; Putterill, 1954; Braverman, 1960; Kenneth, 1962; Shoemaker, 1962; Scott, 1991).

Much confusion still exists regarding the separation of net- and spot-type isolates by means of morphology and other cultural criteria. Ito and Kuribayashi (1931) distinguished *P. teres* from *P. japonica* based on width and colour of conidia and size of ascospores. Furthermore, they indicated few other morphological differences between *P. teres* and *P. japonica* and stated that the main difference was in the symptoms produced on barley. However, Shipton *et al.* (1973) warned that identification on the basis of host specificity and symptomatology is unreliable.

Ito and Kuribayashi (1931) claimed that "claw-like bands" were constant phenomena in *P. teres* cultures in Japan, whereas in *P. japonica* cultures, "fan-like bands" of white or greyish aerial mycelium were formed instead. However, Kenneth (1962) observed fan-like bands in *P. teres* cultures and concluded that differentiation between *P. japonica* from Japan and *P. teres* from Israel by means of these structures was not feasible.

In another study on *Pyrenophora*, Shoemaker (1962) recognised *P. japonica* as a cause of leaf spots on barley and concluded that symptoms could be used to differentiate between *P. teres* and *P. japonica*. Morphologically important differences reported to exist between these two forms were that conidia of *P. japonica* lacked an inflated basal cell, and regularly had secondary conidiophores. McDonald (1967) reported that an isolate similar to *P. japonica* mated with an isolate of *P. teres*. He was therefore of the opinion that the *P. japonica* isolate used by Ito and Kuribayashi (1931) was a mutant strain of *P. teres*.

Smedegård-Petersen (1971) mated isolates producing net- and spot-type symptoms and concluded that the two types were forms of the same biological species, and therefore proposed the names *P. teres* f. *teres* for the net-type producing isolates and *P. teres* f. *maculata* for the spot-type producing isolates. Smedegård-Petersen (1971) found only a few inconspicuous morphological differences between the two forms of *P. teres*. Furthermore, successful matings between *P. teres* and *P. graminea* were produced, resulting in progeny segregating into net, stripe, spot and intermediate forms. Although Karki and Sharp (1986) and Bockelman *et al.* (1983) could not find any differences in conidium morphology or colony characteristics between isolates of *P. teres* f. *teres* and *P. teres* f. *maculata*, Scott (1991) separated these two forms on the basis of morphological differences and described them as *P. teres* and *P. japonica*.

Due to conflicting reports concerning the identity of the *Pyrenophora* isolates producing leaf spots on barley in South Africa, there is still uncertainty about the species involved (Scott, 1991, 1994, 1995; Crous *et al.*, 1995; Louw *et al.*, 1995; Den Breejen *et al.*, 1996). The net-type and spot-type forms of *P. teres* seem to cause disease on different types of cultivars, and are, furthermore, also controlled by different chemical control programmes (Scott, 1995), thereby making identification of the correct species important.

Pathogenicity and sources of inoculum

Isolates of *P. teres* f. *teres* and *P. teres* f. *maculata* are pathogenic to differentially susceptible barley cultivars, i.e., both formae are not pathogenic to the same cultivars. Furthermore, wild grass species that are present in the near vicinity of barley fields may serve as alternative hosts (Scott, 1991; Brown *et al.*, 1993). Shipton *et al.* (1973) identified 47 species in 18 genera of Poaceae that may serve as alternative hosts while Brown *et al.* (1993) identified 38 new species in 16 genera, four of them never reported to contain *P. teres*. A further interesting feature of these alternative hosts reported by Louw *et al.* (1996) was isolates that produced spot-type symptoms on *Hordeum murinum* (wild barley) produced net-type symptoms on susceptible barley cultivars.

The primary sources of inoculum of *P. teres* are infected barley stubble (Shipton *et al.*, 1973; Jordan, 1981; Jordan & Allen, 1984; Van den Berg & Rossnagel, 1991) and seed (Smith & Rattray, 1930; Shipton *et al.*, 1973; Jordan, 1981; Martin, 1985). Ascospores may also serve as a source of inoculum (Smedegård-Petersen, 1972; Jordan, 1981). Localised populations of *P. teres* can spread over short distances in a field or to nearby fields as wind- and rain-borne spores. On the other hand, net blotch has the capacity for long distance dispersal in infected seed (Peever & Milgroom, 1994).

Variation in symptom expression

Worldwide two forms of net blotch are described based on the symptoms produced on susceptible barley cultivars. Net-type isolates of *P. teres* f. *teres* produce dark brown blotches crisscrossed with a net-like venation and accompanied by chlorosis (Smedegård-Petersen, 1971) (Fig. 3). On the other hand, spot-type isolates of *P. teres* f. *maculata* produce spots of various shapes and sizes (Fig. 3), encircled by varying widths of chlorosis and often water-soaked tissue. Symptoms produced by spot-type isolates are often more variable, producing elliptical, fusiform or irregularly shaped necrotic lesions on leaves, or lesions that vary from dark brown spots to solid stripes, spreading longitudinally between leaf veins (Tekauz & Mills, 1974; Scott, 1991).

Symptoms produced by spot-type isolates are also similar to those produced by the spot blotch pathogen *Cochliobolus sativus* (Tekauz & Mills, 1974), cultivars that are resistant to *P. teres* f. *teres*, genetic necrosis (Karki & Sharp, 1986) or boron toxicity (Scott, 1991). Resistant reactions to net-type isolates are exhibited as small spots with no chlorotic halos. There have also been reports that symptoms produced by spot-type isolates of *P. teres* could also differ between different countries (Khan & Tekauz, 1982; Bockelman *et al.*, 1983; Karki & Sharp, 1986). In some countries spot-type isolates are ascribed to *P. japonica* and net-type isolates to *P. teres* (Ito & Kurabayashi, 1931; Scott, 1991).

Smedegård-Petersen (1976, 1977) showed that after successful mating between net- and spot-type isolates of *P. teres*, the progeny produced

not only net and spot symptoms, but also intermediate symptoms on susceptible barley cultivars. Other intermediate symptom types were also produced following successful mating between isolates of *P. teres* and the barley leaf stripe pathogen, *P. graminea* (Smedegård-Petersen, 1977, 1978).

Sexual reproduction

Like many other Ascomycetes, sexual reproduction in *P. teres* occurs during unfavourable growth conditions i.e., low temperatures (10-15°C) and insufficient nutrients, in contrast to asexual reproduction which occurs at more favourable growth temperatures (20-25°C) and when nutrient supplies are in abundance (Shipton *et al.*, 1973).

Pyrenophora teres has a heterothallic mating system (McDonald, 1963, 1967) similar to that of the well-characterised ascomycete, *N. crassa* (Fig. 1). Unlike many other ascomycetes for which the sexual stage can be produced within a reasonably short time span under laboratory conditions, pseudothecia of *P. teres* may take up to nine months to reach maturity (Fig. 3). McDonald (1963) reported that the minimum period required for ascospore formation was two months.

Despite the long incubation period required for successful mating, production of fruiting bodies between isolates of the same type of *P. teres* has been accomplished (McDonald, 1963, 1967; Smedegård-Petersen, 1972; Peever & Milgroom, 1992, 1994)(Fig. 3). Furthermore, Smedegård-Petersen (1976, 1977) and Crous *et al.* (1995) were able to obtain fruiting bodies following successful mating between net- and spot-type isolates of *P. teres*. However, whether net x spot mating occurs under field conditions is still unclear.

Pseudothecia containing spot-type ascospores have been isolated from barley stubble in the Western Cape province of South Africa (Louw *et al.*, 1994), while the net-type sexual stage has been isolated in California (Cartwright *et al.*, 1988).

Resistance breeding

Breeding for disease resistance is one of the main strategies employed in controlling net blotch on barley (Douyssi *et al.*, 1998; Richter *et al.*, 1998; Williams *et al.*, 1999). For such breeding strategies to be successful, especially for broad-based resistance to this pathogen, knowledge of the virulence diversity of populations is required (Jalli & Robinson, 2000). Virulence surveys have shown that both net- and spot-type populations are composed of a large number of pathotypes and furthermore, that susceptible cultivars are differentially susceptible to either net-type or spot-type isolates but rarely to both (Tekauz, 1990; Steffenson & Webster, 1992; Jonsson *et al.*, 1997). Literature concerning screening of populations on different barley cultivars is vast (Khan, 1982; Tekauz, 1990; Ho *et al.*, 1996; Robinson & Jalli, 1996; Jonsson *et al.*, 1997, 1999; Douyssi *et al.*, 1998; Jalli & Robinson, 2000). Furthermore, it has become important to have an international differential set of barley lines for assessing pathogen virulence in different countries (Steffenson & Webster, 1992; Afanasenko *et al.*, 1995; Jonsson *et al.*, 1997). On a global scale this would enable variations in virulence between different net blotch populations to be standardised.

The reaction of barley cultivars to *P. teres* is usually tested at the seedling stage in bioassays when plants are inoculated with the pathogen. A resistant seedling reaction is considered desirable because of a high degree of correlation with adult responses under field conditions (Tekauz, 1986). Resistant genotypes are characterized by having fewer numbers and a reduced size of visible foliar lesions, diminished and delayed sporulation on mature leaves, a restricted growth of fungal mycelium within infected leaf tissue, and an increased production of antifungal substances in the leaves (Keeling & Bantari, 1975; Tekauz & Buchannon, 1977).

Information on the number and chromosomal location of loci controlling resistance to net blotch would greatly facilitate the development of resistant cultivars (Steffenson *et al.*, 1996). Early investigations indicated the presence of one to three genes controlling net-type net blotch resistance. Schaller (1955) first reported that resistance to net blotch in Tifang barley was conditioned by a single dominant gene (*Pt₁*). Later, Mode and Schaller (1958)

identified two additional genes (Pt_2 and Pt_3) responsible for net blotch resistance. Furthermore, it was discovered that Pt_1 and Pt_2 were tightly linked at 2.57 recombination units, but were not linked to Pt_3 . Khan and Boyd (1969) reported a fourth resistance gene (Pt_a) that segregated independantly of the V (two-row) gene. Bockelman *et al.* (1977), using trisomic analysis, identified resistance genes ($Rpt1a$, $Rpt1b$, $Rpt2c$ and $Rpt3d$) in Tifang barley and two other cultivars. In another investigation, Wilcoxson *et al.* (1992) found that net blotch resistance was conditioned by one or possibly two genes. These early studies produced differing results due to the fact that different mixtures of isolates were used in the inoculation tests. However, as pointed out by Steffenson and Webster (1992), these results are expected because pathotypes vary from region to region and virulence varies from pathotype to pathotype.

Little progress has been made in identifying resistance genes against spot-type isolates. Recently, however, Williams *et al.* (1999) identified and mapped a single resistance gene ($Rpt4$) in Australia in a cross between "Galleon" (spot resistant) and "Haruna Nijo" (spot susceptible).

Progress in determining the chromosomal locations of important disease resistance loci has been accelerated by the development of molecular genome maps (Graner *et al.*, 1991; Kleinhofs *et al.*, 1993). With the help of these genome maps, the number and location of loci encoding net blotch resistance, also termed quantitative trait loci (QTLs) has become possible with multiple molecular markers using AFLPs (Richter *et al.*, 1998) and RFLPs (Graner *et al.*, 1996).

Despite the vast literature pertaining to the identification and mapping of resistance genes to net blotch in barley, a single major gene controlling virulence was recently identified in *P. teres* f. *teres* (Weiland *et al.*, 1999). This was achieved by mating two isolates, one with high and another with low virulence. Of the 82 progeny tested, 42 exhibited high virulence and 40 exhibited low virulence on "Harbin" barley, supporting a single major gene theory as indicated by the 1:1 ratio. Furthermore, RAPD markers were obtained that were associated with low virulence.

Induced resistance

Induced resistance is a control strategy whereby host plants can be protected against virulent pathogens by prior inoculation with virulent or avirulent isolates of pathogens from another host (Hammerschmidt & Yang-Cashman, 1995; Steiner & Schönbeck, 1995). Preinoculation of barley leaves with either of two nonbarley pathogens, *Bipolaris maydis* from maize or *Septoria nodorum* from wheat, 24 hours in advance of inoculation with a virulent isolate of *D. teres* f. *maculata* resulted in significantly reduced infection by the latter organism (Lyngs Jørgensen *et al.*, 1996). Reductions in disease severity measured 39-70% and 22-65% after preinoculation with *B. maydis* and *S. nodorum*, respectively. In addition, the disease-reducing capacities exerted by the two organisms were effective against *D. teres* in different barley cultivars.

Chemical control

Fungicides are also routinely used for the control of net blotch on barley (Jordan, 1981; Sutton & Steele, 1983; Khan, 1987; Martin & Sanderson, 1988; Olvång, 1988; Van den Berg & Rossnagel, 1990; Steffenson *et al.*, 1991; Toubia-Rahme *et al.*, 1995). Control is primarily exercised by means of seed treatments or foliar sprays.

Triazole fungicides, a class of sterol demethylating inhibitors (DMIs) that inhibit the C14 demethylation step in fungal ergosterol biosynthesis (Copping *et al.*, 1984; Scheinpflug & Kuck, 1987), have been the most widely used. Propiconazole has been most often used as a foliar spray (Martin & Sanderson, 1988; Van den Berg & Rossnagel, 1990; Scott *et al.*, 1992). In Canada, control of net blotch in susceptible cultivars increased grain yields by 23% with application of propiconazole at ear emergence and 34% when the fungicide was applied at both tillering (Fig. 4) and ear emergence (Martin & Sanderson, 1988). It has also been reported that spot-type is more difficult to control than net-type (Van den Berg & Rossnagel, 1990; Scott *et al.*, 1992) on susceptible cultivars. A double application of propiconazole is necessary for reliable control of spot-type net blotch.

Triadimenol has been used extensively as a seed treatment fungicide for the control of net blotch and other barley pathogens (Sheridan & Grbavac, 1985). However, there have been reports of widespread resistance of net blotch towards triadimenol (Sheridan *et al.*, 1985). The first report was from New Zealand where net-type isolates were isolated from a new barley cultivar that did not respond to triadimenol treatment. *In vitro* investigations using potato dextrose agar amended with triadimenol indicated that several isolates had IC₅₀ values of 50-60 µg/ml. This build-up of resistance was attributed to a resistant isolate that was brought into New Zealand. It was therefore suggested by Sheridan and Grbavac (1985) that new cultivars be screened for their response to disease before a seed treatment is recommended. Furthermore, Peever and Milgroom (1992) reported the inheritance of triadimenol insensitivity in progeny following mating between triadimenol resistant and triadimenol sensitive isolates. From these studies the margin between sensitive and resistant isolates was shown to be 10 µg/ml.

Field isolates of other cereal pathogens including *Erysiphe graminis* (Butters *et al.*, 1984; De Waard *et al.*, 1986) and *Rhynchosporium secalis* (Kendall *et al.*, 1993) have also shown resistance to triadimenol.

Cereal pathogens have also been controlled using benzimidazole fungicides (methyl-benzimidazole-carbamate-generating [MBC] fungicides) that act by affecting tubulin synthesis (Davidse, 1973). Studies have indicated that benomyl and carbendazim may stimulate disease development of *P. teres*, especially at recommended field rate concentrations (Jordan & Best, 1981; Toubia-Rahme *et al.*, 1995). This was partly due to the wide utilisation of benzimidazoles in Europe. Studies have also indicated that stimulation of disease development can differ between the two types of net blotch (Toubia-Rahme, 1992; Toubia-Rahme *et al.*, 1995). Triadimefon inhibited net blotch development of the spot-type at the recommended field rate when applied preventatively. However, when applied curatively, it stimulated disease development of the net-type of *P. teres*. Benomyl applied curatively at the recommended field rate enhanced the sporulation of the spot-type, but had no effect on the net-type.

Resistance to MBC fungicides has been reported in other cereal pathogens such as *Tapesia yallundae* (King & Griffin, 1985), *Septoria tritici* (Griffin & Fisher, 1985) and *Rhynchosporium secalis* (Kendall *et al.*, 1994).

Studies using the herbicides glyphosate and paraquat have also been done (Toubia-Rahme *et al.*, 1995a) to determine their effect in controlling *D. teres*. Although these herbicides had an inhibitory effect on sclerotoid formation for both types, it was indicated that the net-type was more sensitive than the spot-type.

Molecular characterisation of *P. teres*

To date, molecular data pertaining to *P. teres* has focussed primarily on systematic studies and population structure. The first systematic study done involved distinguishing the two morphologically similar species *P. teres* and *P. graminea* using RAPDs (Reeves & Ball, 1991). *P. graminea* is strictly seed-borne, whereas *P. teres* can be seed-borne but is mainly transmitted locally from plant debris. For seed health testing to be effective in the management of these diseases, each pathogen must be unambiguously identified. Identification is difficult using conventional methods such as agar plate and blotter tests, and also time-consuming if pathogenicity testing is done. In another study, Peltonen *et al.* (1996), employing RAPDs, obtained a similarity coefficient of only 42% between *P. teres* and *P. graminea*. Distinction between *P. teres* and other *Pyrenophora* spp. (Louw *et al.*, 1995) as well as other related *Helminthosporium* spp. (Peltonen *et al.*, 1996) using RAPD markers has also been reported. RAPDs have not been able to reveal reliable differences between net- and spot-type isolates of *P. teres* (Louw *et al.*, 1995), although the stability of lesion types suggested them to be genotypic characters (Smedegård-Petersen, 1977). Reeves and Ball (1991) indicated that these two types could be separated based on RAPD fingerprints, but their analysis was based on only one to two isolates. Using A+T-rich DNA banding patterns, it was revealed that *P. japonica* and *P. teres* f. *maculata* were almost identical, after which Crous *et al.* (1995) suggested that *P. japonica* be treated as a synonym of *P. teres*.

The genetic structure of *P. teres* populations from various countries has been investigated using RAPD markers (Peever & Milgroom, 1994; Peltonen *et al.*, 1996; Jonsson *et al.*, 2000). In all three studies the number of genetic loci used for determining allele frequencies varied between eight (Peever & Milgroom, 1994) and 20 (Peltonen *et al.*, 1996). Using Nei's G_{ST} index (Nei, 1973), allele frequencies (allelic diversity) among five populations was partitioned into within- and among-population components (Peever & Milgroom, 1994). A G_{ST} value of 0.46 was obtained among all populations indicating that approximately 46% of the total genetic variability detected was due to differentiation among populations compared with 54% within populations. Furthermore, five to nine multilocus genotypes were found in each population, each of which consisted of 22-35 isolates. Gametic disequilibrium values (nonrandom associations of RAPD loci) were used to assess whether sexual reproduction was occurring within populations. In four of the five populations highly significant values were obtained, thereby indicating that the genetic structures of these populations were consistent with random sexual reproduction. Jonsson *et al.* (2000) also indicated that there was a limited amount of gametic disequilibrium within a field in Sweden. Their results were supported by the fact that all the isolates sampled represented a unique genotype. Jonsson *et al.* (2000) postulated that a larger variation in genotypes obtained in their study than compared to the study carried out by Peever and Milgroom (1994) was due to climatic and growing conditions, which influences the amount of sexual and asexual reproduction, and furthermore, to selection pressure exerted by barley cultivars.

A preliminary study (Wu *et al.*, 1993) to investigate the suitability of RFLP markers in the determination of *P. teres* population structures has been done. Using a randomly selected 0.5 kb fragment from an isolate of *P. teres* f. *teres* as a probe, unique DNA banding patterns were obtained for every isolate in a sample comprising *P. teres* f. *teres*, *P. teres* f. *maculata* and *P. graminea* isolates. DNA probes to distinguish *Pyrenophora* spp. have also been proposed by Baltazar *et al.* (1995).

Taking into consideration the molecular data available on *P. teres* and the current uncertainty with regard to the identification of *Pyrenophora*

species (spot-type isolates) in South Africa, a study with the following objectives was proposed:

1. To set up a series of net-spot matings using verified *P. teres* f. *teres* isolates and unknown isolates causing spot symptoms; following mating to subject the putative hybrids to a series of molecular techniques to determine whether recombination took place; to determine whether putative hybrids displayed a different symptom on susceptible cultivars and to determine if the putative hybrids showed an increased level of resistance to fungicides used against *P. teres*.
2. To ascertain the stability/viability of the putative hybrids following a series of inoculation/isolation procedures on susceptible barley cultivars.
3. To determine the levels of genetic diversity within separate populations of net- and spot-type isolates and thereby predict the presence of the sexual stage under field conditions. Furthermore, to determine if any sexual relationship exists between net- and spot-type isolates.
4. To study the levels of fungicide resistance in South Africa by comparing a local net- and spot-type population of *P. teres* to commercially used fungicides.

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Table 1. Various equations used in the statistical genetic analysis of population structure

| Index (name) | Equation | Description of terms | Statistical use | Reference |
|-------------------------------|--|--|--|------------------------------|
| Gametic disequilibrium index | $D=(p_1q_1)(p_2q_2)-(p_1q_2)(p_2q_1)$ | p_1 and p_2 are the frequencies of alleles 1 and 2 at one locus and q_1 and q_2 are the frequencies of alleles 1 and 2 at another locus | Determination of random mating (sexual recombination) within a field | Hartl & Clark (1989) |
| Allelic diversity index | $H=1-\sum p_i^2$ | p_i is the frequency of the i th allele | Allelic diversity | Nei (1973) |
| Clonal fraction | $Cf=(N-C)/N$ | N is the sample size and C is the number of distinct genotypes (clones) | Fraction of clones in a population | Drenth (1998) |
| Shannon index | $SI=-\sum p_i \ln p_i$ And $SI_M=SI/\ln k$ | p_i is the frequency of isolates with the i th phenotype SI_M is the normalised SI value after normalising the sample size; k is the number of isolates in the population | Genotypic diversity | Goodwin <i>et al.</i> (1993) |
| Genetic differentiation index | $G_{ST}=(H_T - H_S)/H_T$ | H_T is the total gene diversity; H_S is the average gene diversity within a population | Determination of diversity between populations | Nei (1973) |

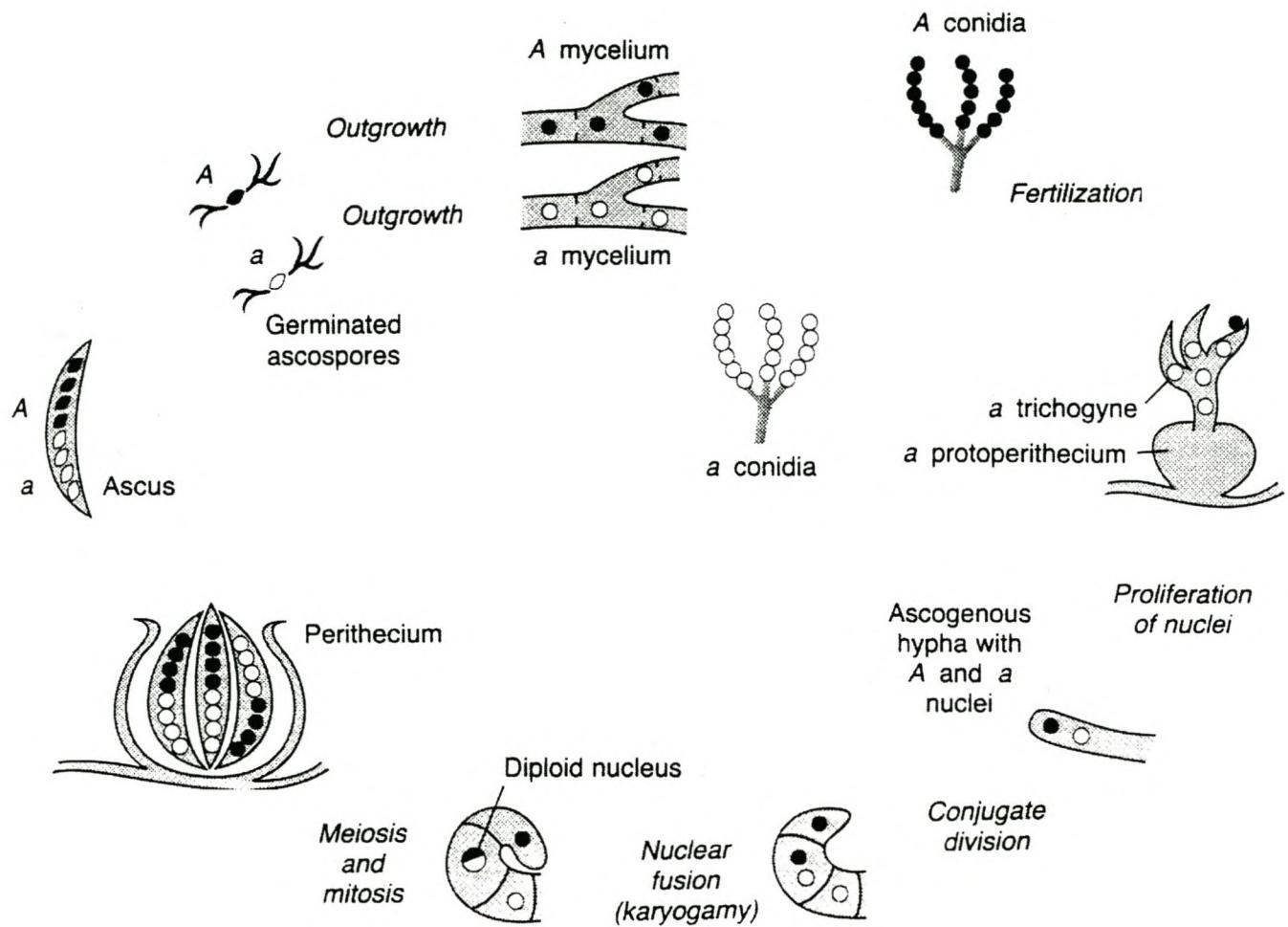


Fig. 1. The life cycle of *Neurospora crassa* (Metzenberg & Glass, 1990).

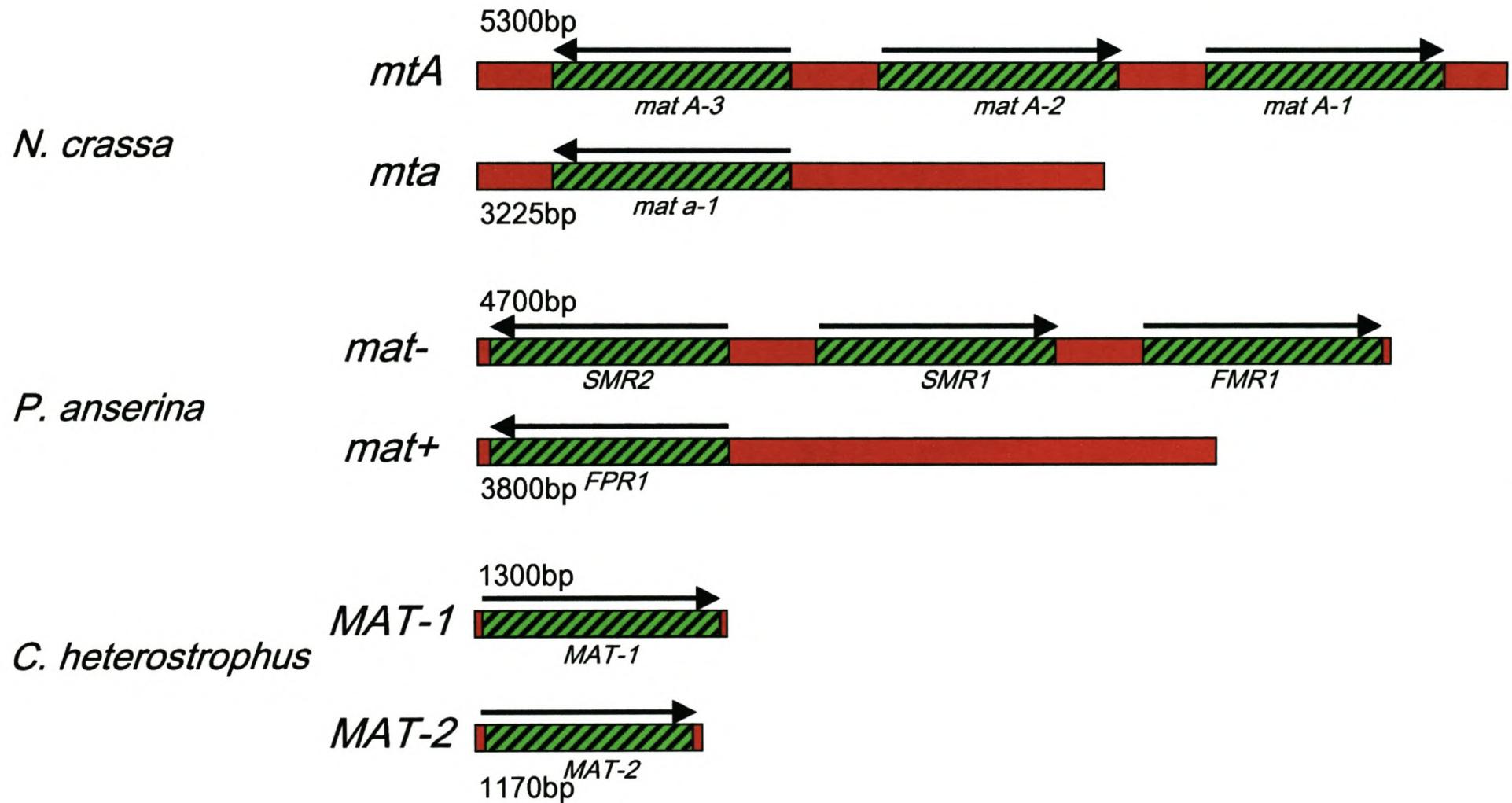


Fig. 2. Comparison of the mating-type idiomorphs in Ascomycetes. Idiomorph sizes are given in basepairs (bp). Arrows indicate direction of coding sequences of genes. Striped boxes indicate genes within the idiomorphs.

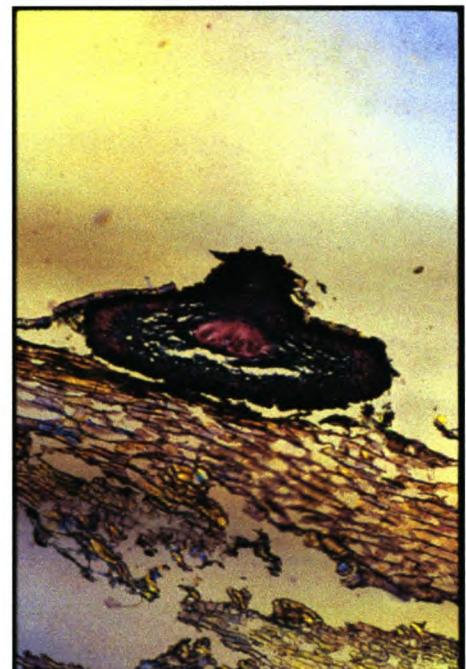
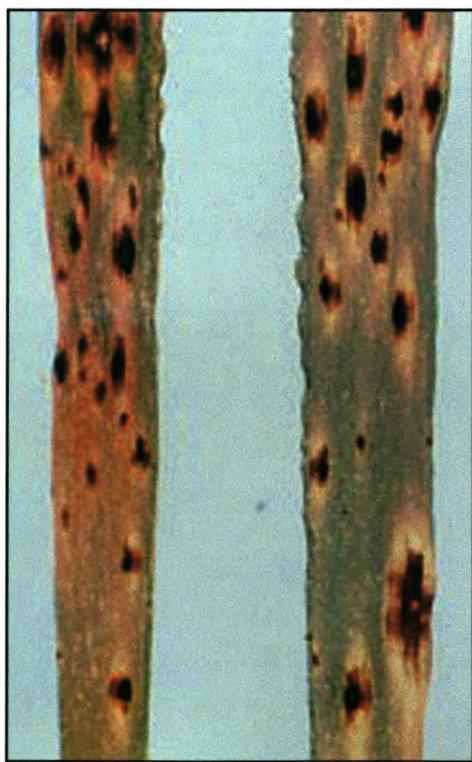


Fig. 3. Leaf symptoms and morphological structures of *Pyrenophora teres*. (a) Net-type symptoms of *P. teres* f. *teres*. (b) Spot-type symptoms of *P. teres* f. *maculata*. (c) 4-8 septate conidia of *P. teres*. (d) Mating studies done with *P. teres* isolates. (e) Vertical section through a perithecium.

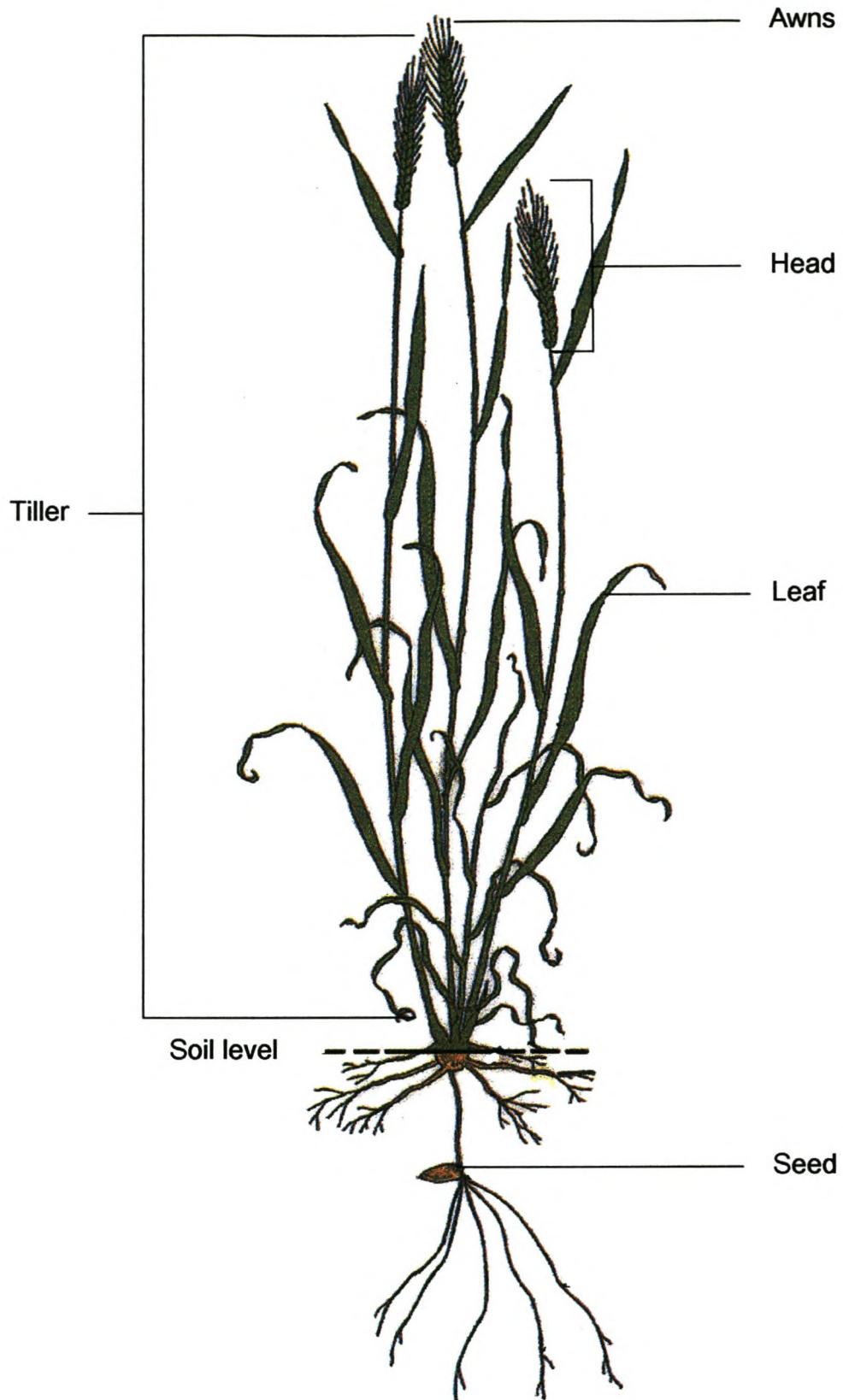


Fig. 4. Graphic representation of a mature barley plant.

2. *Pyrenophora teres* f. *maculata*, the cause of Pyrenophora leaf spot of barley in South Africa*

ABSTRACT

Net blotch caused by *Pyrenophora teres* is a serious disease of barley in many cereal production areas world-wide, including the Western Cape province of South Africa. The pathogen occurs as two forms, namely *P. teres* f. *teres*, which produces net-blotch symptoms, and *P. teres* f. *maculata* which produces leaf spots. *Pyrenophora japonica* and *P. hordei*, which have also been reported in South Africa, also produce spots on susceptible barley cultivars. Using RAPD markers, spot-forming isolates from the South African population were found to be relatively uniform. Single ascospores were obtained from pseudothecia after *in vitro* mating had occurred between a verified *P. teres* net-blotch isolate from Denmark and a representative Pyrenophora leaf spot isolate from South Africa. Using amplified fragment length polymorphism (AFLP) and RAPD markers, recombination was demonstrated in the progeny which had DNA banding patterns different from the two parental isolates. Pathogenicity trials also confirmed that recombination had taken place during mating. Inoculations were conducted on the differential cultivars susceptible to the net-blotch and leaf spot forms. The two parents induced typical net-blotch or leaf spot symptoms whereas the progeny mostly induced a jagged spot symptom on each cultivar. Fungicide sensitivity tests using the ergosterol biosynthesis inhibitors triademolin, bromuconazole and triticonazole showed that, due to recombination, some progeny could have increased resistance to these fungicides. Due to mating

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and subsequent recombination between a net blotch isolate of *P. teres* and a representative leaf spot isolate, it was concluded that the latter was *P. teres* f. *maculata*. These results contrast with the earlier belief that Pyrenophora leaf spot isolates present in the Western Cape are *P. japonica* and *P. hordei*.

INTRODUCTION

Correct identification of the causal agent of a disease on agricultural crops as well as a knowledge of the genetic variability of the pathogen (McDonald & Martinez, 1990) are important for breeding programmes directed at producing resistant cultivars, and for developing strategies to exploit resistance (Leung *et al.*, 1993).

Pyrenophora teres Drechsler (anamorph *Drechsler teres* [Sacc.] Shoemaker) the cause of net blotch of barley (*Hordeum vulgare* L. emend. Bowden) is an economically important pathogen in South Africa and throughout most other barley growing regions in the world (Shipton *et al.*, 1973; Steffenson *et al.*, 1991; Louw *et al.*, 1996). Two types of leaf symptoms are associated with net blotch, a net-like symptom which produces elongated, light brown lesions with dark brown reticulations, and a leaf spot symptom which is dark brown spot with a distinct halo (Smedegard-Petersen, 1971). *P. japonica* S. Ito and Kurib. [anamorph *Drechslera tuberosa* (G. F. Atk.) Shoemaker] was originally identified as causing the leaf spot symptoms, whereas *P. teres* has been associated with net-blotch lesions (Ito & Kurabayashi, 1931; Shoemaker, 1962). McDonald (1967), however, reported after successfully mating net-blotch and leaf spot isolates, that the latter were mutant forms of *P. teres*. Smedegard-Petersen (1971) repeated these matings with Danish isolates, and concluded that the two types were forms of the same biological species, for which he proposed the names *P. teres* f. *teres* for the net-blotch causing isolates, and *P. teres* f. *maculata* Smed.-Pet. for the leaf spot form. Each form is capable of causing economic yield losses (Jordan, 1981; Jordan *et al.*, 1985; Martin, 1985; Deadman & Cooke, 1987; Delserone & Cole, 1987; Steffenson *et al.*, 1991). Scott (1991) identified the cause of leaf spot of barley in South Africa as *P. japonica*. This was

supported by Louw *et al.* (1994), who also identified the teleomorph from barley stubble and from crosses between leaf spot isolates in culture as *P. japonica*. More recently *P. hordei* Wallwork, Lichon & Sivan., which also causes a leaf spot of barley, has been isolated in South Africa (Scott, 1994; Den Breeyen *et al.*, 1996). Using restriction fragment banding patterns of A+T-rich total DNA, Crous *et al.* (1995) showed that verified *P. japonica* and *P. teres* f. *maculata* isolates were almost identical, and that *P. japonica* should be treated as synonym of *P. teres*. Furthermore, Crous *et al.* (1995) reported successful mating between a Danish leaf spot isolate and an Australian net-blotch isolate. When single ascospore isolates were inoculated onto differentially susceptible barley cultivars, net-blotch, leaf spot and intermediate symptoms were produced, suggesting that recombination had occurred. Pathogenicity trials conducted on putative spot x blotch hybrids in other studies by Smedegard-Petersen (1976, 1977) yielded similar results. Molecular genetic evidence for hybridity, however, was not obtained.

Due to conflicting reports concerning the identity of the *Pyrenophora* isolates producing leaf spots on barley in South Africa, there is still uncertainty about the species involved (Scott, 1991, 1994, 1995; Crous *et al.*, 1995; Louw *et al.*, 1995; Den Breeyen *et al.*, 1996). The net-blotch and leaf spot forms of *P. teres* both cause disease on a number of susceptible barley cultivars, but are controlled by different chemical control programmes (Scott, 1995), thereby making identification of the correct species important. The aim of the present study was, therefore, to mate a verified *P. teres* net-blotch isolate with a *Pyrenophora* leaf spot isolate from South Africa, and to subsequently analyse the progeny for recombination using amplified fragment length polymorphisms (AFLPs) (Zabeau & Vos, 1993) and random amplified polymorphic DNA (RAPD) (Williams *et al.*, 1990) as molecular genetic markers. Furthermore, symptom expression of the progeny on two differential barley cultivars was assessed, and the sensitivity of the parental isolates and the progeny to the major triazole fungicides used against this pathogen was also determined.

MATERIALS AND METHODS

Isolate maintenance and mating studies

The isolates used were: Pt90-8a (Danish net-blotch); KH334 (Australian net-blotch); Nap5 (South African net-blotch) and MP4 (South African leaf spot). Isolate MP4 was chosen as a representative of the South African Pyrenophaora leaf spot populations which was characterised using A+T-rich DNA banding patterns (Crous *et al.*, 1995) and RAPDs (unpublished results). Isolates were maintained on potato dextrose agar (PDA) slopes at 4°C. Matings of the four isolates in all possible combinations and as single isolates were made on barley agar (BA) consisting of sterile barley straws lain on water agar in Petri dishes (Louw *et al.*, 1995), which were kept moist in larger containers and incubated at 10° under nuv light for 1 yr. As only a few pseudothecia formed asci with ascospores after this period, plates were subsequently transferred to 4° for a further 6 mo. Protopseudothecia were periodically examined microscopically for the production of asci with ascospores. To induce ascospore release from pseudothecia, fruiting bodies were removed from barley stalks and soaked in water for 2 h. Pseudothecia were subsequently attached to the inner surface of a Petri dish lid with the aid of petroleum jelly. After 24-48 h at 25° in the dark, ascospores were ejected from the pseudothecia on to PDA plates. Single germinated ascospores were subsequently transferred to clean PDA plates, and stored on slants. Ascospore progeny were numbered GC1-GC23, and are maintained in the culture collection of the Department of Plant Pathology at the University of Stellenbosch, South Africa.

DNA isolation

Mycelial plugs from 7-day-old cultures were transferred to flasks containing 100 ml of a yeast extract and glucose medium (YEG) [8 g/l yeast extract and 5 g/l glucose]. Flasks were incubated on a rotary shaker at 150 rpm at 25° for 3 days. Due to excessive polysaccharide production by this fungus in liquid culture harvesting of mycelia with a Buchner funnel was difficult. Mycelia were, therefore, harvested by centrifugation at 5000 rpm for 5 min at 4°. Once

harvested, mycelium was stored at -80° until required, when it was crushed in liquid nitrogen with a mortar and pestle, and transferred to an Eppendorf tube containing 500 µl extraction buffer of 50 mM Tris (pH 7.2), 50 mM NaCl, 50 mM EDTA, and 3% (w/v) SDS. Then 350 µl phenol was added followed by 150 µl chloroform/isoamylalcohol (24:1 (v/v)). The suspension was mixed, incubated while being shaken at room temperature for 15 min, and subsequently centrifuged at 13 000 rpm for 60 min. The aqueous phase was transferred to a clean Eppendorf tube after which 25 µl RNase (10 mg/ml) (Boehringer Mannheim Chemicals, South Africa) was added and incubated for 30 min at 37°. An equal volume of chloroform was added followed by centrifugation at 13 000 rpm for 10 min. The aqueous phase was transferred to another tube and subjected to two more chloroform extraction procedures. DNA was precipitated with 0.54 vol. isopropanol and incubated at -20° for 2 h, and pelleted by centrifugation at 13 000 rpm for 5 min. The DNA was subsequently washed twice with 70% ethanol, dried, resuspended in 100 µl TE buffer [50 mM Tris (pH 8.0), 50 mM EDTA] and stored at -20° for future use. In DNA samples with excessive polysaccharides, the volume was increased to 400 µl with TE buffer. An equal volume of Phenol/chloroform/isoamylalcohol [25: 24: 1 (v/v)] was added followed by centrifugation at 13 000 rpm for 10 min. The aqueous phase was extracted three more times with an equal volume of chloroform/isoamylalcohol. The DNA was subsequently precipitated by the addition of 7.5 M NH₄OAc to a final concentration of 2 M and 2 vol. 100% cold ethanol. Samples were washed with 70% cold ethanol, dried and resuspended in 100 µl TE buffer.

RAPD analysis

Amplification reactions were performed in a final volume of 25 µl of reaction mixture. The reaction mixture contained 2.5 µl of 10X *Taq* DNA polymerase buffer [100 mM Tris HCl (pH 8.3), 15 mM MgCl₂, 500 mM KCl] (Boehringer Mannheim, South Africa); 200 µm of each dNTP; 10 pmol of oligonucleotide primer, 50 ng genomic DNA and 1 U of *Taq* DNA polymerase. The final MgCl₂ concentration was adjusted to 4 mM. Reaction mixtures were overlaid with 50 µl mineral oil to prevent evaporation during thermocycling. Primers

(Operon Technologies Inc., Alameda, U.S.A.) that gave discriminatory banding profiles for the two parental isolates and which could, therefore, reveal genetic recombination in the ascospore progeny were used for RAPD analysis; the primers as well as their respective sequences were:

OPE 7: 5' AGATGCAGCC 3'

OPE 15: 5' ACGCACAAACC 3'

OPM 10: 5' TCTGGCGCAC 3'

OPM 20: 5' AGGTCTTGGG 3'

Amplifications were made in a Biometra TRIO-Thermoblock TB1 (Gottingen, Germany). Reactions underwent an initial denaturation process at 96° for 120 s, followed by 30 cycles of 92° for 30 s, 38° for 30 s and 72° for 60 s. After the last cycle a final extension step was conducted at 72° for 120 s. Amplification products were separated through 1.5% (w/v) agarose gels in TAE buffer (Sambrook *et al.*, 1989).

AFLP analysis

Generation and selection of fragments. The AFLP analysis was performed following the procedure of Zabeau and Vos (1993). For preparation of primary template DNA, approximately 50 ng genomic DNA was digested at 37° for 1 h using 20 U of *Pst*I, 4 U of *Mse*I and 5 µl reaction buffer (One Phor All buffer, Pharmacia Biotech) in a final vol. of 50 µl. Following digestion the adapter molecules were ligated to the cleaved DNA fragments. This was done by adding 10 µl of a mixture containing 5 pmol of *Pst*I adapter; 50 pmol of *Mse*I adapter; 1.2 µl of a 10 mM ATP solution; One Phor All buffer and 1 U of T4-ligase (Pharmacia Biotech). This 60 µl restriction fragment ligation mixture was subsequently incubated at 37° for 2 h. The sequence of the *Pst*I adapters were:

5'-biotin-CTCGTAGACTGCGTACATGCA-3'

3'-CATCTGACGCATGT-5'

The sequence of the *Msel* adapters were:

5'-GACGATGAGTCCTGAG-3'

3'-TACTCAGGACTCAT-5'

Dynabeads M-280 streptavidin (Dynal, Oslo, Norway) were used to select biotinylated DNA fragments. Before use, the beads were washed with 1 vol. TE buffer and resuspended in 1 vol. TE buffer. To each DNA sample (60 μ l) 10 μ l of resuspended beads was added. Samples were subsequently incubated at room temperature for 30 min. The beads were then collected using a magnet (Dynal MPC). The supernatant was removed and the beads were washed 3 times with 200 μ l TE buffer. After the final washing step the beads were resuspended in 50 μ l TE buffer.

PCR amplification. Selective amplification was done with four AFLP primers specific for *PstI* adapters. Each primer contained two selective nucleotides at the 3' end. A total of four primer combinations was used as the sequence of the *Msel* primer was kept constant. The sequences of the *PstI* primers was:

PstI.1 5' GACTGCGTACATGCAGAC 3'

PstI.2 5' GACTGCGTACATGCAGAA 3'

PstI.3 5' GACTGCGTACATGCAGCC 3'

PstI.4 5' GACTGCGTACATGCAGCA 3'

The sequence of the *Msel* primer was:

5' GATGAGTCCTGAGTAAACA 3'

One primer was labelled according to the manufacturer's recommendations using 1 μ Ci gamma ^{33}P -ATP (Amersham) and 0.2 U of T4-kinase (Pharmacia Biotech). Each amplification was performed with 30 ng of labelled and 30 ng of unlabelled selective primer in the reaction mixture.

Each PCR reaction contained the following: 2 μ l PCR buffer [100 mM Tris-HCl (pH 8.0), 15 mM MgCl₂, 500 mM KCl], 1 μ l of template DNA, 1 μ l labelled *Msel* primer (30 ng), 1 μ l *PstI* primer (30ng), 3 μ l of a 1.25 mM solution, 0.2 μ l *Taq* polymerase (Gibco BRL) and 13 μ l ddH₂O. Amplifications were done in a Stratagene Robocycler Gradient 40. Reactions underwent an initial denaturation process at 94° for 60 s. This was followed by 10 cycles of 94° for 40 s, 62° for 60 s and 72° 60s. A further 25 cycles was conducted in

which the annealing temperature was lowered to 56° for 60 s. After the last cycle a final extension step was conducted at 72° for 20 min.

Reaction products were denatured at 95° for 3 min, snap-cooled on ice and run through 6% (w/v) denaturing polyacrylamide sequencing gels at 35 W for 3 h in 1X TBE buffer (Sambrook *et al.*, 1989) using a Model S2 sequencing gel system (Life Technologies, Inc., Gaithersburg, MD). The gels were dried with a Model 583 Bio-Rad gel dryer and exposed to Kodak Biomax MR1 X-ray films for 12 h at room temperature.

Data analysis

For RAPD and AFLP analyses, DNA fingerprints were evaluated by visual inspection of photographs of the gels, and autoradiographs respectively. All monomorphic and polymorphic fragments which were observed as dark intense bands were used for analysis. Data was scored in the form of the presence or absence of each fragment within each individual isolate and then pooled over all fragments and primer(s) (combinations) in order to assess the degree of (co)segregated fragments. Recombination in progeny was identified by the cosegregation of bands that distinguished the parents or by the absence of a band present in both parental isolates. The proportion of cosegregated bands in the progeny to the total number of bands was also assessed.

Pathogenicity trials

Mycelial plugs from 7-day-old cultures were transferred to flasks containing 100 ml YEG medium. Flasks were incubated at 25° on a rotary shaker at 150 rpm for 3 days. Mycelia were harvested by centrifugation at 5000 rpm for 5 min and subsequently resuspended in 25 ml dH₂O. This was followed by homogenisation of the mycelium with a Virtis homogenizer (Virtis Company Inc., U.S.A.).

The two differentially susceptible barley cvs Stirling and B87/14 (susceptible to net-blotch and leaf spot, respectively) were used in the pathogenicity trials. Plants were incubated at the two leaf stage in a glasshouse (20-15°, day/night temperature) using the technique described by

Louw *et al.* (1994). Plants were initially sprayed with a solution of 0.01% (v/v) Tween 20 to reduce the leaf surface tension, and subsequently sprayed to runoff with the mycelial suspensions. Plastic bags were then placed over the inoculated plants for 48 h to create moisture chambers. Control plants were sprayed with distilled water without inoculum. Plants were examined for symptom expression 10 days after inoculation.

Fungicide sensitivity

Both parental isolates and all the progeny were tested for their sensitivity towards the major triazole fungicides used to control net-blotch. The technique described by Robbertse *et al.* (1996) was followed. Mycelial plugs (3 mm in diam.) from 7-day-old cultures were placed in the centre of PDA plates amended with either triademinol, bromuconazole or triticonazole at various concentrations; these were 0, 1.0, 10.0, 30.0 and 60 µg/ml for triademinol and 0, 0.1, 0.3, 1.0, 3.0 and 10.0 µg/ml for the other two fungicides. All isolates were tested in triplicate at each concentration for each fungicide. Stock solutions were prepared for each fungicide by dissolving the fungicide in 70% ethanol. For each fungicide, control plates were amended to contain the same amount of solvent as plates containing the highest concentration of fungicide. Plates were incubated inverted at 25° for 5 days. Colony diameters were subsequently derived by averaging two perpendicular measurements and subtracting the diameter of the agar plug. The % inhibition for each isolate at each specific concentration was expressed as the proportion of radial growth on the fungicide-amended plates compared to growth on the control plates. After visual inspection a best curve was fitted to this data. The SAS/STAT software version 6.04 package was used to calculate the IC₅₀ values for each isolate. Non-linear regression was carried out to calculate the IC₅₀ for each isolate by regressing radial growth (as a proportion of the control) against log-transformed fungicide concentrations and using the fitted regression line to estimate IC₅₀ values.

RESULTS

Mating studies

Although protopseudothecia were formed in all combinations between isolates, fertile pseudothecia containing mature ascospores were produced only by the MP4 X Pt90-8a mating. From these, 23 single ascospore cultures were obtained.

RAPD analysis

The results of the RAPD analysis of the parental isolates and their progeny are shown in Fig. 1 and Table 1. Of the primers screened OPE 7, OPE 15 and OPM 10 and OPM 20 revealed DNA polymorphisms (unique bands) between the parents. All the primers with the exception of OPE 15 yielded unique marker bands in each parent. Recombination in the progeny was observed as the (co)segregation of unique parental RAPD marker bands. In the case of primer OPE 15 the two parental isolates were distinguish by the presence of a single band in the leaf spot parent which was absent in the net-blotch parent. Of the 23 progeny, 18 contained (co)segregating markers from both parents. The number of visible bands that were scored for analysis ranged from 3 to 8 for the different combinations while the range of polymorphic bands varied from 1 to 2. When all four primers were scored together 7 out of a total of 20 bands revealed differences between the parental isolates.

AFLP analysis

AFLPs were used to conclusively show that recombination had taken place during mating, and therefore, that the putative hybrids produced were actually true hybrids. Recombination was confirmed by the cosegregation of parental AFLP marker bands in the progeny. Four primer combinations were used in which AFLP markers were produced for all pairs of combinations (Fig. 2).

Tabulated electrophoretic data are depicted in Table 2. The number of visible bands that were scored for analysis ranged from 16 to 28 for the different combinations while the range of polymorphic bands was between 6

and 11. When all four primer combinations were taken into account, 36 out of a total of 81 bands revealed differences between the parents. All primer combinations revealed genetic markers which could be observed to have cosegregated in the progeny isolates. Of the 23 progeny tested, three of the primer combinations showed recombination in more than 20 while the combination containing *PstI.2* showed 16. When the primer combinations were pooled together the range of cosegregated bands amongst the 23 progeny isolates was between 8 and 26 (mean of 15.75). Primer combinations which yielded segregating bands from only one parent and not the other were not included as cosegregating bands which could reveal recombination. For the primer combination containing *PstI.4* recombination was observed in some isolates as the absence of a band which was present in both parental isolates.

Symptom expression

The verified parental net-blotch and leaf spot isolates produced typical symptoms on the two differentially susceptible cultivars (Figs 3a, b). Most of the progeny produced symptoms that were intermediate between typical net-blotch and leaf spot symptoms (Figs 3c, d). In comparison to the well-defined spots produced on the cv. B87/14 by MP4, the intermediate symptoms caused by progeny on either cultivar displayed jagged, elongated, brown spots. Around the edges of the intermediate symptoms were areas of chlorotic tissue.

Fungicide sensitivity

The results of the fungicide sensitivity trials are shown in Table 3 and Figs 4a-c. For the three fungicides tested several ascospore isolates showed enhanced *in vitro* resistance compared to their net- and spot-type parents. Increased resistance to triademolin was displayed by ascospore isolates GC19 and GC21 while isolates GC18 and GC19 showed resistance to triticonazole. Four isolates showed strong resistance to bromuconazole compared to the parental isolates, namely GC16 and GC18-GC20. Ascospore isolate GC19 showed increased resistance to all three fungicides tested.

DISCUSSION

The taxonomic status of the *Pyrenophora* leaf spot genotype associated with barley in South Africa has been uncertain. On the basis of the morphological descriptions by Sivanesan (1987), Scott (1991) reported that the *Pyrenophora* sp. causing leaf spot in South Africa was *P. japonica*. Louw *et al.* (1994) concluded that the name of the newly collected teleomorph state for the leaf spot *Pyrenophora* in South Africa was *P. japonica*. The present study supports the claims of Louw *et al.* (1995) that leaf spot isolates identified as *P. japonica* (Scott, 1991) are in fact *P. teres* f. *maculata*. Various other studies have been conducted on *P. teres* involving morphological criteria and mating tests. Smedegard-Petersen (1971) could not distinguish the two pathotypes of *P. teres* using morphological characteristics, and therefore proposed them as two forms, namely *P. teres* f. *teres* (net-blotch) and *P. teres* f. *maculata* (leaf-spot). Furthermore, Smedegard-Petersen (1976, 1977) reported that net-blotch and leaf spot isolates could be mated in culture, thereby confirming their being the same species. In addition, putative hybrid progeny subjected to pathogenicity trials showed that as a result of recombination occurring during mating, intermediate symptom types between net-blotch and leaf spot were obtained. In the present study a verified *P. teres* net-blotch isolate from Denmark mated with a South African *Pyrenophora* leaf spot isolate thereby confirming the identity of the local isolate as *P. teres* f. *maculata*. Pathogenicity trials with progeny from the mating revealed intermediate symptoms similar to those obtained by Smedegard-Petersen (1977).

Evidence that recombination occurred during mating between the net-blotch and leaf spot isolates in the present study was demonstrated by using RAPD and AFLP markers to identify novel DNA banding patterns, by comparing the parental genotypes with the novel genotypes of the progeny. The demonstration of novel genotypes in the progeny suggested that the pseudothecia formed during mating were the product of a true cross. Recombination of RAPD markers during the sexual cycle has also been demonstrated in other phytopathogenic fungi (Dyer *et al.*, 1993, 1994; Daniels *et al.*, 1995; Nicholson *et al.*, 1995) as well as in agriculturally important food

crops (Echt *et al.*, 1992; Heun & Helentjaris, 1993). The advantage of AFLPs over RAPDs in being able to identify cosegregating markers has been shown in several crop species (Hill *et al.*, 1996; Maughan *et al.*, 1996; Sharma *et al.*, 1996; Maheswaran *et al.*, 1997; Paul *et al.*, 1997).

It is of note that only one mating was successful. The viable progeny obtained were the result of mating between a Danish net-blotch and South African leaf spot isolate. Both the net-blotch and leaf spot as well as the teleomorph of the leaf spot form are present in South Africa (Louw *et al.*, 1994, 1995, 1996; Crous *et al.*, 1995). The fact that no viable progeny were produced from the mating between the South African net-blotch and leaf spot isolates as well as for the other matings might therefore be due to sub-optimal laboratory conditions. Alternatively, isolates used in some of the matings might have been of the same mating-type, as *P. teres* has a two-allele heterothallic mating system (McDonald, 1963).

The presence of intermediate symptom types due to recombination has several implications with regards to the identification of net-blotch under field conditions. The symptoms caused by all the ascospore progeny were clearly different to the symptoms produced by the parental isolates. Although other *Pyrenophora* spp. such as *P. hordei* Wallwork, Lichon & Sivan., *D. wirreganensis* Wallwork, Lichon & Sivan. and *P. graminea* Ito & Kurib. have also been reported from South Africa, recombination between the net-blotch and leaf spot forms of *P. teres* could explain the great variation of symptoms frequently observed under field conditions (Scott, 1995; Den Breeyen *et al.*, 1996). As this study was mainly concerned with confirming hybridity the actual extent of disease which these novel genotypes might cause has still to be assessed. It has been reported that the spot symptoms caused by *P. teres* f. *maculata* vary widely with regards to their form and colour and may lead to confusion with atypical lesions induced by other barley pathogens (Toubia-Rahme *et al.*, 1995a). It is conceivable that symptoms caused by ascospores produced as a result of a cross between a net-blotch and leaf spot isolate may also lead to confusion in the identification of this fungus with other *Pyrenophora* spp. pathogenic to barley.

The present study also demonstrated that recombination between net-blotch and leaf spot isolates may have affected fungicide sensitivity. Tests of sensitivity to the three triazole fungicides detected altered dose responses among the progeny. The IC₅₀ values for several isolates could not be obtained as they fell below the lowest concentrations used. In the present study both the net-blotch and leaf spot parents were sensitive to triademinoil, with resistant isolates growing at 10 µg/ml (Sheridan *et al.*, 1985; Peever & Milgroom, 1992). Three of the progeny, however, showed increased resistance, of which two were highly resistant. No published data are available on the activity of bromuconazole and triticonazole against *P. teres*. For both fungicides, however, progeny with increased resistance by comparison with parental isolates were again obtained. These results suggest that should sexually compatible net-blotch and leaf spot isolates be present in a mating population in the field, progeny with resistance to fungicides might arise, despite the sensitivity of parental isolates. Isolate GC19 showed increased resistance to each of the three fungicides. This suggests that recombination between isolates of *P. teres* can potentially lead to multiple resistance towards different triazoles.

Steffenson and Webster (1992) reported a large number of pathotypes present in populations of *P. teres* in California where the teleomorph is commonly produced under field conditions. The present study demonstrated that sexual recombination in *P. teres* can result in substantial changes in both disease expression in the host and to fungicide sensitivity in the pathogen. This is important with regard to the epidemiology and control of net blotch in that it provides the pathogen with the genetic flexibility to respond to selection pressures such as fungicide applications (Peever & Milgroom, 1992). In the Western Cape province there has been a shift in the *P. teres* population from the net-blotch to the leaf spot form (Louw *et al.*, 1996). Whether this shift was due to the introduction of new cultivars with increased susceptibility to leaf spot or the occurrence of mating between net-blotch and leaf spot isolates, followed by some other mode of selection for leaf spot isolates has not been established. Although the teleomorph of the leaf spot form of *P. teres* has been collected from stubble in South Africa (Louw *et al.*, 1994) it is still not

clear what the implications of a net x spot mating would be to field populations which differ regarding their cultivar (Tekauz, 1990; Arabi *et al.*, 1992; Afanasenko *et al.*, 1995) and fungicide responses (Toubia-Rahme *et al.*, 1995b)

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Table 1. Genetic recombination observed in progeny isolates of *Pyrenophora teres* as revealed by RAPD analysis. Recombination is shown as the (co)segregation of parental bands in the progeny isolates using four different primers

| Isolate | Primers ^d | | | | | | | | Total (co)segregating bands | Total bands scored ^b | (Co)segregation (%) | | | |
|---------|----------------------|----------------|--------|---|--------|---|--------|---|-----------------------------|---------------------------------|---------------------|--|--|--|
| | OPE 7 | | OPE 15 | | OPM 10 | | OPM 20 | | | | | | | |
| | N ^a | S ^a | N | S | N | S | N | S | | | | | | |
| Net | 1 ^c | | 0 | | 1 | | 1 | | - | 19 | - | | | |
| Spot | 1 ^c | | 1 | | 1 | | 1 | | - | 20 | - | | | |
| GC1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 3 | 21 | 14.3 | | | |
| GC2 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 20 | 10.0 | | | |
| GC3 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 4 | 22 | 18.2 | | | |
| GC4 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 19 | 10.5 | | | |
| GC5 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 3 | 20 | 15.0 | | | |
| GC6 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 4 | 21 | 19.0 | | | |
| GC7 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 4 | 19 | 21.1 | | | |
| GC8 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 5 | 21 | 23.8 | | | |
| GC9 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 2 | 17 | 11.8 | | | |
| GC10 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 2 | 18 | 11.1 | | | |
| GC11 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 2 | 19 | 10.5 | | | |
| GC12 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 2 | 19 | 10.5 | | | |
| GC13 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 3 | 19 | 15.8 | | | |
| GC14 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 4 | 20 | 20.0 | | | |
| GC15 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 3 | 20 | 15.0 | | | |
| GC16 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 2 | 20 | 10.0 | | | |
| GC17 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 3 | 19 | 15.8 | | | |
| GC18 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 3 | 20 | 15.0 | | | |
| GC19 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 2 | 19 | 10.5 | | | |
| GC20 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 4 | 19 | 21.1 | | | |
| GC21 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 5 | 20 | 25.0 | | | |
| GC22 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 4 | 21 | 19.0 | | | |
| GC23 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 3 | 20 | 15.0 | | | |

^a Net-blotch (N) and leaf spot (S) parental isolates.

^b Total number of bands scored for all four primers.

^c Number of bands unique to parental isolates.

^d Dominant and (co)segregating bands.

Table 2. Genetic recombination observed in progeny of *Pyrenophora teres* as revealed by AFLP analysis. Recombination is shown as the cosegregation of parental bands in the progeny using four different primer combinations

| Isolate | Primer combinations ^f | | | | | | | | Total | Total bands scored ^d | Cosegregation (%) | | | |
|---------|----------------------------------|----------------|-----------------|---|-----------------|---|-----------------|---|-------|---------------------------------|-------------------|--|--|--|
| | <i>PstI</i> 1.1 | | <i>PstI</i> 1.2 | | <i>PstI</i> 1.3 | | <i>PstI</i> 1.4 | | | | | | | |
| | N ^a | S ^a | N | S | N | S | N | S | | | | | | |
| Net | 5 ^e | | 2 | | 5 | | 3 | | - | 60 | - | | | |
| Spot | 6 ^e | | 6 | | 3 | | 6 | | - | 67 | - | | | |
| | 4 | 4 | 1 | 2 | 4 | 1 | 2 | 4 | 22 | 68 | 32.4 | | | |
| GC2 | 4 | 2 | 2 | 6 | 3 | 2 | 1 | 4 | 1 | 24 | 67 | | | |
| GC3 | 2 | 5 | 1 | 1 | 2 | 1 | 2 | 2 | 16 | 61 | 26.2 | | | |
| GC4 | 4 | 2 | 2 | 6 | 2 | 0 | 1 | 4 | 1 | 19 | 68 | | | |
| GC5 | 3 | 4 | 1 | 2 | 1 | 1 | 1 | 5 | 1 | 18 | 63 | | | |
| GC6 | 5 | 3 | 2 | 4 | 4 | 0 | 2 | 4 | | 20 | 70 | | | |
| GC7 | 1 | 4 | 0 | 5 | 2 | 2 | 1 | 2 | | 12 | 62 | | | |
| GC8 | 1 | 3 | 0 | 4 | 2 | 2 | 1 | 2 | | 11 | 61 | | | |
| GC9 | 3 | 3 | 0 | 5 | 4 | 1 | 2 | 5 | | 18 | 73 | | | |
| GC10 | 2 | 1 | 1 | 2 | 3 | 2 | 1 | 2 | | 14 | 59 | | | |
| GC11 | 4 | 3 | 1 | 3 | 4 | 1 | 2 | 4 | | 22 | 68 | | | |
| GC12 | 4 | 1 | 2 | 1 | 3 | 1 | 2 | 3 | | 17 | 64 | | | |
| GC13 | 2 | 3 | 0 | 0 | 4 | 3 | 1 | 3 | | 16 | 63 | | | |
| GC14 | 1 | 2 | 0 | 3 | 1 | 1 | 2 | 2 | | 9 | 55 | | | |
| GC15 | 3 | 5 | 1 | 4 | 5 | 3 | 3 | 3 | | 27 | 72 | | | |
| GC16 | 1 | 3 | 1 | 1 | 6 | 2 | 1 | 2 | | 17 | 59 | | | |
| GC17 | 2 | 2 | 1 | 4 | 2 | 2 | 1 | 4 | 1 | 18 | 63 | | | |
| GC18 | 2 | 3 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 12 | 55 | | | |
| GC19 | 2 | 3 | 1 | 1 | 1 | 1 | 1 | 4 | 1 | 14 | 60 | | | |
| GC20 | 1 | 4 | 0 | 1 | 2 | 1 | 1 | 2 | 1 | 11 | 59 | | | |
| GC21 | 4 | 4 | 1 | 1 | 4 | 2 | 1 | 3 | | 20 | 68 | | | |
| GC22 | 4 | 5 | 1 | 1 | 1 | 1 | 0 | 3 | | 13 | 63 | | | |
| GC23 | 4 | 1 | 2 | 3 | 5 | 1 | 2 | 5 | | 23 | 67 | | | |

^a Net-blotch (N) and leaf spot (S) parental isolates.

^b Recombination revealed using primer combination *PstI* 1.4 was shown by the absence of a band present in both parental isolates.

^c Primer combinations in which one of the parental isolates yielded no segregating bands were not included in the total number of cosegregated bands.

^d Total number of bands scored for all four primer combinations.

^e Number of bands unique to parental isolates.

^f Dominant and (co)segregating bands.

Table 3. IC₅₀ values of the net-blotch and leaf spot parents and their progeny determined after fungicide testing with the triazoles triademinol, bromuconazole and triticonazole

| Isolate | Triademinol | Bromuconazole | Triticonazole |
|-------------------|-------------|---------------|---------------|
| Net-blotch parent | 0.01 | 0.01 | 4.91 |
| Spot parent | 5.97 | 0.14 | 0.72 |
| GC1 | 0.01 | 0.12 | 0.08 |
| GC2 | 0.85 | 0.19 | 0.64 |
| GC3 | 0.81 | * | 0.18 |
| GC4 | 5.49 | 0.14 | 0.47 |
| GC5 | 10.56 | 0.20 | 1.64 |
| GC6 | 6.61 | 0.20 | 1.02 |
| GC7 | 0.85 | * | 0.33 |
| GC8 | 0.11 | 0.01 | 0.50 |
| GC9 | 0.12 | * | 0.07 |
| GC10 | 3.36 | 0.12 | * |
| GC11 | * | 0.01 | 0.12 |
| GC12 | 4.01 | 0.16 | * |
| GC13 | 5.42 | 0.24 | 0.16 |
| GC14 | 3.67 | * | 0.46 |
| GC15 | 0.77 | 0.06 | 0.22 |
| GC16 | 0.70 | 5.23 | 0.53 |
| GC17 | * | 0.21 | 0.26 |
| GC18 | 5.31 | 2.49 | 18.78 |
| GC19 | 39.47 | 2.51 | 22.86 |
| GC20 | * | 1.99 | * |
| GC21 | 37.65 | * | 0.25 |
| GC22 | 5.19 | 0.08 | 0.21 |
| GC23 | 0.97 | 0.09 | 0.16 |

* IC₅₀ values fell below the range of concentrations used.

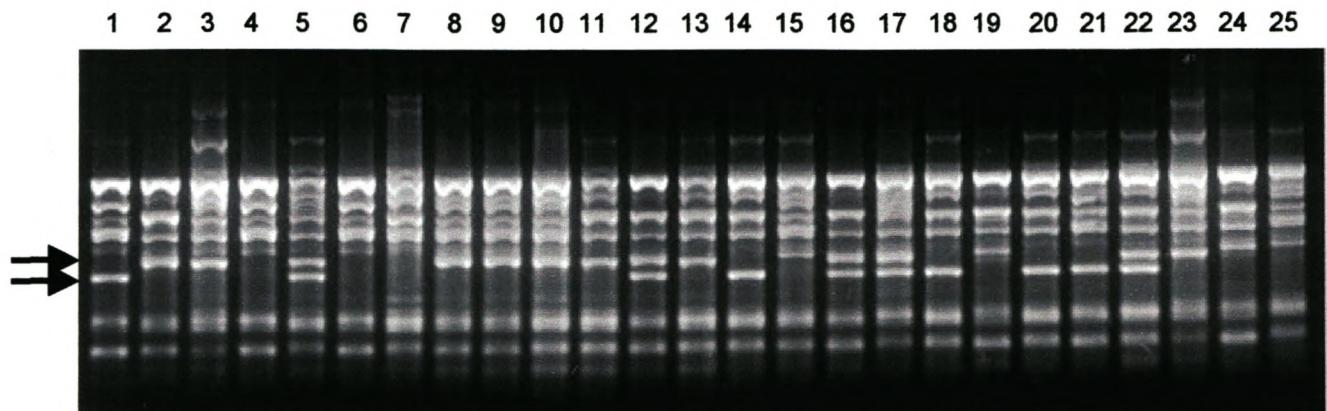


Fig. 1. Segregation pattern of the RAPD marker bands for primer OPM 10. Lanes 1 and 2 are respectively the net-blotch and leaf spot parents while lanes 3-25 show the banding patterns of the progeny. The arrows indicate the bands discriminating the net-blotch and leaf spot parents.

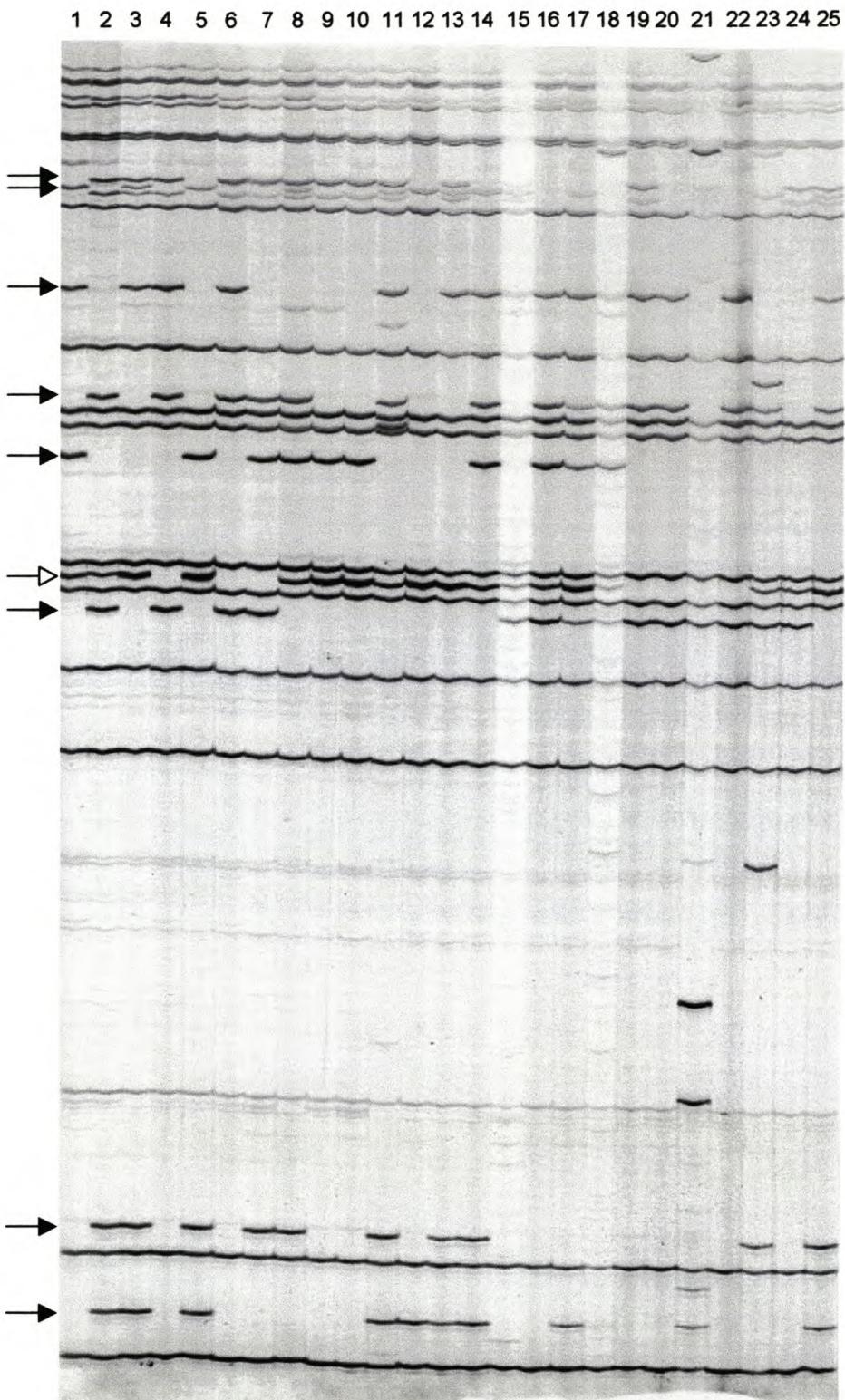


Fig. 2. Segregation patterns of the AFLP marker bands for the primer combination *Mse*1/*Pst*1.4. Lanes 1 and 2 are respectively the net-blotch and leaf spot parents while lanes 3-25 indicate the banding patterns of the progeny. Solid arrows indicate polymorphic AFLP bands which cosegregate as dominant markers (presence or absence). The open arrow indicates recombination in the progeny due to the loss of a fragment present in both parents.

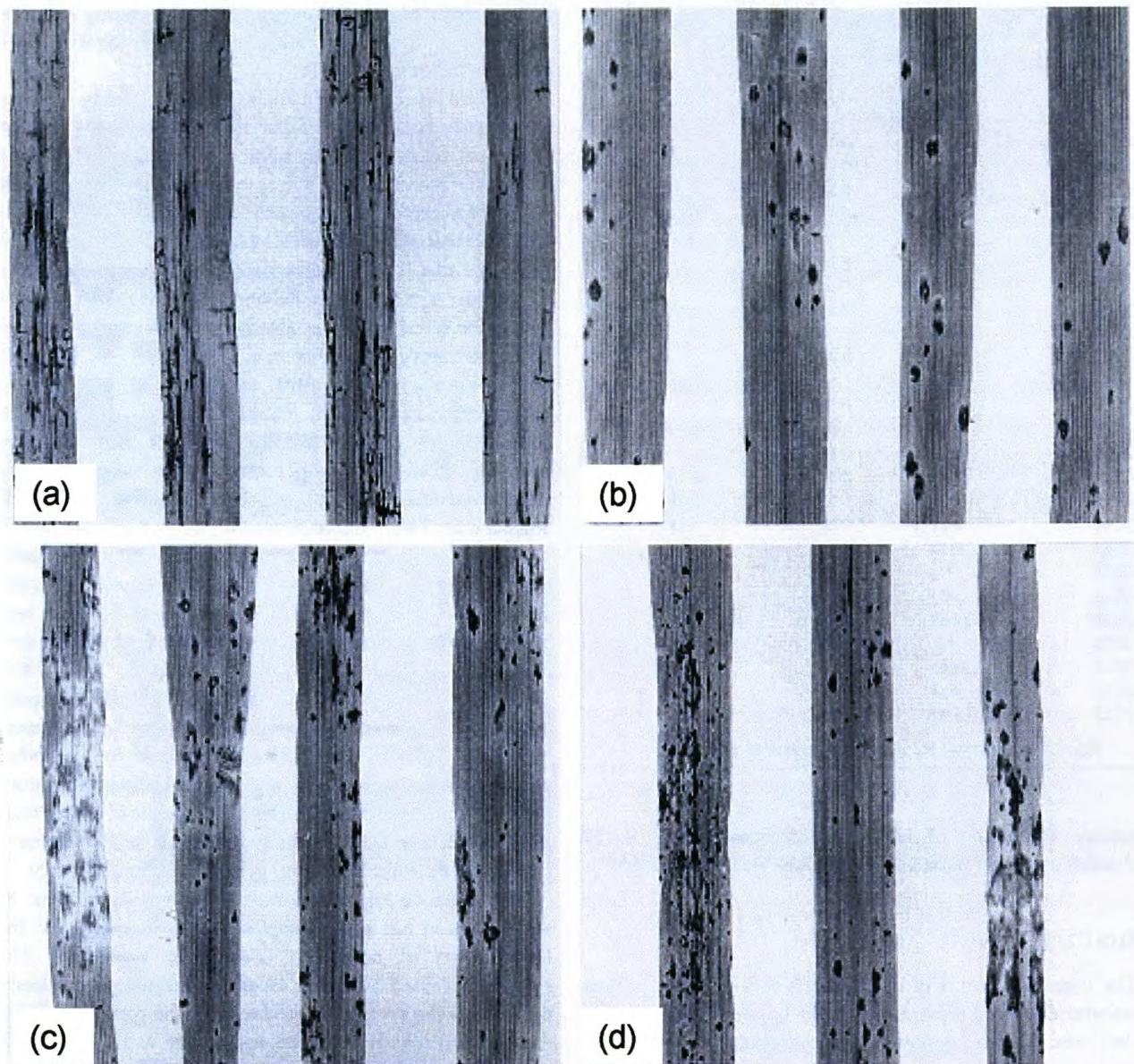


Fig. 3. Symptom expression on cvs Stirling and B87/14. (a) Pt90-8a (net-blotch parent) on Stirling, (b) MP4 (leaf spot parent) on B87/14, (c) isolate GC19 on Stirling and (d) GC19 on B87/14.

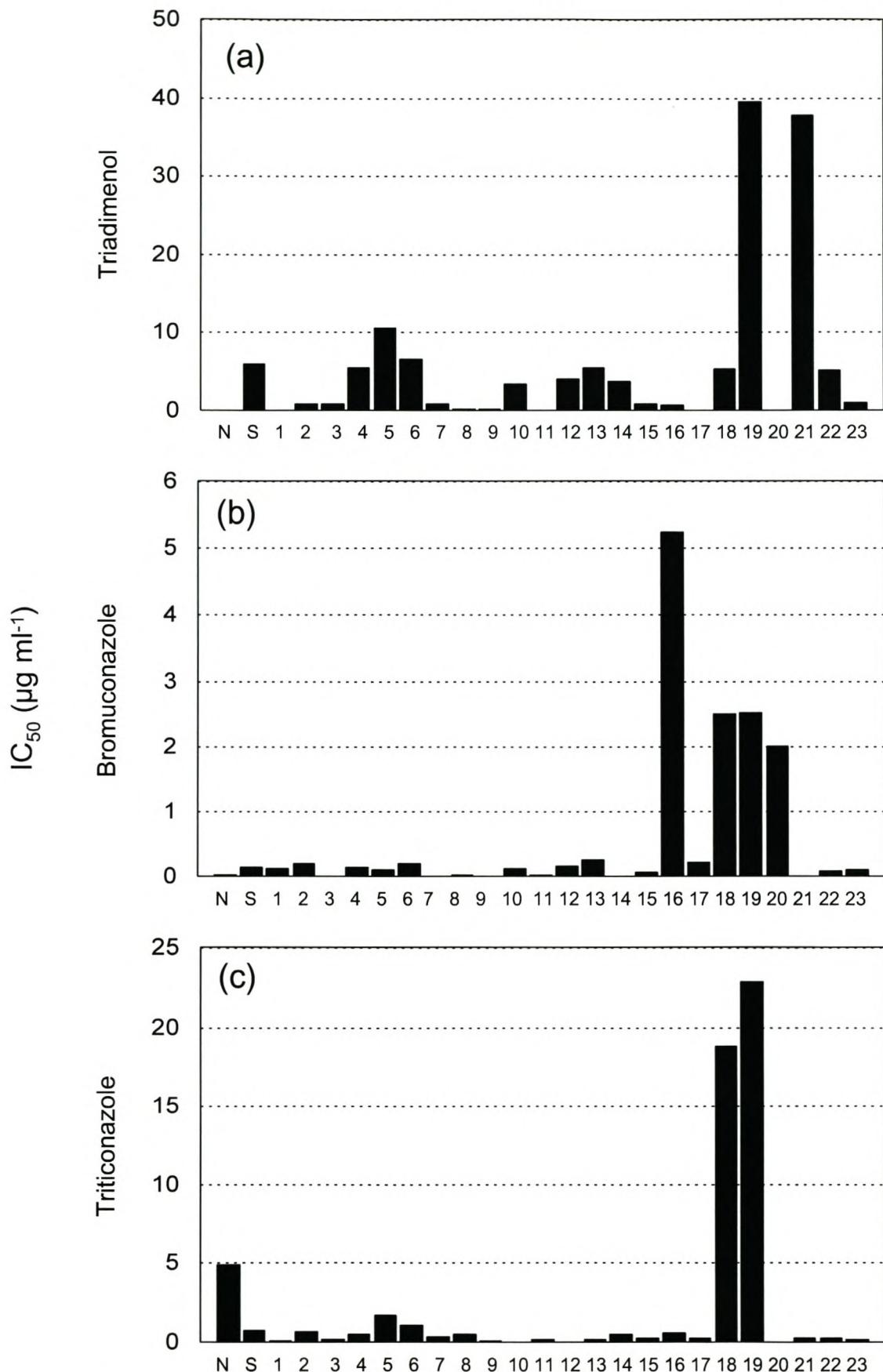


Fig. 4. IC_{50} values for net-blotch and leaf spot parents and the progeny for fungicides (a) Triadimenol, (b) Bromuconazole and (c) Triticonazole.

3. Genetic stability of hybrid progeny of *Pyrenophora teres* causing intermediate net x spot symptoms on barley

ABSTRACT

Hybrid progeny produced from a mating between two isolates of the barley net blotch pathogen *Pyrenophora teres*, causing either net- or spot-type symptoms were screened to assess their viability and genetic stability. Hybrid progeny (F_1) inoculated onto seedlings of the barley cultivars Stirling (differentially susceptible to net-type isolates), B87/14 and Clipper (both differentially susceptible to spot-type isolates) produced intermediate symptoms on all cultivars. Axenic cultures (F_{1-1}) isolated from foliar lesions, followed by repeated inoculation and isolation (F_{1-2}) onto a healthy set of seedlings produced similar intermediate symptoms. RAPDs produced with a 10-mer primer on all isolates of F_{1-1} and F_{1-2} progeny revealed profiles similar to those obtained for F_1 isolates. These molecular data, therefore, indicated that hybrid progeny of this net x spot mating were genetically stable after two cycles of repetitive inoculation and reisolation. Phylogenetic analysis of DNA sequences of the internal transcribed spacers (ITS1 and ITS2) flanking 5.8S nuclear ribosomal RNA gene and the 5' end partial histone H3 gene have confirmed the genetic stability of the hybrid progeny. These results also indicated that the hybrid progeny produced consistent symptoms throughout the series of experiments, and maintained their virulence to the differential cultivars screened. Contrary to earlier expectations, stable hybrids between net- and spot-type isolates of *P. teres* may therefore play a significant role in net blotch epidemiology in barley production areas.

INTRODUCTION

Net blotch, caused by the fungus *Pyrenophora teres* Drechsler [anamorph *Drechslera teres* (Sacc.) Shoemaker] is a destructive foliar disease of barley (*Hordeum vulgare* L. emend. Bowden) in South Africa and throughout most other barley growing regions of the world (Shipton *et al.*, 1973; Steffenson *et al.*, 1991; Louw *et al.*, 1996). Two types of leaf symptoms are associated with net blotch, a net-like symptom which produces elongated, light brown lesions with dark brown reticulations, and a spot symptom which is dark brown with a distinct halo (Smedegård-Petersen, 1971). Both types are capable of causing economic yield losses (Jordan, 1981; Jordan *et al.*, 1985; Martin, 1985; Deadman & Cooke, 1987; Delserone & Cole, 1987; Steffenson *et al.*, 1991). *P. japonica* S. Ito & Kurib. [anamorph *Drechslera tuberosa* (G.F. Atk.) Shoemaker] was originally described as the pathogen causing spot-type symptoms, whereas *P. teres* was associated with net-type lesions (Ito & Kurabayashi, 1931; Shoemaker, 1962). Smedegård-Petersen (1971) mated net blotch and leaf spot isolates and concluded that they were two formae of the same biological species for which he proposed the names *P. teres* f. *teres* (net-type symptoms) and *P. teres* f. *maculata* Smedegård-Petersen (spot-type symptoms).

Contrary to earlier belief that Pyrenophora leaf spot isolates in the Western Cape were *P. japonica* (Scott, 1991; Louw *et al.*, 1994), Campbell *et al.* (1999) showed that these Pyrenophora leaf spot isolates are actually *P. teres* f. *maculata*. This was shown by demonstrating that recombination, confirmed using molecular markers, had taken place following mating between a net-type and spot-type isolate of *P. teres*. Although mating between net- and spot-type isolates of *P. teres* has been reported in various studies (McDonald, 1967; Smedegård-Petersen, 1971, 1976, 1977; Louw *et al.*, 1995; Campbell *et al.*, 1999) it is difficult to initiate mating under laboratory conditions (Peever & Milgroom, 1992; Campbell *et al.*, 1999). Not only have net x spot hybrids been shown to cause an intermediate symptom on susceptible cultivars (Smedegård-Petersen, 1977; Crous *et al.*, 1995; Campbell *et al.*, 1999), as a percentage of hybrid progeny were found to be more resistant to commercially used fungicides following mating between two

sensitive parents (Campbell *et al.*, 1999). The role and genetic stability of these hybrid progeny in net blotch epidemics, however, remain unclear.

Various DNA-based techniques can be used to assess genetic stability of hybrids. RFLPs are the most popular due to the fact that they exhibit a higher number of co-dominant markers than other DNA markers such as RAPDs that are normally dominant in nature. However, if co-dominant RAPD markers are available then RAPD analysis has the advantage of being more cost efficient than RFLPs (Williams *et al.*, 1990). RAPD markers have been used to demonstrate the formation of hybrids during the sexual cycle in various phytopathogenic fungi (Dyer *et al.*, 1993, 1994; Peever & Milgroom, 1994; Daniels *et al.*, 1995; Nicholson *et al.*, 1995; Schoch *et al.*, 2000) as well as monitoring the genetic stability of plants regenerated from suspension cultures (Isabel *et al.*, 1993; Vallés *et al.*, 1993; Rani *et al.*, 1995; Gavidia *et al.*, 1996; Wallner *et al.*, 1996). The application of rDNA sequences in phylogenetic studies has been reviewed previously (Bruns *et al.*, 1991; Hibbett, 1992; Kohn, 1992; Kurtzman, 1992; Samuels & Seifert, 1995), and the 5.8S rDNA and flanking internal transcribed spacers (ITS1 and ITS2) and histone genes are commonly employed to study the phylogeny of fungal plant pathogens (Lee & Taylor, 1992; Morales *et al.*, 1993; Zambino & Szabo, 1993; Kang *et al.*, 2001).

The aim of the present paper was, therefore, to study the genetic stability of net x spot hybrid progeny of *P. teres* by screening isolates for pathogenicity, reconfirming their genetic fingerprints using RAPDs, and phylogenetic analysis using DNA sequence data of ITS regions of rRNA and histone genes.

MATERIALS AND METHODS

Experimental design and isolates

Hybrid progeny (F_1) produced from a mating between a net- and spot-type isolate of *P. teres* (Campbell *et al.*, 1999) was used to monitor their genetic stability over a series of pathogenicity trials (Fig. 1). Isolates were maintained on potato dextrose agar (PDA) at 4°C. For cultural growth, inoculated PDA culture plates were incubated at 25° for 7 days. In the present study, the

following 14 hybrid isolates were used from Campbell *et al.* (1999) for the pathogenicity trials and RAPD stability analyses: GC2, 3, 4, 6, 7, 9, 10, 12, 15, 16, 17, 19, 20 and 22.

For phylogenetic analyses, GC18 from the F₁ generation and GC4, 15, 16 and 19 from the F₁₋₂ generation were randomly selected as representative isolates of the hybrid progeny to be sequenced. In addition, field isolates Cal1, Swe1 (spot-type isolates) and Nap8 (net-type isolate), obtained from the culture collection of the Department of Plant Pathology at the University of Stellenbosch, were also sequenced. For further sequence alignment purposes, 37 Genbank entries representing different species of *Pyrenophora*, *Drechslera*, *Bipolaris* and *Pleospora* were used.

This study consisted of the following phases: phase 1 – inoculation of hybrid progeny (F₁) onto barley seedlings; phase 2 – isolation of the fungal pathogen from diseased leaves to obtain F₁₋₁; phase 3 – inoculation of F₁₋₁ onto barley seedlings and finally, phase 4 – re-isolation of the fungal pathogen from diseased leaves to obtain F₁₋₂.

Pathogenicity

The three differentially susceptible barley cultivars Stirling (susceptible to net-type isolates), B87/14 and Clipper (susceptible to spot-type isolates) were used in the pathogenicity trials. Production and processing of mycelia for inoculation onto seedlings was done according to the method set out by Campbell *et al.* (1999). Plants were incubated at the two-leaf stage in a glasshouse (15–20°, night/day temperature) using the technique as explained by Louw *et al.* (1994). A solution of 0.01% (v/v) Tween 20 was initially sprayed onto the plants to reduce leaf surface tension, after which the mycelial suspensions were sprayed to runoff. Moisture chambers were created by placing plastic bags over the inoculated plants for 48 h. Plants were examined for symptom expression 10 days after inoculation.

Diseased barley leaves with net blotch symptoms were removed from the plants 5–7 days after inoculation. Leaves were surface-sterilised by immersion in 70% ethanol for 30 s followed by transfer to 2% NaOCl for 60 s and finally 70% ethanol for 30 s. Sterilised leaves were subsequently left to air-dry in a laminar air-flow cabinet. Air-dried leaves were placed onto glass

slides in moisture chambers and incubated at 10° under near-UV light to induce sporulation. After approximately 3–4 days single conidia were transferred to PDA culture plates.

DNA isolation, RAPDs, gel electrophoresis and analysis

Isolation of DNA, RAPD analysis and gel electrophoresis were performed according to standard procedures (Sambrook *et al.*, 1989) essentially as described by Campbell *et al.* (1999) with the following modification: the reaction mixture for RAPD analysis contained 2.5 µl of 10X NH₄ buffer [160 mM (NH₄)₂SO₄, 670 mM Tris-HCl (pH 8.8), 0.1% Tween-20] [Bioline Ltd, London, UK and Whitehead Scientific, South Africa]; 200 µm of each dNTP; 10 pmol of oligonucleotide primer, 50 ng genomic DNA and 1.0 unit of BIOTAQ DNA polymerase. RAPD analysis was done with two different primers that exhibited co-dominant markers (DNA bands from both parental types). The sequences of the primers were the following:

OPE7: 5' AGATGCAGCC 3'

OPM10: 5' TCTGGCGCAC 3'

Amplifications were done in a Perkin Elmer GeneAmp PCR system 2400 cycler. Reactions underwent an initial denaturation process at 96° for 120 s, followed by 45 cycles of 92° for 30 s, 38° for 30 s and 72° for 60 s. After the last cycle a final extension step was conducted at 72° for 120 s. Amplification products were separated through 1.5% (w/v) agarose gels in TAE buffer (Sambrook *et al.*, 1989).

DNA fingerprints were observed in a SYNGENE Darkroom S/N: SYDR/1318 linked to a desktop computer. Fingerprints were captured using the SYNGENE programme GeneSnap. A molecular weight marker (λ DNA digested with *Hind*III) was used as a reference for comparing samples from different gels. Using the F₁ fingerprints (OPE7 and OPM10) as references, genetic stability at the molecular level was assessed in isolates from F₁₋₁ and F₁₋₂.

Gene amplification, sequencing and phylogenetic analysis

Template DNA was amplified in a 25 µl PCR reaction mixture consisting of 2.0 units of *Taq* polymerase (Boehringer Mannheim, Germany), PCR reaction

buffer supplied by the manufacturer, additional 1.25 mM MgCl₂, 500 µM of each dNTP, with 60 pmol ITS1 and ITS4 primers (White *et al.*, 1990), or H3-1a and H3-1b (Glass & Donaldson, 1995). The reaction profile was set up as follows: initial denaturation at 96° for 120 s, followed by 30 cycles of denaturation at 94° for 30 s, annealing at 55° for 30 s, extension at 75° for 120 s, and a final extension step at 75° for 7 min. Amplifications were done in a Rapidcycler (Idaho Technology Idaho, U.S.A.). A negative control using water instead of template DNA was run for each experiment. PCR products were separated through 0.8 % (w/v) agarose (Promega, Madison, Wisconsin) gels in TAE buffer, and subsequently purified using a QIAquick PCR Purification Kit (Qiagen GmbH, Germany). The purified PCR products were sequenced using an ABI Prism 377 DNA Sequencer (Perkin-Elmer, Norwalk, Connecticut) with an ABI PRISM™ Dye Terminator Cycle sequencing Ready Reaction Kit (Perkin Elmer, Warrington, UK).

The nucleotide sequences of the 5.8S rRNA gene and the flanking internal transcribed spacers (ITS1 and ITS2) and histone H3 gene were assembled using Sequence Navigator™ version 1.0.1. (Perkin Elmer, Applied Biosystems, Inc., Foster City, CA). Alignment of the sequence files was conducted using the CLUSTAL W software (Thompson *et al.*, 1994). Adjustments for improvement were made by eye where necessary. Alignment gaps were treated as missing data in the parsimony analysis. The parsimony and neighbour-joining analyses were performed for the ITS sequences with PAUP* (Phylogenetic Analysis Using Parsimony) version 4.0b6 (Swofford, 2001). Clade stability was assessed using 500 parsimony and 1000 neighbour-joining bootstrap replications. Decay indices were also calculated using AutoDecay (Eriksson, 1998) to verify the robustness of the branches of the tree. The tree scores including tree length, consistency index, retention index, rescaled consistency index and homoplasy index (CI, RI, RC and HI) were also calculated for the parsimony tree topology. The best-fit maximum likelihood tree was also calculated with 100 random sequence input orders and global rearrangement to test the parsimony and neighbour-joining tree topologies.

RESULTS

Symptom expression

Disease symptom expression on the three differentially susceptible barley cultivars inoculated with F_{1-1} and F_{1-2} isolates is depicted in Fig. 2A-D. Symptoms were obtained on all three cultivars for all F_{1-1} and F_{1-2} isolates. Intermediate symptom expression characterised by jagged, elongated brown lesions were produced to varying degrees depending on the cultivar used. These results indicated that the *Pyrenophora teres* net x spot hybrids studied remained virulent and stable, being able to induce disease following a series of inoculation and re-isolation steps.

RAPD analysis

The results of the RAPD analysis for F_1 (reference isolates), F_{1-1} and F_{1-2} using OPE7 and OPM10 are depicted in Figs. 3A-B. Using OPE7, two marker bands (indicated as 1 and 2) were produced that distinguished the net- and spot-type parental isolates. Marker band 2, characteristic of the spot-type parent, is present in six of the hybrid progeny in F_1 , and is also present in the respective isolates in F_{1-1} and F_{1-2} . Marker band 1, characteristic of the net-type parent, is present in seven of the hybrid progeny in F_1 , as well as in the respective isolates in F_{1-1} and F_{1-2} .

Using primer OPM10, two marker bands (indicated as 3 and 4) are produced that distinguish the net- and spot-type parental isolates. Isolates where both these bands are present (codominant) in the hybrid progeny in F_1 , are also present in the respective isolates in both F_{1-1} and F_{1-2} .

The results obtained using these two primers clearly indicated that the hybrid progeny are genetically stable as the net- and spot-type marker bands were retained through a series of inoculation and re-isolation cycles.

Phylogenetic analysis

Sequence data of isolates studied were lodged in GeneBank (XXX–XXX) (Appendix 1) and the alignment in TreeBase (SN XXX). The nucleotide sequences of histone H3 gene of 17 isolates were aligned, but proved to be identical. This data set was therefore excluded from further analysis. The

alignment of the ITS sequence data set of 8 isolates, and 37 GenBank entries spanned 665 sites including 275 ambiguous characters at the 5' end of ITS1 region. This resulted in 25800 equally most parsimonious trees (MPTs) and an unacceptably long tree with 981 steps being produced in a preliminary parsimony analysis. This region was therefore excluded from the subsequent analysis. The remaining 390 characters used contained 37 variable and 56 parsimony-informative characters.

The maximum parsimony analysis of the remaining 390 characters using the heuristic search option in PAUP* with 1000 randomisations of sequence input orders generated 48 MPTs and a shortest tree of 213 steps. *Pleospora herbarum* (Pers.: Fr.) Rabenh. was used as an outgroup to root the tree. The best tree topology of the 48 MPTs was selected as the phylogenetic tree (Fig. 4) through the Kishino-Hasegawa likelihood test (data not shown), and evaluated with 500 bootstrap replications in a heuristic search for clade stability. The neighbour-joining analysis of the data subset with 1000 bootstrap replications in PAUP* produced a majority-rule consensus tree (data not shown) similar to the phylogenetic tree (Fig. 4). Maximum-likelihood analysis was also performed in PAUP* for the data set, which produced a tree topology (data not shown) resembling the phylogenetic tree (Fig. 4) in clustering the same major groups of isolates.

The ITS gene tree (Fig. 4) generated two major clades. The three *Bipolaris* Shoemaker species clustered in a clade with strong bootstrap (parsimony/neighbour-joining) and decay indices support (94/95, d4). All the *Pyrenophora* Fr. isolates along with *Drechslera* S. Ito. anamorphs formed a strongly supported clade (100/99, d9), indicating the same genealogical origin of the genus. In this clade, 1 net-type isolate, 2 spot-type isolates and 5 hybrid progeny isolates clustered together with isolates of *P. graminea* S. Ito, *P. japonica* and *P. teres* with high support (87/98, d2), which further substantiated the genetic stability of the hybrid progeny isolates from the net x spot mating, and furthermore also indicated that all the isolates were synonymous with *P. teres*.

DISCUSSION

In a previous study, Campbell *et al.* (1999) demonstrated recombination between a net- and spot-type isolate of *P. teres* using RAPD and AFLP analyses. The aim of the present study was to determine if hybrid progeny (F_1) of a *P. teres* net x spot-type cross from that study would remain genetically stable, i.e., if RAPD markers inherited from each parental isolate would still be present in the hybrid progeny following a series of glasshouse experiments involving inoculation and re-isolation cycles. Using pathogenicity and RAPD markers to investigate these questions, evidence has been provided that net x spot hybrids of *P. teres* can remain virulent and genetically stable following a series of inoculation trials.

The results obtained in this study may have plant pathological significance with regards to the epidemiology of net blotch disease. Firstly, evidence obtained through glasshouse pathogenicity trials suggests that hybrid forms of the pathogen may be able to survive from season to season on straw remaining in fields after harvest. Crops in subsequent seasons could then be infected by means of rain splash (Jordan, 1981) or by wind-borne conidia (Shipton *et al.*, 1973). Straw remaining in fields after harvest is regarded as the primary inoculum source of *P. teres* (Jordan, 1981; Piening, 1968). Overwintering structures such as pseudothecia may also play a role in the epidemiology of net x spot hybrid isolates. Campbell *et al.* (1999) reported that hybrid pseudothecia induced by means of mating on barley stubble produced genetically stable ascospores when tested on differential cultivars. Furthermore, symptomatic leaves were found to sporulate within 2–3 days after being placed in moisture chambers. These findings support those of Tekauz and Buchannon (1977) who reported that sporulation occurred within 5 days following infection, indicating a rapid build-up of secondary inoculum.

The viability, virulence and genetic stability of hybrid net x spot isolates may also be significant from a fungicide resistance perspective. In a previous study Campbell *et al.* (1999) showed that hybrid progeny may be produced that are more resistant than parental isolates to the active component in commercially used fungicides. When interpreted with the data obtained in this

study, indications are that hybrid resistant isolates can also proliferate asexually from season to season. In so doing, it might increase the proportion of inoculum resistant to fungicides.

Three differentially susceptible barley cultivars were used in pathogenicity trials in the present study. Stirling is highly susceptible to net-type isolates and slightly susceptible to spot-type isolates, while B87/14 and Clipper are only susceptible to spot-type isolates. However, the hybrid isolates infected all cultivars in both sets of pathogenicity trials indicating that due to recombination the normal constraints on host range had been overcome. Furthermore, it has been reported by Tekauz (1990) that two malting cultivars in Canada showed equal susceptibility to both net- and spot-type isolates, indicating some sort of physiological specialisation (Tekauz, 1990). This was attributed to various factors, one being the possibility for sexual recombination between net- and spot-type isolates (Tekauz, 1990). The mating studies between net- and spot-type isolates conducted by Campbell *et al.* (1999), as well as the molecular proof of recombination in hybrid progeny suggested that Smedegård-Petersen (1971) was correct in naming them *formae* of the same species. The genetic stability of these hybrid progeny as demonstrated in the present study, as well as the high percentage of DNA sequence homology in relation to other species of *Pyrenophora* (Fig. 4), provide further evidence to support the fact that the net- and spot-type isolates should still be treated as one biological species, which is undergoing speciation.

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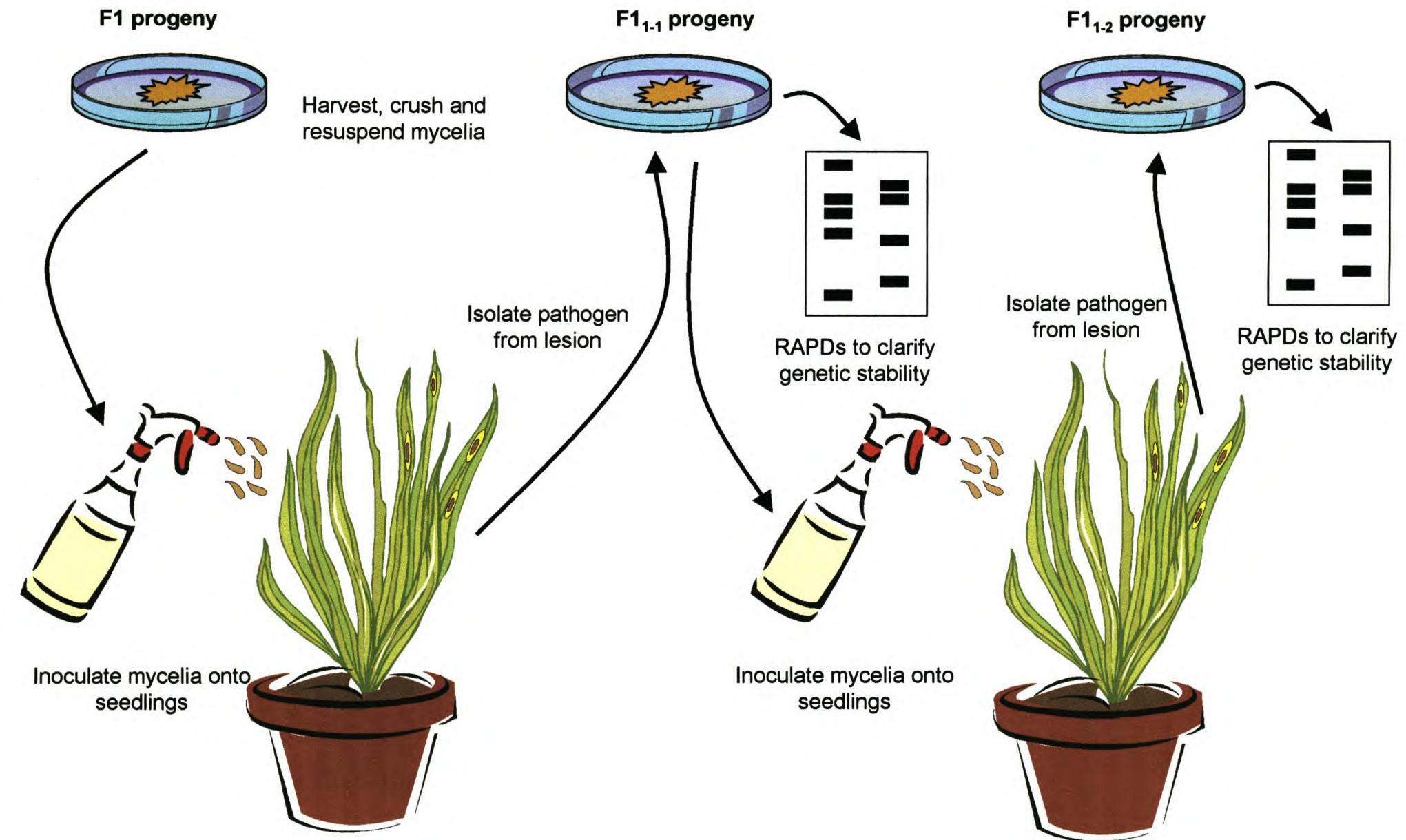


Fig. 1. Schematic representation showing design of study.

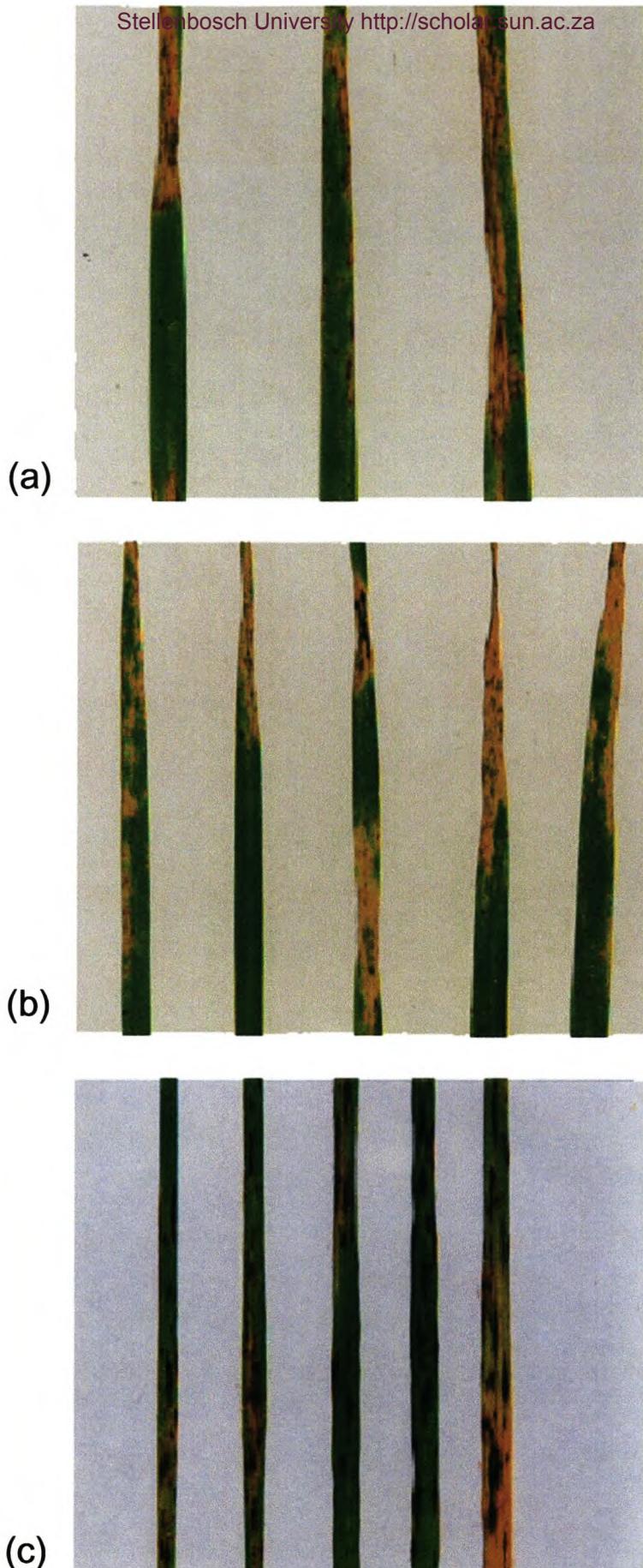


Fig. 2A. Symptom expression for hybrid isolate GC9 ($F1_{1-1}$) on the cultivars
(a) Stirling, (b) B87/14 and (c) Clipper.

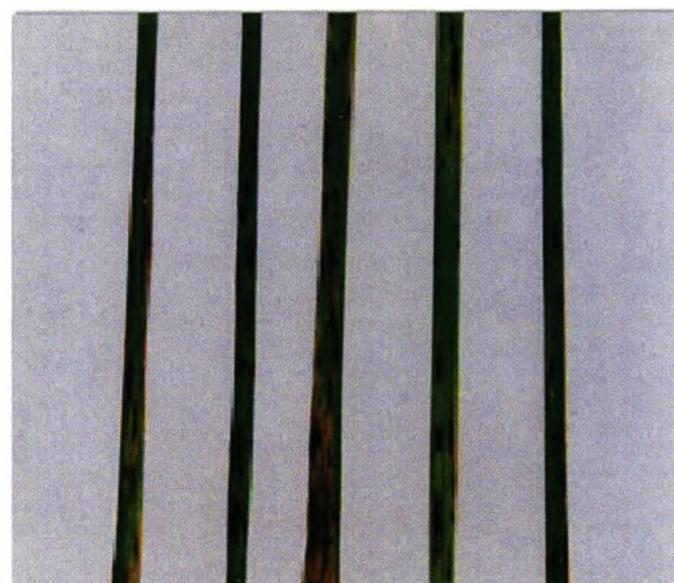
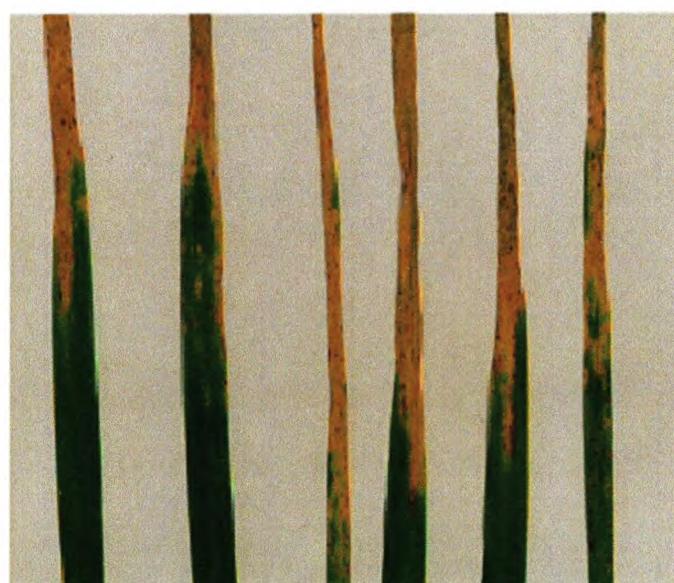
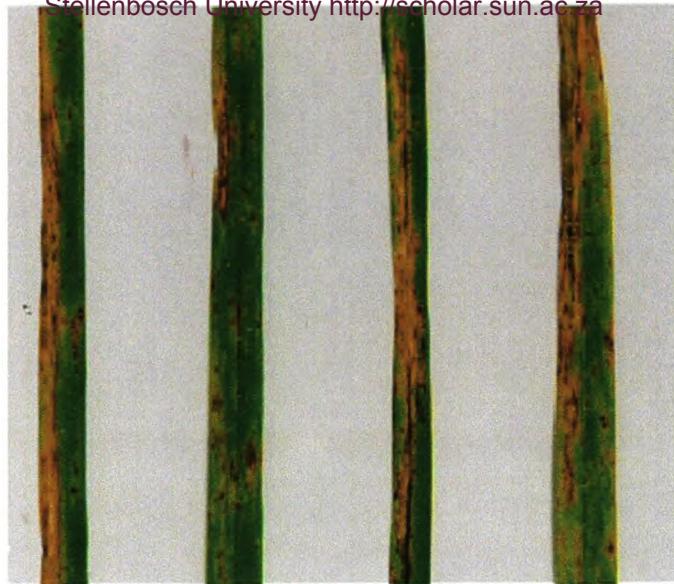


Fig. 2B. Symptom expression for hybrid isolate GC9 ($F1_{1-2}$) on the cultivars
(a) Stirling, (b) B87/14 and (c) Clipper.

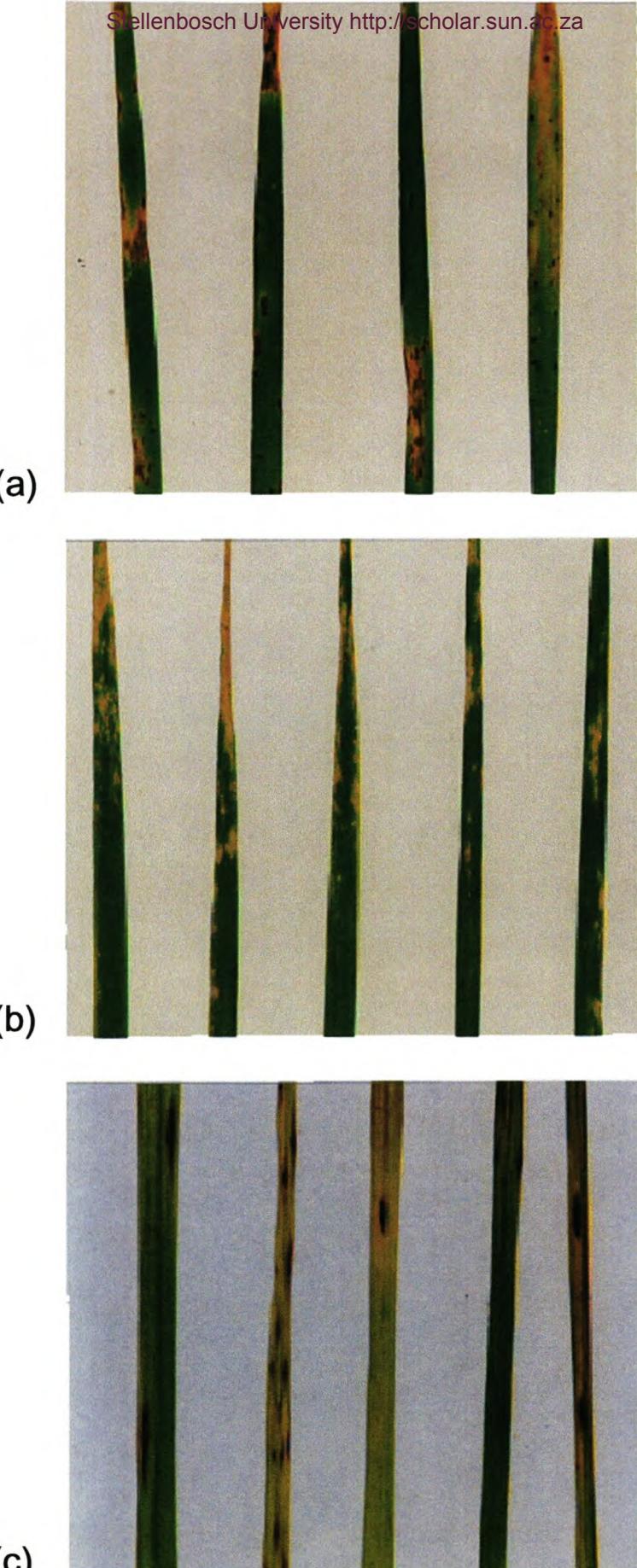


Fig. 2C. Symptom expression for hybrid isolate GC19 ($F1_{1-1}$) on the cultivars (a) Stirling, (b) B87/14 and (c) Clipper.

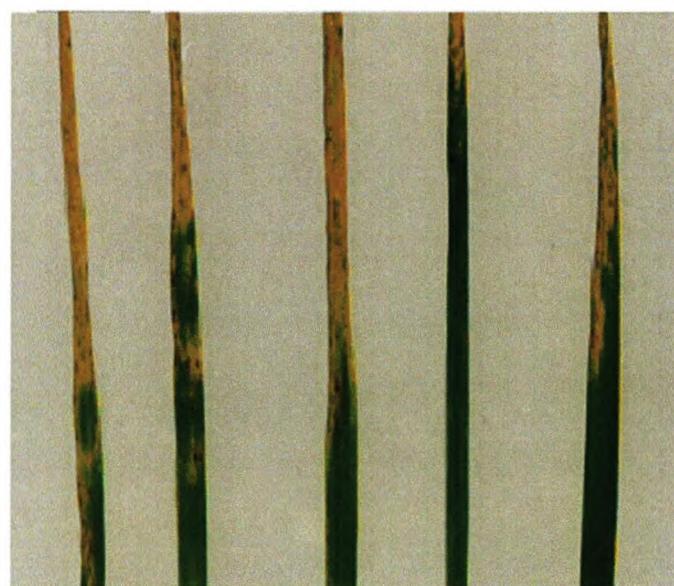
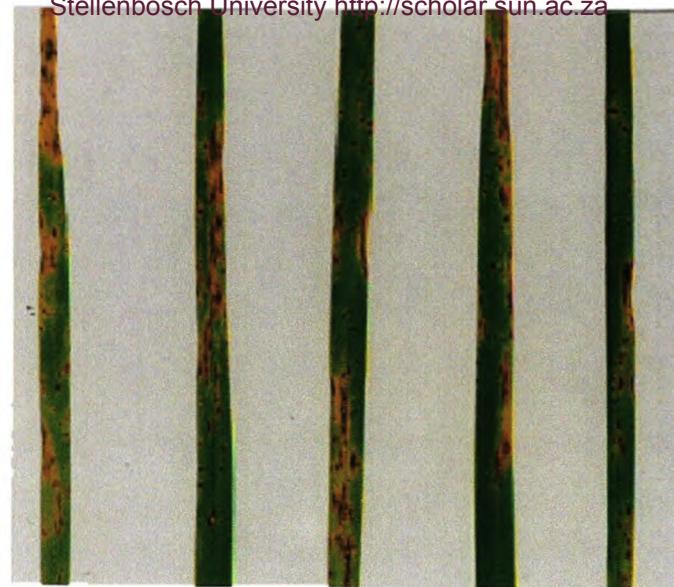


Fig. 2D. Symptom expression for hybrid isolate GC19 ($F1_{1-2}$) on the cultivars (a) Stirling, (b) B87/14 and (c) Clipper.

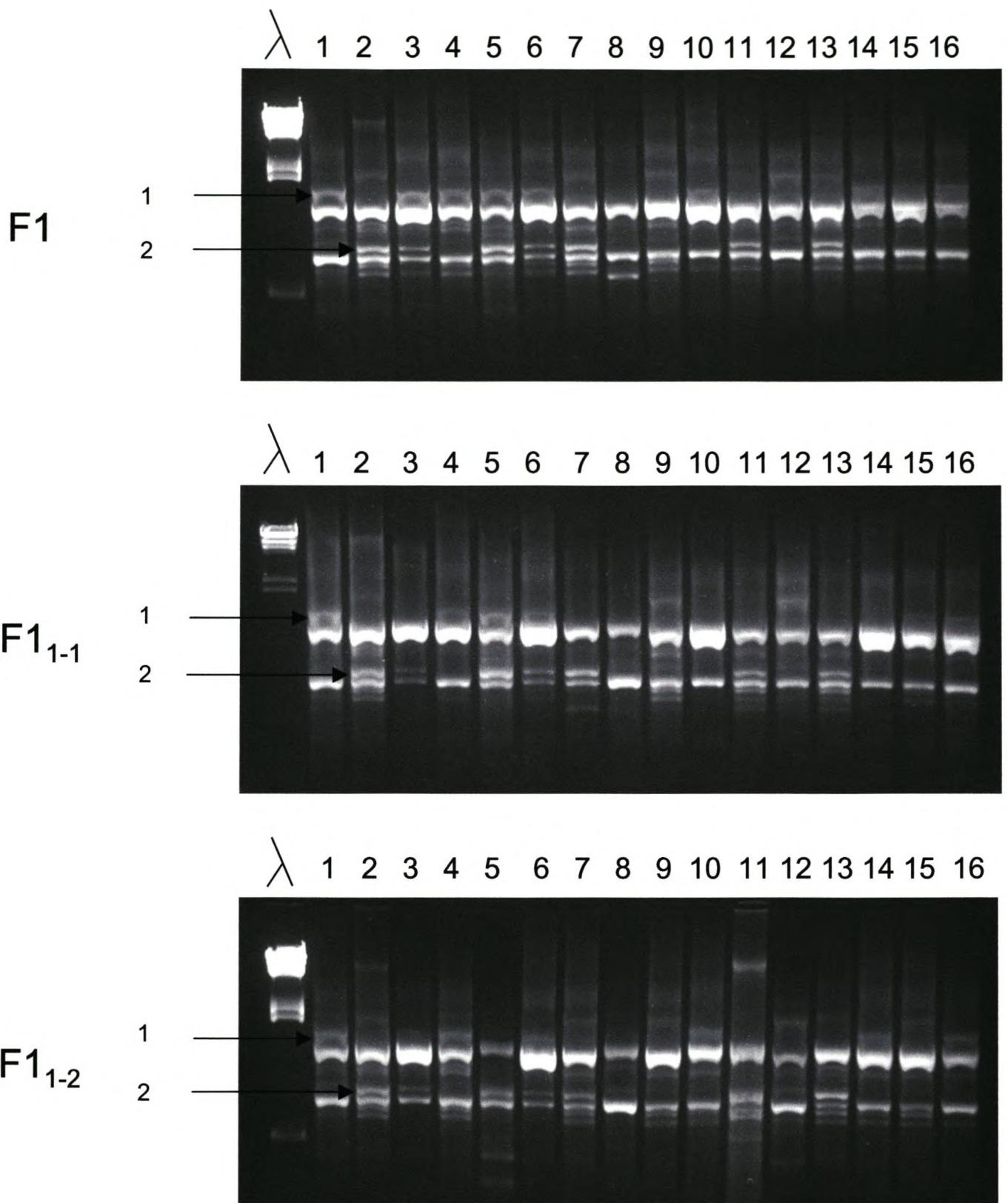


Fig. 3A. RAPD fingerprints for F1, F1₁₋₁ and F1₁₋₂ using primer OPE7. The first lane is λ DNA digested with *Hind*III. Lanes 1 and 2 are respectively the net- and spot-type parental isolates. Lanes 3-16 are the hybrid isolates. Marker bands 1 and 2 are indicated with arrows.

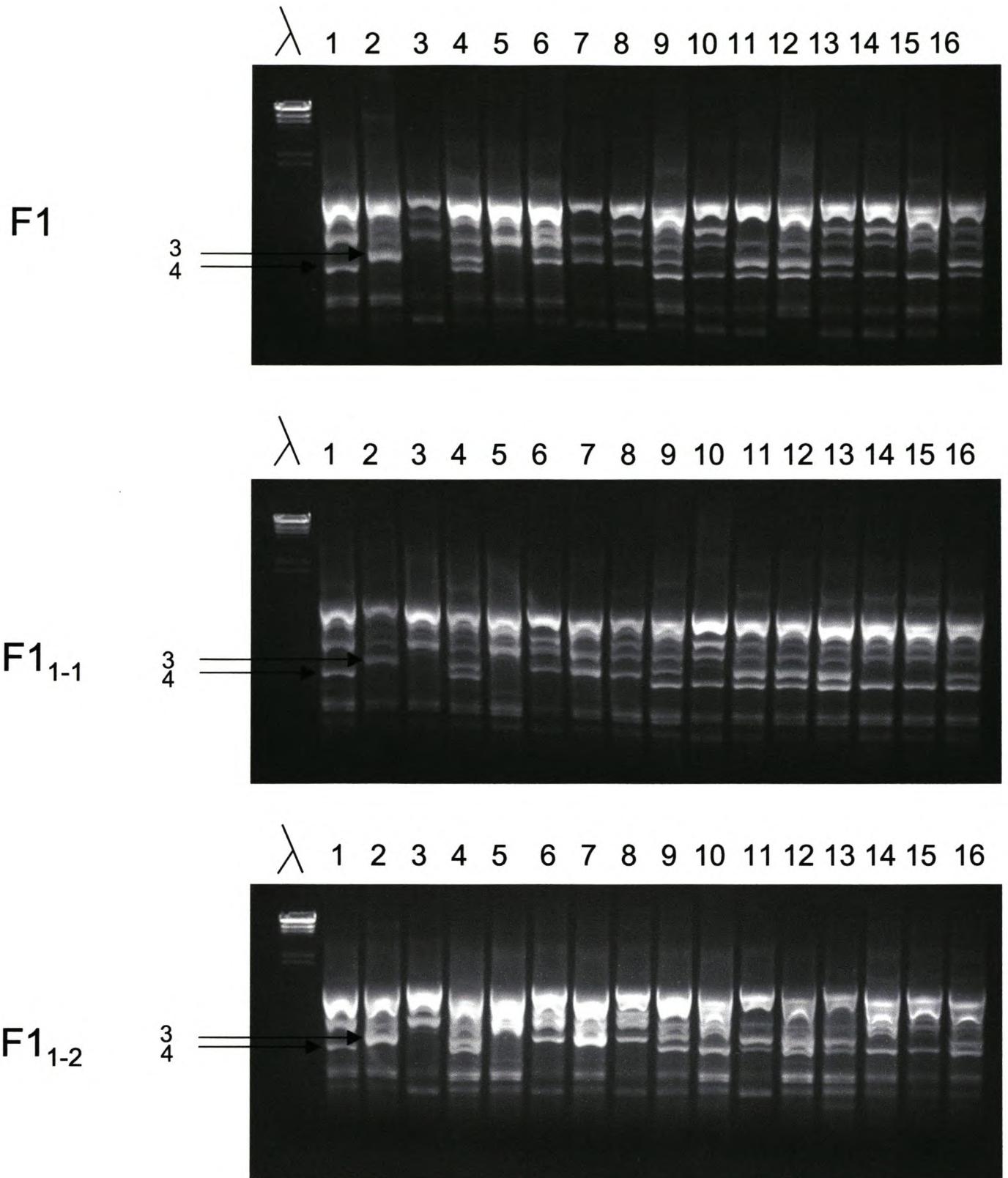


Fig. 3B. RAPD fingerprints for F1, F1₁₋₁ and F1₁₋₂ using primer OPE10. The first lane is λ DNA digested with *Hind*III. Lanes 1 and 2 are respectively the net- and spot-type parental isolates. Lanes 3-16 are the hybrid isolates. Marker bands 3 and 4 are indicated with arrows.

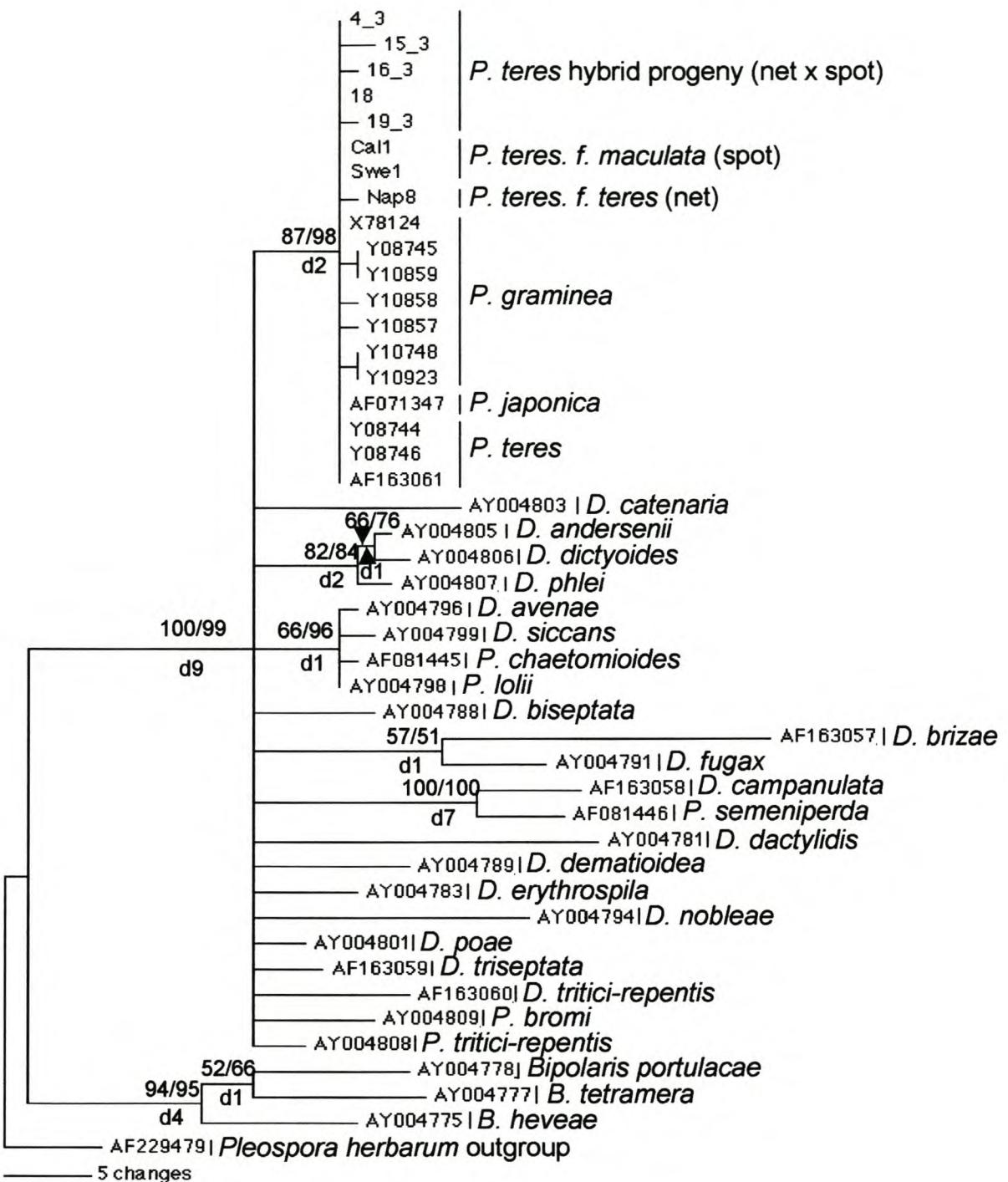


Fig. 4. One of the 48 MPTs obtained from the alignment of the ITS dataset using the heuristic search with a 1000 random sequence input orders of maximum parsimony. The tree is rooted with the outgroup *Pleospora herbarum* (TL = 252 steps, CI = 0.552, RI = 0.577, RC = 0.318, HI = 0.448). Bootstrap values of 500 replications in parsimony/1000 replications in neighbour-joining are indicated above the branches, while decay indices are indicated below. Isolate 18 is from the F_1 generation, while 4_3, 15_3, 16_3 and 19_3 are from the F_{1-2} generation.

4. Genetic diversity as determined by RAPD analysis amongst *Pyrenophora teres* populations causing net- and spot-type symptoms on barley

ABSTRACT

The genetic structure of *Pyrenophora teres*, the causal agent of net blotch of barley was examined in two fields 30 km apart in the Western Cape province of South Africa. The two fields respectively represented a net- and spot-type population, the two types being distinguished on the basis of symptom expression on differentially susceptible cultivars. The number of isolates sampled from each field was 36 for the net-type population and 29 for the spot-type population. Samples were collected from infected barley leaves from two separate quadrants in each field, the two quadrants positioned in corners of the fields, diagonal to one another. Of the 40 10-mer random oligonucleotide primers screened, five produced scorable, reproducible DNA bands suitable for the determination of population structure. A total of 65 loci were produced of which 54 were polymorphic. Genetic analysis of bands produced by one of the primers has revealed single locus segregation in a mating between a net- and spot-type isolate, indicating that RAPD bands can be interpreted as alleles at genetic loci. Total gene diversities determined for all loci resulted in mean indices of 0.063 and 0.082 being obtained respectively for the net- and spot-type populations. Genetic diversity among the two populations was divided into within- (variation between sampling sites) and among population components using Nei's G_{ST} . A coefficient of genetic differentiation (G_S) of 0.0149 was obtained between sites within populations while a coefficient (G_T) of 0.63 was obtained between the two populations. Genotypic variation revealed 13 distinct multilocus genotypes (haplotypes) in the net-type population while there were 12 in the spot-type population. UPGMA cluster analysis done on the two populations together with six

progeny from the mating between a net- and spot-type isolate resulted in three main clusters being produced, one for each population and one for the progeny. One isolate collected from the net-type population that did not cluster with the other net-type isolates clustered directly next to the cluster containing the sexual progeny. This isolate also contained a unique spot-type DNA band. This suggested that sexual recombination may be occurring between net- and spot-type isolates under field conditions.

INTRODUCTION

Genetic structure refers to the amount and distribution of genetic variation within and between populations (McDonald & McDermott, 1993). Knowledge of the genetic structure of fungal pathogen populations has direct applications to agricultural ecosystems. For instance, the amount of genetic variation being maintained within a population indicates how rapidly a pathogen can evolve, and this information may eventually be used to predict how long a control measure is likely to be effective. Molecular markers are being used on an ever-increasing scale for quantifying genetic variation, superseding more conventional characters such as virulence phenotype and fungicide sensitivity that are under strong selection pressures in agricultural systems (Michelmore & Hulbert, 1987). RAPD markers have been used extensively to quantify genetic variation in fungal pathogens associated with food crops (Zheng & Ward, 1998; Meng *et al.*, 1999; Nyassé *et al.*, 1999; Vicente *et al.*, 1999; Morris *et al.*, 2000).

Pyrenophora teres Drechsler (anamorph *Drechsler teres* [Sacc.] Shoemaker), the causal agent of net blotch disease of barley (*Hordeum vulgare* L. emend. Bowden) is an economically important disease of this crop in South Africa and throughout most other barley production regions in the world (Shipton *et al.*, 1973; Steffenson *et al.*, 1991; Louw *et al.*, 1996). Yield losses attributed to net blotch, ranging between 26-77%, have been reported in various countries (Jordan, 1981; Khan, 1989; Jordan *et al.*, 1985; Martin, 1985; Deadman & Cooke, 1987; Delserone & Cole, 1987; Steffenson *et al.*, 1991). Two types of leaf symptoms are associated with net blotch disease, namely net and spot blotch (Smedegård-Petersen, 1971). *P. japonica* S. Ito

and Kurib. [anamorph *Drechslera tuberosa* (G. F. Atk.) Shoemaker] was originally described as the pathogen causing spot-type symptoms, whereas *P. teres* was associated with net-type lesions (Ito and Kurabayashi, 1931; Shoemaker, 1962). After successful mating between net- and spot-type isolates by McDonald (1967) and Smedegård-Petersen (1971) it was concluded that the two types were formae of the same biological species and were subsequently named *P. teres* f. *teres* (net-type isolates) and *P. teres* f. *maculata* (spot-type isolates). Both types occur within close proximity to one another in the Western Cape province of South Africa (Scott, 1995; Louw *et al.*, 1995, 1996).

Under laboratory conditions, mating has been induced between net- and spot-type isolates of *P. teres* (Smedegård-Petersen, 1976; Crous *et al.*, 1995; Campbell *et al.*, 1999). Furthermore, recombination, can alter the fungicide sensitivity of progeny (Peever & Milgroom, 1992a,b; Campbell *et al.*, 1999). However, to date no reports have been made of net x spot recombinant isolates existing under field conditions.

Preliminary studies have been done to determine the effectiveness of RAPDs as intraspecific markers in *P. teres* (Reeves & Ball, 1991; Louw *et al.*, 1995). RAPD markers have also been used to indicate genetic recombination following mating between net- and spot-type isolates (Campbell *et al.*, 1999). Comprehensive studies investigating genetic diversity within populations of *P. teres* have also been done in various countries (Peever & Milgroom, 1994; Peltonen *et al.*, 1999; Jonsson *et al.*, 2000). However, only populations comprising net-type isolates were evaluated in these studies. In the Western Cape province of South Africa barley cultivars susceptible to either the net- or spot-type of *P. teres* are grown within close proximity of one another. As a result of crop rotation, fields sown with cultivars susceptible to the spot-type are subsequently replanted with cultivars susceptible to the net-type. Coupled with this there has been a major population shift from the spot- to the net-type (Louw *et al.*, 1996). The sexual stage of the spot-type of *P. teres* has also been isolated from barley stubble in the Western Cape province (Louw *et al.*, 1994), thereby supporting the fact that meiosis and recombination are occurring under field conditions. There are, therefore, various evolutionary forces that may be changing the genetic structure of net blotch populations.

The aim of this study, therefore, was to investigate the genetic structure of *P. teres* populations in the Western Cape province using RAPDs, and furthermore, to ascertain if recombination occurs between the two types under field conditions.

MATERIALS AND METHODS

Sampling methods

Plant leaves from two winter barley fields with cultivars Stirling (30 ha) and Clipper (25 ha) were sampled respectively for net- and spot-type symptoms of *P. teres* in October 1997. One field was treated as a net-type and the other as a spot-type population. These two fields were located approximately 30 km apart in the Western Cape province, the major barley production region in South Africa. Leaves were sampled from two separate quadrants from each field, the quadrants being in the corners of each field diagonal to one another. The size of each quadrant was 25 m x 25 m. One infected leaf was collected per plant, with plants being 1-2 m from one another. The number of leaves sampled from each quadrant was 14 and 22 from the net-type field, and 14 and 15 from the spot-type field. The leaves were placed into separate brown paper bags and subsequently air-dried in the laboratory for 3 d.

Fungal isolation

Leaves were surface sterilised by immersion for 30 s in 70% ethanol, followed by 60 s in 2% NaOCl and 30 s in 70 % ethanol. Sterilised leaves were air-dried in a laminar airflow cabinet. Sporulation of *Pyrenophora* was achieved by placing the sterilised leaves in moisture chambers which were then incubated at 4°C for 3-4 days. Single conidia were subsequently inoculated onto potato dextrose agar (PDA) and incubated for 7 days. Cultures were maintained on PDA slants at 4°.

DNA isolation

Mycelial plugs from 7-day-old cultures were transferred to flasks containing 100 ml of a yeast extract and glucose medium (YEG) [8 g/l yeast extract and 5

g/l glucose]. Flasks were incubated on a rotary shaker at 150 rpm at 25° for 2 days. Further procedures were conducted as described by Campbell *et al.* (1999).

RAPD analysis

Amplification reactions were performed in a final volume of 25 µl of reaction mixture. The reaction mixture contained 2.5 µl of 10X NH₄ buffer [160 mM (NH₄)₂SO₄, 670 mM Tris-HCl (pH 8.8), 0.1% Tween-20] [Bioline Ltd, London, UK and Whitehead Scientific, South Africa]; 200 µm of each dNTP; 10 pmol of oligonucleotide primer, 50 ng genomic DNA and 1.0 U of BIOTAQ DNA polymerase. The MgCl₂ concentration was adjusted to a final concentration of 4 mM. Sequences of primers used in separate RAPD reactions were as follows:

| | |
|---------|-------------------|
| GFC 1: | 5' CGTCGCTGTT 3' |
| GFC 2: | 5' ATACGGGCAA 3' |
| OPE 15: | 5' ACGCACAAACC 3' |
| OPE 18: | 5' GGACTGCAGA 3' |
| OPM 10: | 5' TCTGGCGCAC 3' |

Sequences for primers GFC 1 and GFC 2 were obtained from Peever and Milgroom (1994) and primers OPE 15, OPE 18 and OPM 10 were obtained from Operon Technologies Inc. Alameda, USA.

Amplifications were conducted in a Perkin Elmer GeneAmp 2400 PCR System. Reactions underwent an initial denaturation process at 96° for 120 s, followed by 45 cycles of 92° for 30 s, 38° for 30 s and 72° for 60 s. After the last cycle a final extension step was conducted at 72° for 120 s. Amplification products were separated through 1.5% (w/v) agarose gels in TAE buffer (Sambrook *et al.*, 1989).

Data analysis

DNA gel analysis. DNA gels were observed in a SYNGENE Darkroom S/N: SYDR/1318 linked to a desktop computer. Fingerprints were captured using the SYNGENE programme GENESNAP. A 100 base pair ladder (DNA molecular weight marker XIV, Boehringer Mannheim Chemicals, South Africa)

was used as a molecular weight reference for comparing samples from different gels.

DNA gels were scored only for reproducible fragments. RAPD bands at each locus were treated as a 1 (present) or a 0 (absent) and entered into a binary matrix. Furthermore, all bands that were present at each locus were assumed to be the same fragment in all isolates. A multilocus DNA banding pattern or haplotype was generated for each isolate by combining each individual banding pattern for each primer into one data set.

Population structure analyses. For the population structure analyses the progeny from the net x spot mating were excluded, as these had been produced from a mating between a net- and spot-type isolate under laboratory conditions. These progeny were therefore deemed not to be field isolates. Population structure was analysed using four different measures of genetic diversity. The measures used were the proportion of polymorphic loci, gene diversity, the coefficient of genetic differentiation, and lastly the number and frequency of haplotypes in each population.

The proportion of polymorphic loci for each sampling site was calculated by dividing the number of polymorphic loci over the total number of loci. A locus was considered to be polymorphic when the most common allele had a frequency of no greater than 95%.

The frequencies of the alleles at each polymorphic RAPD locus and mean allele frequencies for each locus amongst the net- and spot populations (weighted by population sample size) were determined. The gene diversity at each polymorphic RAPD locus for each individual sampling site was determined by $H_i = 2p_i(1-p_i)$ where p_i is the allele frequency at locus i (Crow, 1986). For l loci the mean genetic diversity is given by $\bar{H} = \frac{1}{l} \sum_{i=1}^l H_i$ (Weir, 1996). Estimation of H_i was done by using the $n/(n-1)$ correction for small samples of size n (Nei, 1987).

The genetic structure within and between populations was determined using the coefficient of genetic differentiation according to Nei's G-statistics (Nei, 1973). In this study, the analysis was carried out at two levels. The

proportion of genetic divergence between sites within populations was determined using $G_s = \frac{\bar{H}_s - \bar{H}}{\bar{H}_s}$ and the proportion between populations was determined using $G_r = \frac{\bar{H}_r - \bar{H}}{\bar{H}_r}$ (Hartl & Clark, 1997).

An analysis of variance (ANOVA) was performed on the data with populations, sites within populations and loci as main effects. This analysis was done on gene diversities transformed to $\arcsin \sqrt{\frac{np}{n+3}}$ as is standard practise for analysis of variance of frequency data, which can take values of 0 and 1 (Sokal & Rolf, 1995).

Lastly, the number and frequencies of each multilocus banding pattern (haplotype) was determined for each sampling site and each population. This was to obtain a measure of genotypic diversity.

Cluster analysis. Simple matching coefficients (S_{sm}) were generated for each pair of isolates for RAPD markers. The simple matching coefficients were calculated by the formula described by Sneath and Sokal (1973):

$S_{sm} = m/(m+u)$, where m is the number of bands found in common between two isolates and u is the total number of bands unique to each isolate. A phenogram for each type of data was constructed after cluster analysis of the similarity coefficients by unweighted pair-group method using arithmetic averages (UPGMA) (Sneath & Sokal, 1973). These calculations were performed with the programs SIMQUAL and SAHN of the software package NTSYS-pc version 1.80 (Exeter Software, Setauket, NY).

RESULTS

Forty 10-mer arbitrary oligonucleotide primers were screened for their capacity to identify DNA polymorphism amongst 10 randomly selected net- and spot-type isolates of *P. teres*. Thirty-six primers produced bands for all isolates. Of these, five primers that produced strong, reproducible polymorphic bands were selected for further analysis. A total of 65 RAPD loci

were produced after PCR-amplification using these five primers. Both monomorphic and polymorphic loci were used for all analyses. Examples of agarose gels containing DNA banding patterns obtained using five primers are shown in Fig. 1a-e. Independent DNA preparations from the same isolate used in separate PCR amplifications produced similar banding patterns.

The two populations were each represented by two sites with 11-26 monoconidial isolates per site. The proportion of polymorphic RAPD loci at each of these two sites was 30.8% and 13.8% in population A (net-type) while values of 58.8% and 16.9% were obtained in population B (spot-type).

Allele frequencies for the presence of bands at the 65 loci and the corresponding gene diversities are listed in Table 1. The mean gene diversities (H) were 0.0625 and 0.082 in populations A and B respectively. Nei's diversity statistics are summarised in Table 2. The proportion of diversity between sites within populations (G_S) was 0.0149. Furthermore, the proportion of diversity between the two populations (G_T) was 0.6301.

An analysis of variance (Table 3) of the gene diversities indicated significant differences between the two populations and loci ($P \leq 0.01$) while highly significant differences were obtained for sites within populations ($P \leq 0.001$).

A total of 25 unique multilocus haplotypes were obtained following pooling together of all five primers for each isolate. Thirteen haplotypes were from population A while 12 were from population B (Table 4).

A dendrogram based on simple matching coefficients amongst the field isolates from the two populations and the progeny from the net x spot mating is presented in Fig. 2. All progeny isolates, all net-type isolates except one, and all spot-type isolates except two grouped into three main clusters. The net-type isolate that did not cluster with the other net-type isolates (isolate 2) contained a unique spot-type RAPD band and therefore clustered directly next to the progeny cluster. The two spot-type isolates that did not cluster within the spot-type cluster clustered by themselves. These two isolates exhibited 12 and 9 unique RAPD bands, respectively, that were not observed in any of the other isolates.

DISCUSSION

Knowledge of the population structure of phytopathogenic organisms of major agricultural crops is important when formulating strategies for disease control (McDonald & McDermott, 1993). Investigations of the genetic structure of *P. teres* populations have to date only involved net-type populations (Peever & Milgroom, 1994; Jonsson *et al.*, 2000). However, in the Western Cape province cultivars susceptible to net- and spot-type isolates of *P. teres* are grown within very close proximity of one another. The aim of the present study was, therefore, to determine the population structures of local net- and spot-type populations occurring in barley fields, and also to determine if a relationship exists between the two types.

The mean gene diversities obtained in the present study for the two populations (0.062 and 0.082) are lower than those obtained in other *P. teres* population studies. Gene diversities of 0.182 and 0.216 were observed in two net blotch populations in Sweden (Jonsson *et al.*, 2000) while values of 0.08-0.17 were obtained amongst five populations in North America and Germany (Peever & Milgroom, 1994). This may be attributed to the fact that in the present study polymorphic as well as monomorphic RAPD loci were taken into consideration during the calculation of gene diversities. The gene diversities obtained by Peever and Milgroom (1994) were based only on polymorphic loci. Furthermore, in the present study, gene diversities were based on 65 loci which is in contrast to Peever and Milgroom (1994) and Jonsson *et al.* (2000) who respectively used 8 and 19 loci for their calculations.

In the present study the coefficient of genetic differentiation between the net- and spot-type populations was 0.63. Peever and Milgroom (1994) obtained a value of 0.46 that was reduced to 0.33 when a revised figure was calculated only including the four North American populations. Furthermore, between two Alberta populations which were only 20 km apart a value of 0.05 was obtained which is the same as the figure of 0.053 observed by Jonsson *et al.* (2000) between the two Swedish populations that were also 20 km apart. The G_T value of 0.63 obtained in the present study therefore indicates that net- and spot-type isolates are genetically distinct to one another. Further evidence that the two types are genetically distinct can be

determined through the use of conventional markers such as symptom expression on differentially susceptible cultivars (Smedegård-Petersen, 1971).

The genotypic variation observed in the present study requires clarification. At one sampling site where 26 isolates were collected 10 unique multilocus genotypes haplotypes were obtained. This is very similar to results obtained by Peever and Milgroom (1994) who concluded that four out of the five populations they studied were sexually reproducing based on the presence of 5-9 multilocus genotypes among the 23-35 isolates collected from each population. Other fungal populations have also shown a relatively low degree of genotypic diversity amongst isolates within a single population (McDonald & Martinez, 1990). In a single wheat field 22 haplotypes were observed among 93 isolates of *Mycosphaerella graminicola*. This is in contrast to other studies in which a high degree of genotypic variation has been observed. Milgroom *et al.* (1992) were able to differentiate 33 different genotypes out of a sample of 39 isolates of the chestnut blight fungus *Cryphonectria parasitica*, while Kohn *et al.* (1991) were able to show that a canola field containing a population of *Sclerotinia sclerotiorum* had 88 distinct genotypes.

Mating and subsequent recombination between net- and spot-type isolates of *P. teres* has been demonstrated in various studies under laboratory conditions (Smedegård-Petersen, 1976; Campbell *et al.*, 1999). However, there are no reports to date indicating sexual reproduction between net- and spot-type isolates under field conditions.

Evidence of sexual reproduction within fungal ecosystems can be assessed in a number of ways (Milgroom, 1996). One strategy that can be employed is to assess the genotypic composition of a population to identify heterozygotes. The advantage of using molecular markers as opposed to conventional markers to investigate genotypic composition is that they are often present as codominant characters (McDonald & McDermott, 1993). This allows heterozygous individuals to be distinguished from homozygotes. RAPD markers are generally dominant (Lynch & Milligan, 1994), whereas isozyme and RFLP markers frequently exhibit codominant markers (McDonald, 1997). However, in a previous study, Campbell *et al.* (1999) used codominant RAPD markers to demonstrate genetic recombination between a net- and spot-type

isolate. Genetic recombination was furthermore verified using amplified fragment length polymorphisms (AFLPs) (Campbell *et al.*, 1999). In the present study, using the same RAPD primer, a field isolate exhibiting unique net- and spot-type DNA bands was identified. This was not entirely surprising as barley cultivars that are susceptible to either spot- or net-type respectively are grown within very close proximity of each other i.e., in adjacent fields. Therefore, net- and spot-type isolates have the potential for coming into contact with each other and as a result the potential for sexual reproduction exists. In Canada, where net- and spot-type isolates occur in all major barley growing regions, Tekauz (1990) reported that the increase in the number of *P. teres* pathotypes could be due to one or a combination of factors, including the opportunity for sexual recombination between the two types. Furthermore, Tekauz (1990) also indicated that some barley cultivars were susceptible to both types of *P. teres* that included 11 pathotypes of the net-type and seven pathotypes of the spot-type. Louw *et al.* (1996) also reported the susceptibility of barley cultivars in South Africa to both forms of the pathogen.

The presence of net x spot recombinant isolates under field conditions may therefore be significant. Firstly, it could make identification of the pathogen difficult when foliar symptoms are assessed. Intermediate symptom types (Campbell *et al.*, 1999; Smedegård-Petersen, 1976, 1977), variation within spot-type symptoms incited by *P. teres* f. *maculata* (Scott, 1991; Skou & Haahr, 1987; Tekauz & Mills, 1974), spot blotch symptoms produced by *Cochliobolus sativus* (Tekauz & Mills, 1974), genetic necrosis (Karki & Sharp, 1986) and boron toxicity (Scott, 1991) all produce similar foliar symptoms that could make identification of net-spot recombinants difficult without further laboratory analysis using more established molecular techniques. Secondly, fungal sexual recombination may lead to an increased rate of fungicide resistance (Crute, 1989; Milgroom, 1996). Campbell *et al.* (1999) reported fungicide resistance in progeny following sexual reproduction between a net- and spot-type isolate of *P. teres*. This means that net x spot recombinants resistant to commercially used fungicides may potentially occur under field conditions. Asexual reproduction may then subsequently lead to a high level of these recombinant net x spot genotypes. Furthermore, as reported in Chapter 3, recombinant net x spot isolates can remain genetically stable and

pathogenic through repeated cycles of isolation from foliar lesions and subsequent re-inoculation onto healthy barley seedlings.

The sexual stage of the spot-type of *P. teres* has been isolated from barley fields in the Western Cape province (Louw *et al.* 1994). Further evidence of sexual recombination occurring under field conditions was indicated by the fact that many of the spot-type isolates from sampling site B1 had distinct multilocus haplotypes. These isolates also had several unique DNA bands that were not present in any of the other spot-type isolates tested. Sexual reproduction breaks up allelic associations and causes allelic variants to occur independently relative to each other. This may also provide an explanation for the manner in which the two spot-type isolates (isolates 61 and 64 in dendrogram) clustered in the dendrogram. Interestingly, field isolate 2 that clustered directly between these two isolates and the hybrid progeny (N x S cluster) was isolated from a net-type foliar symptom. However, RAPD analysis using primer OPM10 indicated that this isolate contained unique net- and spot-type bands, thereby indicating that sexual reproduction had occurred between net- and spot-type isolates under field conditions. Therefore, on the basis that sexual reproduction breaks up allelic associations, it is not entirely surprising that the two spot-type isolates clustered next to the hybrid field isolate and the hybrid progeny where sexual reproduction had clearly occurred. This is in contrast to asexual reproduction that produces a limited number of genotypes, showing strong allelic associations between loci (Jonsson *et al.* 2000).

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Table 1. Frequencies of the presence of bands for 65 RAPD loci in isolates of *P. teres* f. *teres* and *P. teres* f. *maculata* sampled from 2 sites in each of two barley fields. Sites A1 and A2 were sampled from a field grown from cv. Stirling, and B1 and B2 from cv. Clipper

| Locus | Population sites | | | | | | | |
|-----------|--------------------|-------|-------|-------|----------------------|-------|-------|-------|
| | Allele frequencies | | | | Gene diversities (H) | | | |
| | A1 | A2 | B1 | B2 | A1 | A2 | B1 | B2 |
| GFC 1 01 | 0.818 | 1.000 | 1.000 | 1.000 | 0.327 | 0.000 | 0.000 | 0.000 |
| GFC 1 02 | 1.000 | 1.000 | 1.000 | 1.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| GFC 1 03 | 1.000 | 1.000 | 1.000 | 1.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| GFC 1 04 | 0.909 | 1.000 | 1.000 | 1.000 | 0.182 | 0.000 | 0.000 | 0.000 |
| GFC 1 05 | 1.000 | 0.808 | 1.000 | 1.000 | 0.000 | 0.323 | 0.000 | 0.000 |
| GFC 1 06 | 0.000 | 0.000 | 0.929 | 1.000 | 0.000 | 0.000 | 0.143 | 0.000 |
| GFC 1 07 | 0.091 | 0.000 | 0.000 | 0.000 | 0.182 | 0.000 | 0.000 | 0.000 |
| GFC 1 08 | 1.000 | 1.000 | 0.929 | 1.000 | 0.000 | 0.000 | 0.143 | 0.000 |
| GFC 1 09 | 0.455 | 0.154 | 0.000 | 0.000 | 0.545 | 0.271 | 0.000 | 0.000 |
| GFC 1 10 | 0.000 | 0.038 | 0.857 | 0.867 | 0.000 | 0.077 | 0.264 | 0.248 |
| GFC 1 11 | 0.727 | 1.000 | 0.929 | 1.000 | 0.436 | 0.000 | 0.143 | 0.000 |
| GFC 1 12 | 0.000 | 0.038 | 0.000 | 0.000 | 0.000 | 0.077 | 0.000 | 0.000 |
| GFC 1 13 | 0.000 | 0.000 | 0.214 | 0.000 | 0.000 | 0.000 | 0.363 | 0.000 |
| GFC 1 14 | 0.000 | 0.000 | 0.071 | 0.000 | 0.000 | 0.000 | 0.143 | 0.000 |
| GFC 1 15 | 0.091 | 0.000 | 0.071 | 0.000 | 0.182 | 0.000 | 0.143 | 0.000 |
| GFC 2 01 | 1.000 | 1.000 | 1.000 | 1.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| GFC 2 02 | 0.091 | 0.000 | 1.000 | 1.000 | 0.182 | 0.000 | 0.000 | 0.000 |
| GFC 2 03 | 0.818 | 1.000 | 0.357 | 0.067 | 0.327 | 0.000 | 0.495 | 0.133 |
| GFC 2 04 | 0.273 | 0.000 | 1.000 | 1.000 | 0.436 | 0.000 | 0.000 | 0.000 |
| GFC 2 05 | 0.727 | 1.000 | 0.000 | 0.000 | 0.436 | 0.000 | 0.000 | 0.000 |
| GFC 2 06 | 1.000 | 1.000 | 0.714 | 1.000 | 0.000 | 0.000 | 0.440 | 0.000 |
| GFC 2 07 | 1.000 | 0.962 | 0.429 | 0.400 | 0.000 | 0.077 | 0.527 | 0.514 |
| GFC 2 08 | 0.000 | 0.000 | 0.786 | 0.933 | 0.000 | 0.000 | 0.363 | 0.133 |
| GFC 2 09 | 0.909 | 1.000 | 0.071 | 0.067 | 0.182 | 0.000 | 0.143 | 0.133 |
| GFC 2 10 | 0.000 | 0.000 | 0.214 | 0.000 | 0.000 | 0.000 | 0.363 | 0.000 |
| GFC 2 11 | 0.000 | 0.000 | 0.143 | 0.000 | 0.000 | 0.000 | 0.264 | 0.000 |
| GFC 2 12 | 0.000 | 0.000 | 0.214 | 0.000 | 0.000 | 0.000 | 0.363 | 0.000 |
| GFC 2 13 | 0.000 | 0.000 | 0.071 | 0.000 | 0.000 | 0.000 | 0.143 | 0.000 |
| GFC 2 14 | 0.000 | 0.000 | 0.071 | 0.000 | 0.000 | 0.000 | 0.143 | 0.000 |
| OPE 15 01 | 1.000 | 1.000 | 1.000 | 1.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| OPE 15 02 | 0.909 | 1.000 | 0.071 | 0.000 | 0.182 | 0.000 | 0.143 | 0.000 |
| OPE 15 03 | 1.000 | 1.000 | 1.000 | 1.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| OPE 15 04 | 1.000 | 1.000 | 1.000 | 1.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| OPE 15 05 | 1.000 | 1.000 | 0.929 | 1.000 | 0.000 | 0.000 | 0.143 | 0.000 |
| OPE 15 06 | 0.000 | 0.000 | 0.929 | 1.000 | 0.000 | 0.000 | 0.143 | 0.000 |
| OPE 15 07 | 0.909 | 1.000 | 0.857 | 1.000 | 0.182 | 0.000 | 0.264 | 0.000 |
| OPE 15 08 | 0.091 | 0.000 | 0.000 | 0.000 | 0.182 | 0.000 | 0.000 | 0.000 |
| OPE 15 09 | 0.000 | 0.000 | 0.071 | 0.000 | 0.000 | 0.000 | 0.143 | 0.000 |
| OPE 15 10 | 0.000 | 0.000 | 0.143 | 0.000 | 0.000 | 0.000 | 0.264 | 0.000 |
| OPE 18 01 | 1.000 | 1.000 | 1.000 | 1.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| OPE 18 02 | 1.000 | 1.000 | 1.000 | 1.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| OPE 18 03 | 1.000 | 0.962 | 1.000 | 1.000 | 0.000 | 0.077 | 0.000 | 0.000 |
| OPE 18 04 | 0.818 | 1.000 | 1.000 | 1.000 | 0.327 | 0.000 | 0.000 | 0.000 |
| OPE 18 05 | 0.273 | 0.038 | 0.000 | 0.000 | 0.436 | 0.077 | 0.000 | 0.000 |
| OPE 18 06 | 0.636 | 0.423 | 0.286 | 0.000 | 0.509 | 0.508 | 0.440 | 0.000 |
| OPE 18 07 | 0.000 | 0.000 | 0.143 | 0.000 | 0.000 | 0.000 | 0.264 | 0.000 |
| OPE 18 08 | 0.000 | 0.000 | 0.071 | 0.000 | 0.000 | 0.000 | 0.143 | 0.000 |
| OPE 18 09 | 0.000 | 0.038 | 0.000 | 0.000 | 0.000 | 0.077 | 0.000 | 0.000 |
| OPM 10 01 | 0.727 | 1.000 | 1.000 | 1.000 | 0.436 | 0.000 | 0.000 | 0.000 |
| OPM 10 02 | 1.000 | 1.000 | 0.857 | 1.000 | 0.000 | 0.000 | 0.264 | 0.000 |
| OPM 10 03 | 1.000 | 1.000 | 0.071 | 0.067 | 0.000 | 0.000 | 0.143 | 0.133 |
| OPM 10 04 | 0.000 | 0.000 | 0.714 | 0.933 | 0.000 | 0.000 | 0.440 | 0.133 |
| OPM 10 05 | 1.000 | 1.000 | 1.000 | 1.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| OPM 10 06 | 0.727 | 1.000 | 0.071 | 0.067 | 0.436 | 0.000 | 0.143 | 0.133 |
| OPM 10 07 | 1.000 | 1.000 | 0.929 | 1.000 | 0.000 | 0.000 | 0.143 | 0.000 |
| OPM 10 08 | 1.000 | 1.000 | 1.000 | 1.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| OPM 10 09 | 0.273 | 0.000 | 1.000 | 0.933 | 0.436 | 0.000 | 0.000 | 0.133 |
| OPM 10 10 | 0.000 | 0.000 | 0.929 | 0.933 | 0.000 | 0.000 | 0.143 | 0.133 |
| OPM 10 11 | 1.000 | 1.000 | 0.000 | 0.067 | 0.000 | 0.000 | 0.000 | 0.133 |
| OPM 10 12 | 1.000 | 1.000 | 0.929 | 1.000 | 0.000 | 0.000 | 0.143 | 0.000 |
| OPM 10 13 | 1.000 | 1.000 | 0.929 | 1.000 | 0.000 | 0.000 | 0.143 | 0.000 |
| OPM 10 14 | 0.000 | 0.000 | 0.071 | 0.000 | 0.000 | 0.000 | 0.143 | 0.000 |
| OPM 10 15 | 0.000 | 0.000 | 0.071 | 0.000 | 0.000 | 0.000 | 0.143 | 0.000 |
| OPM 10 16 | 0.000 | 0.000 | 0.071 | 0.000 | 0.000 | 0.000 | 0.143 | 0.000 |
| OPM 10 17 | 0.000 | 0.000 | 0.071 | 0.000 | 0.000 | 0.000 | 0.143 | 0.000 |
| Mean | 0.512 | 0.515 | 0.543 | 0.528 | 0.101 | 0.024 | 0.133 | 0.030 |

Table 2. Polymorphism and genetic diversity (\bar{H})^a for 65 RAPD loci between and within populations and sites of *P. teres*

| | Sites | | | | Total | |
|--------------------------|--------------|-----------|--------------|------------|--------|--|
| | Population A | | Population B | | | |
| | A1 | A2 | B1 | B2 | | |
| Isolates | 11 | 26 | 14 | 15 | | |
| Polymorphic loci | 20 (30.8%) | 9 (13.8%) | 38 (58.8%) | 11 (16.9%) | | |
| \bar{H} | 0.1007 | 0.0240 | 0.1332 | 0.0302 | 0.0614 | |
| St. error ^d | 0.0209 | 0.0102 | 0.0181 | 0.0101 | 0.0080 | |
| \bar{H}_s ^b | 0.0489 | | 0.0794 | | 0.0623 | |
| St. error | 0.0112 | | 0.0119 | | 0.0081 | |
| \bar{H}_T ^c | | | | | 0.1659 | |
| St. error | | | | | 0.0247 | |

^a \bar{H} is mean gene diversity within sites, calculated directly from allele frequencies and then averaged over all loci. All averaging takes account of variations in isolate numbers by the standard procedure of weighting, i.e., mean frequencies weighted by sample size.

^b \bar{H}_s is expected gene diversity within populations calculated from mean allele frequencies averaged over sites, and then averaged over all loci.

^c \bar{H}_T is expected gene diversity in the total population, calculated from mean allele frequencies averaged over sites and populations, and then over loci.

^d Standard errors are calculated from the variances of gene diversity over loci.

Table 3. Analysis of variance for effects of populations, sites within populations and loci as inferred by differences in gene diversity in *P. teres*

| Effect | df | Mean square | F |
|---|-----|-------------|-------------|
| Populations | 1 | 0.1394 | 6.6726 ** |
| Sites within populations | 2 | 0.7845 | 37.5561 *** |
| Loci | 64 | 0.0333 | 1.5945 ** |
| Loci x Populations | 64 | 0.0401 | 1.9174 * |
| Loci x Sites within populations (error) | 128 | 0.0209 | |

* $P \leq 0.05$

** $P \leq 0.01$

*** $P \leq 0.001$

Table 4. Frequencies and distribution of the 25 multilocus haplotypes (1=presence, 0=absence) found in separate net- and spot-type populations of *P. teres*

| Multilocus haplotype | | | | | | | | | | n | Population site |
|-----------------------|-------------------------------|-----------------------------------|-------------------|---------------------------------|----|----|--|--|--|---|-----------------|
| 1 0 1 1 1 0 0 1 0 0 | 0 1 1 0 1 0 1 1 0 0 0 0 0 0 1 | 0 1 1 0 1 0 1 1 1 0 1 1 1 0 0 0 0 | 1 1 1 0 1 0 0 0 0 | 1 1 0 1 0 1 1 0 0 0 0 0 0 0 0 0 | 1 | A1 | | | | | |
| 1 1 1 1 1 0 1 0 0 0 | 1 1 1 1 1 0 0 1 1 0 1 0 0 0 0 | 1 1 1 0 1 1 1 1 0 0 1 1 1 0 0 0 0 | 1 1 1 0 0 1 0 0 0 | 1 0 1 0 1 1 1 0 1 0 0 0 0 0 | 1 | A1 | | | | | |
| 1 1 1 1 1 1 0 1 0 0 0 | 1 1 1 1 1 0 0 1 1 0 1 0 0 0 0 | 1 1 1 0 1 0 1 1 0 0 1 1 1 0 0 0 0 | 1 1 1 1 0 1 0 0 0 | 1 0 1 0 1 1 1 0 1 0 0 0 0 0 | 1 | A1 | | | | | |
| 1 1 1 1 1 1 0 1 0 0 0 | 1 1 1 1 1 0 0 1 0 0 1 0 0 0 0 | 1 1 1 0 1 1 1 1 0 0 1 1 1 0 0 0 0 | 1 1 0 1 1 0 0 0 1 | 1 0 1 0 1 1 1 0 1 0 0 0 0 0 | 1 | A2 | | | | | |
| 1 1 1 1 1 1 0 1 0 0 0 | 1 1 1 1 0 0 0 1 0 0 1 0 0 0 0 | 1 1 1 0 1 1 1 1 0 0 1 1 1 0 0 0 0 | 1 1 1 1 0 1 0 0 0 | 1 0 1 0 1 1 1 0 1 0 0 0 0 0 | 2 | A2 | | | | | |
| 1 1 1 1 1 1 0 1 0 0 0 | 1 1 1 1 0 0 0 1 0 0 1 0 0 0 0 | 1 1 1 0 1 1 1 1 0 0 1 1 1 0 0 0 0 | 1 1 1 1 0 0 0 0 0 | 1 0 1 0 1 1 1 0 1 0 0 0 0 0 | 3 | A2 | | | | | |
| 1 1 1 1 1 1 0 1 0 0 0 | 1 1 1 1 1 0 0 1 0 0 1 1 0 0 0 | 1 1 1 0 1 1 1 1 0 0 1 1 1 0 0 0 0 | 1 1 1 1 0 0 0 0 0 | 1 0 1 0 1 1 1 0 1 0 0 0 0 0 | 1 | A2 | | | | | |
| 1 1 1 1 1 1 0 1 0 0 0 | 1 1 1 1 1 0 0 1 0 0 1 0 0 0 0 | 1 1 1 0 1 1 1 1 0 0 1 1 1 0 0 0 0 | 1 1 1 1 0 0 0 0 0 | 1 0 1 0 1 1 1 0 1 0 0 0 0 0 | 8 | A2 | | | | | |
| 1 1 1 1 1 1 0 1 0 0 0 | 1 1 1 1 1 0 0 1 0 1 1 0 0 0 | 1 1 1 0 1 1 1 1 0 0 1 1 1 0 0 0 0 | 1 1 1 1 0 1 0 0 0 | 1 0 1 0 1 1 1 0 1 0 0 0 0 0 | 1 | A2 | | | | | |
| 1 1 1 1 1 1 0 1 0 0 0 | 1 1 1 1 1 0 0 1 0 0 1 0 0 0 0 | 1 1 1 0 1 1 1 1 0 0 1 1 1 0 0 0 0 | 1 1 1 1 0 1 0 0 0 | 1 0 1 0 1 1 1 0 1 0 0 0 0 0 | 9 | A2 | | | | | |
| 1 1 1 1 1 1 0 1 0 0 0 | 1 1 1 1 1 0 0 1 1 0 1 0 0 0 | 1 1 1 0 1 1 1 1 0 0 1 1 1 0 0 0 0 | 1 1 1 1 0 0 0 0 0 | 1 0 1 0 1 1 1 0 1 0 0 0 0 0 | 1 | A2 | | | | | |
| 1 1 1 1 1 1 0 1 0 0 0 | 1 1 1 1 1 0 0 1 1 0 1 0 0 0 | 1 1 1 0 1 1 1 1 0 0 1 1 1 0 0 0 0 | 1 1 1 1 0 0 0 0 0 | 1 0 1 0 1 1 1 0 1 0 0 0 0 0 | 2 | A2 | | | | | |
| 1 1 1 1 1 1 0 1 0 0 0 | 1 1 1 1 1 0 0 1 1 0 1 0 0 0 | 1 1 1 0 1 1 1 1 0 0 1 1 1 0 0 0 0 | 1 1 1 1 0 1 0 0 0 | 1 0 1 0 1 1 1 0 1 0 0 0 0 0 | 4 | A2 | | | | | |
| 1 0 1 1 0 0 0 0 1 1 | 1 1 1 1 1 0 0 0 0 0 0 1 1 1 | 1 0 1 0 1 1 0 1 1 0 0 0 0 1 1 1 0 | 1 1 1 1 0 0 0 0 0 | 1 1 1 1 0 0 0 0 0 1 1 1 0 1 | 1 | B1 | | | | | |
| 1 1 1 1 1 1 0 0 0 1 | 1 1 1 1 1 1 0 1 0 0 1 0 1 0 | 1 0 0 1 1 0 1 1 1 1 0 1 1 0 0 1 | 1 1 1 1 0 0 1 1 0 | 1 1 1 1 0 0 0 0 0 1 1 1 1 0 | 1 | B1 | | | | | |
| 1 0 1 1 1 1 1 0 0 0 | 1 1 1 1 1 1 0 1 0 1 1 0 1 0 | 1 1 0 0 1 0 1 1 1 1 0 1 1 0 0 0 | 1 1 1 1 0 1 1 0 0 | 1 1 0 1 0 0 0 0 0 1 0 1 0 0 | 1 | B1 | | | | | |
| 1 0 1 1 1 1 1 1 0 0 0 | 1 1 1 1 1 1 0 1 0 1 1 0 0 0 | 1 1 0 0 1 0 1 1 1 1 0 1 1 0 0 0 | 1 1 1 1 0 1 0 0 0 | 1 1 1 1 0 0 1 1 0 0 0 0 0 0 | 1 | B1 | | | | | |
| 1 0 1 1 1 1 1 1 0 0 0 | 1 1 1 1 1 1 0 1 0 1 1 0 0 0 | 1 1 0 0 1 0 1 1 1 1 0 1 1 0 0 0 | 1 1 1 1 0 1 0 0 0 | 1 1 1 1 0 1 1 1 0 0 0 0 0 0 | 1 | B1 | | | | | |
| 1 0 1 1 1 1 1 1 0 0 0 | 1 1 1 1 1 1 0 1 0 1 1 0 0 0 | 1 1 0 1 1 0 1 1 1 1 0 1 1 0 0 0 | 1 1 1 1 0 1 0 0 0 | 1 1 1 1 0 1 1 1 1 0 0 0 0 0 | 1 | B1 | | | | | |
| 1 0 1 1 1 1 1 1 0 0 0 | 1 1 1 1 1 1 0 1 0 1 1 0 0 0 | 1 1 0 1 1 0 1 1 1 1 0 1 1 0 0 0 | 1 1 1 1 0 0 0 0 0 | 1 1 0 1 0 1 1 1 1 0 0 0 0 0 | 1 | B1 | | | | | |
| 1 0 1 1 1 1 1 1 0 0 0 | 1 1 1 1 1 1 0 1 0 1 1 0 0 0 | 1 1 0 1 1 0 1 1 1 1 0 1 1 0 0 0 | 1 1 1 1 0 0 0 0 0 | 1 1 0 1 0 1 0 1 0 0 0 0 0 0 | 12 | B1 | | | | | |
| 1 0 1 1 1 1 1 1 0 0 0 | 1 1 1 1 1 1 0 1 0 1 1 0 0 0 | 1 1 1 0 1 1 0 1 1 1 1 0 1 1 0 0 0 | 1 1 1 1 0 0 0 0 0 | 1 1 0 1 0 1 0 1 0 0 0 0 0 0 | 1 | B2 | | | | | |
| 1 0 1 1 1 1 1 1 0 0 0 | 1 1 1 1 1 1 0 1 0 1 0 0 0 0 | 1 1 0 1 1 0 1 1 1 1 0 1 1 0 0 0 | 1 1 1 1 0 0 0 0 0 | 1 1 1 1 0 1 0 1 0 0 0 0 0 0 | 1 | B2 | | | | | |
| 1 0 1 1 1 1 1 1 0 0 0 | 1 1 1 1 1 1 0 1 0 1 1 0 0 0 | 1 1 0 1 1 0 1 1 1 1 0 1 1 0 0 0 | 1 1 1 1 0 0 0 0 0 | 1 1 0 1 0 1 1 1 1 0 0 0 0 0 0 | 7 | B2 | | | | | |
| 1 0 1 1 1 1 1 1 0 0 0 | 1 1 1 1 1 1 0 1 0 0 1 0 0 0 | 1 1 0 1 1 0 1 1 1 1 0 1 1 0 0 0 | 1 1 1 1 0 0 0 0 0 | 1 1 0 1 0 1 1 1 0 0 0 0 0 0 0 | 1 | B2 | | | | | |

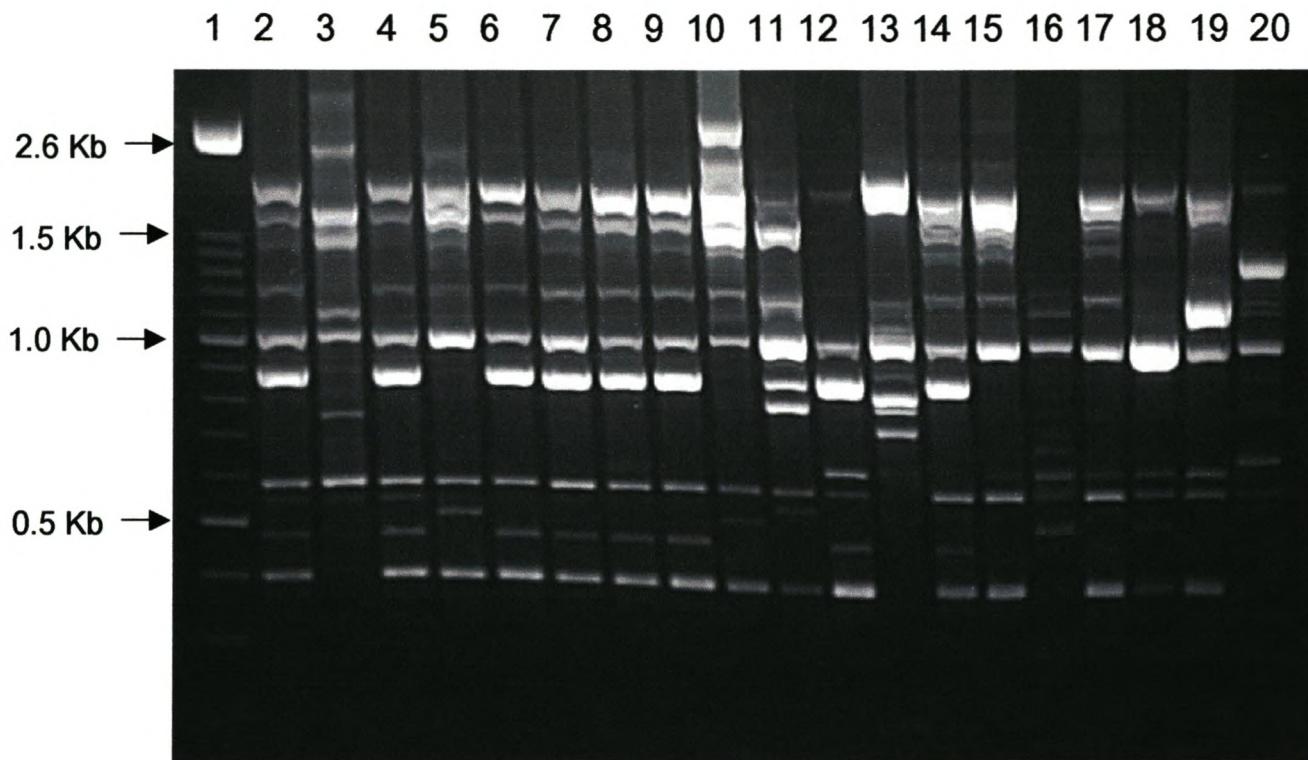


Fig. 1(a). RAPD fingerprint using primer GFC1. Lane 1 is a DNA molecular weight marker (100 base pair ladder); lanes 2-20 are different net- and spot-type isolates of *Pyrenophora teres*.

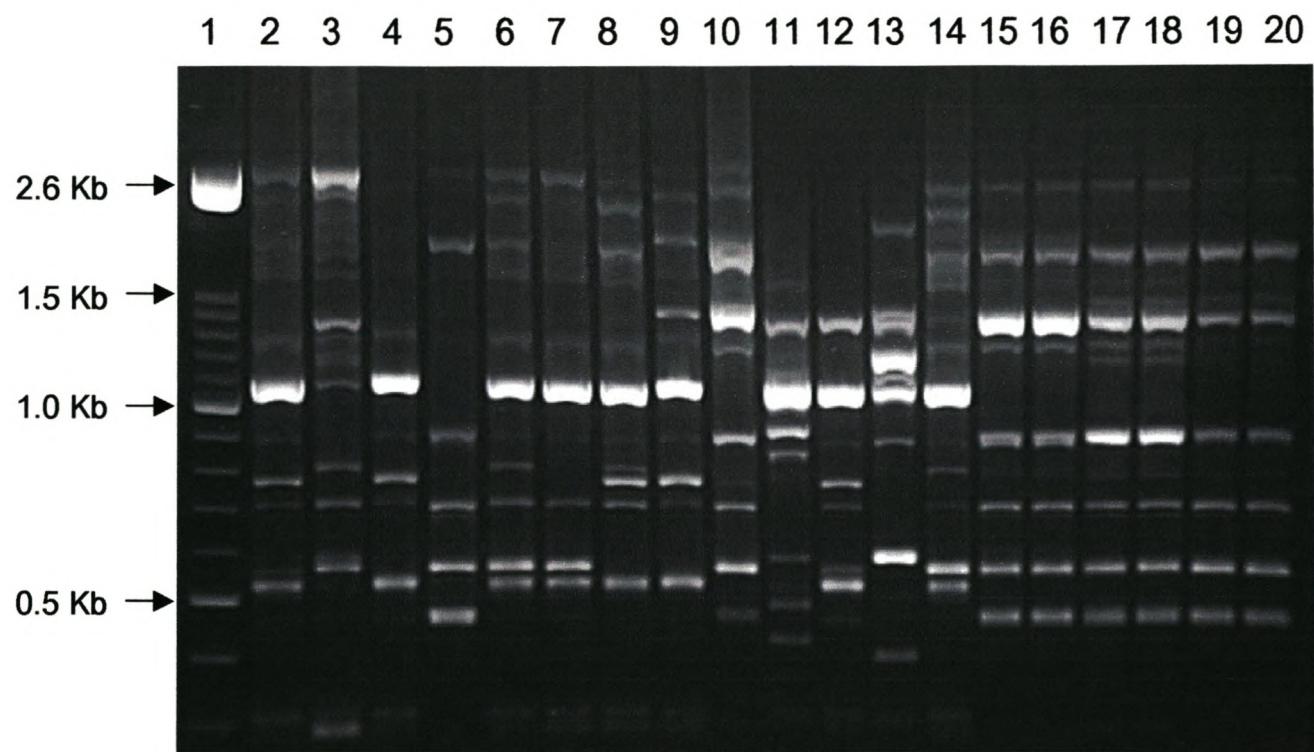


Fig. 1(b). RAPD fingerprint using primer GFC2. Lane 1 is a DNA molecular weight marker (100 base pair ladder); lanes 2-20 are different net- and spot-type isolates of *Pyrenophora teres*.

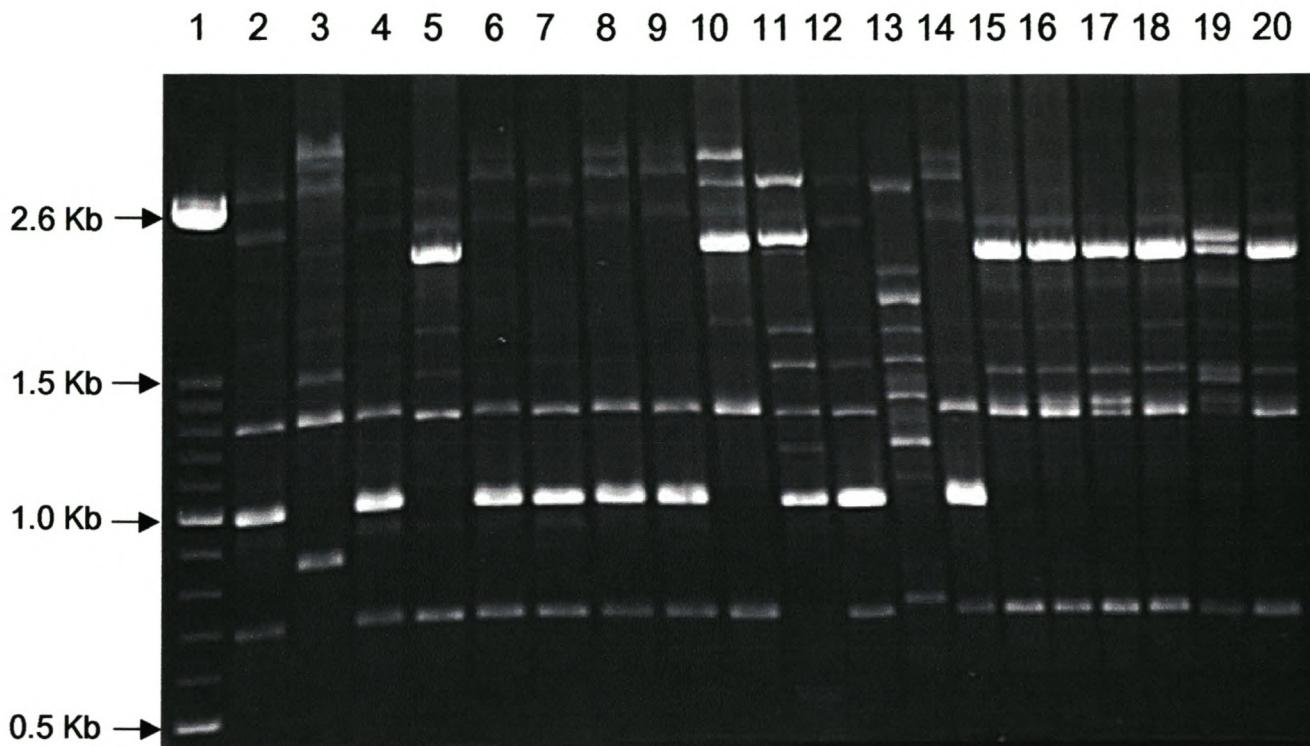


Fig. 1(c). RAPD fingerprint using primer OPE15. Lane 1 is a DNA molecular weight marker (100 base pair ladder); lanes 2-20 are different net- and spot-type isolates of *Pyrenophora teres*.

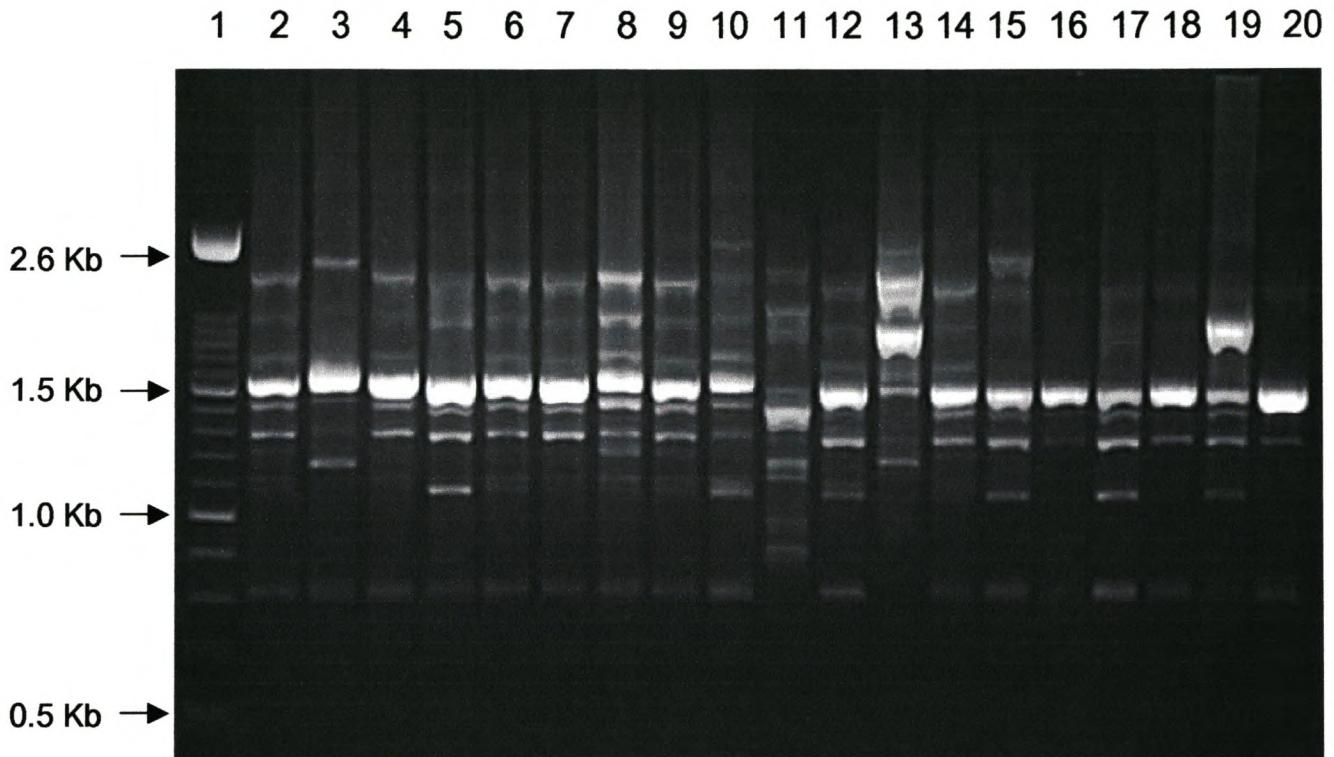


Fig. 1(d). RAPD fingerprint using primer OPE18. Lane 1 is a DNA molecular weight marker (100 base pair ladder); lanes 2-20 are different net- and spot-type isolates of *Pyrenophora teres*.

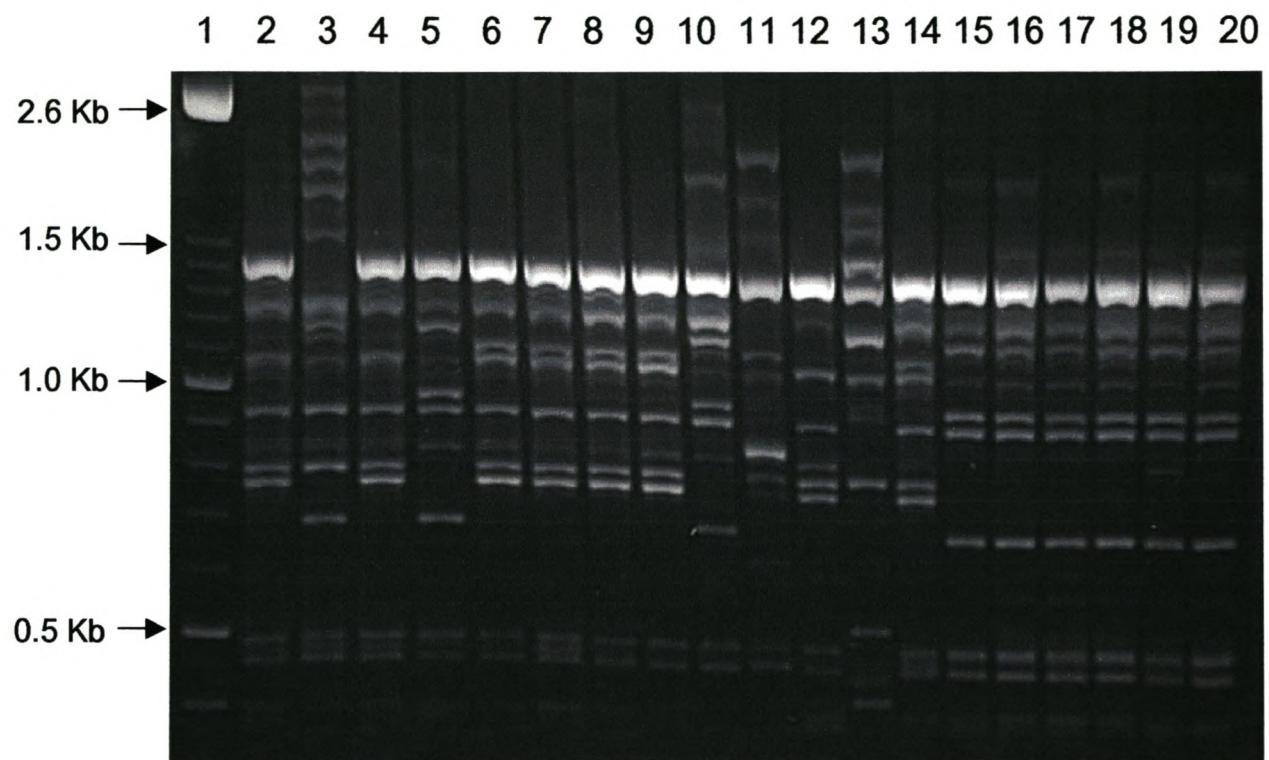


Fig. 1(e). RAPD fingerprint using primer OPM10. Lane 1 is a DNA molecular weight marker (100 base pair ladder); lanes 2-20 are different net- and spot-type isolates of *Pyrenophora teres*.

ISOLATE

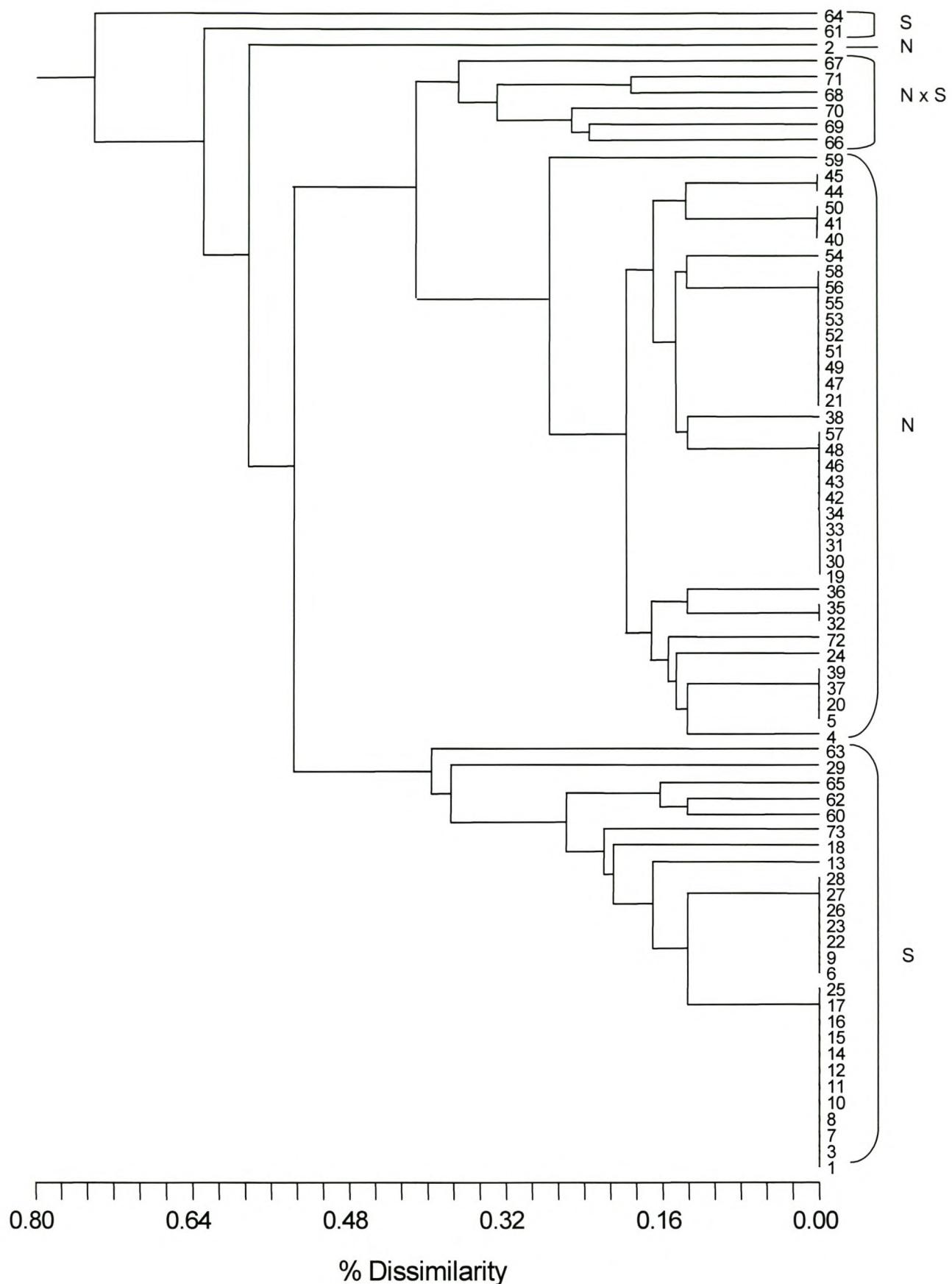


Fig. 2. Dendrogram showing relationships among 73 isolates of *Pyrenophora teres* based on RAPD banding patterns. The dendrogram was constructed from dissimilarity coefficients using UPGMA. Brackets with N or S represent net- and spot-type clusters. The N x S cluster represents hybrid progeny from a net x spot mating.

5. Fungicide sensitivity of South African net- and spot-type isolates of *Pyrenophora teres* to ergosterol biosynthesis inhibitors

ABSTRACT

Pyrenophora teres is a foliar pathogen of barley that occurs as two distinct types as indicated by symptom expression on differentially susceptible cultivars. *Pyrenophora teres* f. *teres* produces a net-type symptom while *P. teres* f. *maculata* produces a spot-type symptom. Fungicide sensitivities (IC_{50} values) of 89 monoconidial isolates of *P. teres* to sterol demethylation inhibiting fungicides were determined, based on the inhibitory effect on radial mycelial growth. These isolates were evaluated *in vitro* to determine their sensitivity to triadimenol, bromuconazole, flusilazole, propiconazole and tebuconazole. Infected leaves displaying either net- or spot-type symptoms were sampled from four fields with two fields representing each symptom type. Both net- and spot-type isolates revealed strong resistance to triadimenol, the mean IC_{50} value being 25.7 µg/ml. Flusilazole was shown to be the strongest inhibitor of fungal growth with a mean IC_{50} value of 0.71 µg/ml. Spot-type isolates showed a higher resistance than net-type isolates to all five fungicides screened ($P = 0.0001$). The IC_{50} values indicated significant differences ($P = 0.0001$) among four of the fungicides (triadimenol, tebuconazole, flusilazole and propiconazole). The IC_{50} values between propiconazole and bromuconazole were not significantly different. The overall conclusion of this study is that spot-type isolates showed a higher degree of resistance to commercially used fungicides than net-type isolates.

INTRODUCTION

Sterol demethylation inhibitors (DMIs) constitute a modern class of fungicides with a broad spectrum of fungal activity (Scheinpflug & Kuck, 1987). Irrespective of their diverse chemical structures, all DMIs have been identified as effective inhibitors of the C-14 demethylation of 24-methylenedihydrolanosterol, a precursor of fungal sterol biosynthesis (Copping *et al.*, 1984; Buchenauer, 1987; Köller, 1988). It is generally accepted that a small proportion of a population contains naturally occurring resistant genotypes in a pathogen population before the first fungicide applications (Brent, 1992). However, under continuous selection pressure due to fungicide application, a fungal population can shift towards a state of reduced sensitivity, and the proportion of resistant phenotypes may reach a level where satisfactory disease control is no longer achieved.

Pyrenophora teres Drechsler (anamorph *Drechslera teres* [Sacc.] Shoemaker) the causal agent of net blotch disease of barley (*Hordeum vulgare* L. emend. Bowden), is an economically important disease of this crop in South Africa and throughout most other barley growing regions in the world (Shipton *et al.*, 1973; Steffenson *et al.*, 1991; Louw *et al.*, 1996). Yield losses attributed to net blotch, ranging between 26-77%, have been reported from different countries (Jordan, 1981; Khan, 1989; Jordan *et al.*, 1985; Martin, 1985; Deadman & Cooke, 1987; Delsignore & Cole, 1987; Steffenson *et al.*, 1991). Two types of leaf symptoms are associated with net blotch disease, namely a net-type symptom which produces elongated, light brown lesions with dark brown reticulations, and a spot-type symptom which produces dark brown spots with distinct halos (Smedegård-Petersen, 1971). *P. japonica* S. Ito and Kurib. [anamorph *Drechslera tuberosa* (G. F. Atk.) Shoemaker] was originally described as the pathogen causing spot-type symptoms, whereas *P. teres* was associated with net-type lesions (Ito and Kurabayashi, 1931; Shoemaker, 1962). After successful mating between net- and spot-type isolates by McDonald (1967) and Smedegård-Petersen (1971), it was concluded the two types were formae of the same species and were subsequently named *P. teres* f. *teres* (net-type isolates) and *P. teres* f. *maculata* (spot-type isolates). Both types occur within close proximity of each

other in the Western Cape province of South Africa (Scott, 1995; Louw *et al.*, 1995, 1996).

Fungicides are used routinely for the control of net blotch (Van den Berg & Rossnagel, 1990; Scott, van Niekerk & Paxton, 1992; Toubia-Rahme *et al.*, 1995b). In South Africa, triadimenol has been used since 1979 as a seed treatment to control various barley fungal pathogens including net blotch. The foliar-applied fungicides, propiconazole, flusilazole and tebuconazole were introduced during 1984, 1988 and 1989, respectively. Although these fungicides have been used extensively for almost two decades in South Africa, no information is available on the fungicide sensitivity of net blotch populations to these compounds.

Published data concerning fungicide application programmes on barley in the Western Cape is very limited. Information on fungicide sensitivity amongst local isolates of net blotch towards the different commercially used fungicides is urgently required, therefore, to assist in the formulation of strategic fungicide programmes. The aim of the present study was to determine the sensitivity of local populations of *P. teres* to five commonly used fungicides, namely triadimenol, bromuconazole, flusilazole, propiconazole and tebuconazole. A further aim was to determine if net- and spot-type isolates from fields with similar fungicide histories differed significantly in their sensitivity towards these fungicides.

MATERIALS AND METHODS

Sampling methods

Leaves containing net- or spot-type symptoms of *P. teres* were collected from barley fields in the Western Cape, the major barley producing area in South Africa. Leaves were sampled from four fields. Two fields, each being approximately 30 ha in size and 15 km apart were chosen for each symptom type (Table 1). Each field was treated as a separate population. Within each field isolates were sampled within a transect of 25 x 25 m. A single diseased leaf was sampled per plant at 1 m intervals.

Fungal isolation

Fungal isolates used in this study are listed in Table 1. Following sampling the leaves were air-dried to reduce the probability of bacterial contamination during isolation. Symptomatic leaves were surface-sterilised by immersion for 30 s in 70% (v/v) ethanol, followed by 60 s in 2% (v/v) sodium hypochlorite and finally again for 30 s in 70% (v/v) ethanol. Air-dried leaf sections were mounted onto glass slides with petroleum jelly for adhesion, placed in moist chambers and incubated at 15°C under continuous nuv for 3-4 days to induce sporulation. Conidia were removed microscopically and dispersed with sterile water onto 2% water agar (WA, Biolab, Merck, South Africa) plates and left to germinate. Single germinated conidia were removed, inoculated onto potato dextrose agar (PDA, Biolab, Merck, South Africa) and incubated at 25°C.

Fungicide sensitivity

The technique described by Robbertse *et al.* (1996) was followed. Mycelial plugs (3 mm in diam.) from 7-day-old cultures were placed in the centre of PDA plates amended with either triadimenol, bromuconazole, flusilazole, propiconazole or tebuconazole at various concentrations; these were 0, 1.0, 10.0, 30.0 and 60 µg/ml for triadimenol and 0, 0.1, 0.3, 1.0, 3.0 and 10.0 µg/ml for the other fungicides. All isolates were tested in triplicate at each concentration for each fungicide. Stock solutions were prepared by dissolving the respective fungicides in 70% (v/v) ethanol. For each fungicide, control plates were amended to contain the same amount of solvent as plates containing the highest concentration of fungicide. Plates were incubated inverted at 25°C for 5 days. Colony diameters were subsequently derived by averaging two perpendicular measurements and subtracting the diameter of the agar plug. The degree of inhibition (% inhibition) for each isolate at each specific concentration was then expressed as the proportion of radial growth on the fungicide-amended plates compared to growth on the control plates. After visual inspection a best curve was fitted to these data. The SAS/STAT software version 6.04 package was used to calculate the IC₅₀ values for each isolate. Non-linear regression was carried out to calculate the IC₅₀ for each isolate by regressing radial growth (as a proportion of the control) against log-

transformed fungicide concentrations and using the fitted regression line to estimate IC₅₀ values.

RESULTS

Fungicide sensitivity between net- and spot-type isolates

Quantification of sensitivities of *P. teres* to DMI fungicides was based on the inhibition of mycelial growth. Fungicide sensitivity ranges and mean IC₅₀ values from the four fields to the different fungicides tested are listed in Table 2 and diagrammatically represented in Fig. 2. Differences were highly significant ($P = 0.0001$) with regard to fungicide sensitivities between net- and spot-type isolates (Fig. 1 and Table 3). Spot-type isolates were less sensitive than net-type isolates towards all the fungicides screened.

Inhibiting capacities of different fungicides

Highly significant differences ($P = 0.0001$) were obtained for the different fungicides that were tested (Table 3). Using the triadimenol concentration of 10 µg/ml as the discriminating concentration between sensitive and insensitive (resistant) isolates to this fungicide (Peever & Milgroom, 1992a), it was found that 64% of the *P. teres* isolates tested in this study were resistant to this fungicide. Isolates of both net- and spot-types were shown to be the least sensitive towards triadimenol. On the other hand, isolates of both types were the most sensitive towards flusilazole. The average IC₅₀ values were 25.7 µg/ml, 3.32 µg/ml, 1.49 µg/ml, 1.62 µg/ml and 0.71 µg/ml when tested against triadimenol, tebuconazole, propiconazole, bromuconazole and flusilazole respectively (Fig. 3). The difference in fungal growth inhibitory capacities between the fungicides (indicated by IC₅₀ values) was highly significant ($P = 0.0001$, Table 3), except between propiconazole and bromuconazole.

DISCUSSION

The aim of the present study was twofold. The first objective was to determine on the basis of the inhibition of mycelial growth on fungicide

amended media whether *P. teres* f. *teres* or *P. teres* f. *maculata* was more resistant to commercially used DMI fungicides in South Africa. The second objective was to determine the relative inhibiting capacities of the different fungicides tested. In other barley producing countries, different fungicides are continually being tested to determine their ability to inhibit the growth of *P. teres* (Martin & Sanderson, 1988; Scott et al., 1992; Sheridan et al., 1985; Van den Berg & Rossnagel, 1990; Steffenson et al., 1991; Toubia-Rahme et al., 1995a,b). The majority of these studies have focussed mainly on two DMI fungicides, namely triadimenol and propiconazole. The actual aspects of focus have been the use of triadimenol as a seed treatment and propiconazole as a foliar spray. However, there are several reports of extensive resistance to triadimenol that was first detected in New Zealand in the mid 1980's (Sheridan & Grbavac, 1985; Sheridan et al., 1985). These reports were later validated by Peever and Milgroom (1992a,b), who proved that by mating triadimenol sensitive and insensitive isolates, progeny inherited triadimenol resistance from parental isolates at specific loci. Furthermore, when crosses were made between isolates from different geographical areas, it was demonstrated that there was a lack of fertility barriers amongst isolates from these areas and that resistance to triadimenol is conferred by alleles at the same locus (Peever & Milgroom, 1992a). Isolates of *P. teres* growing on triadimenol amended laboratory media at concentrations of 25-50 µg/ml have been reported (Sheridan & Grbavac, 1985; Peever & Milgroom, 1992a). The results in the present study compare favourably with those obtained in other studies. That such high IC₅₀ values were obtained in all the fields sampled can almost certainly be attributed to triadimenol being used extensively as a seed treatment in the Western Cape province for the past two decades.

Propiconazole has been tested as a foliar spray for the control of net blotch on barley in various countries including South Africa (Martin & Sanderson, 1988; Sutton & Steele, 1983; Scott et al., 1992). However, there is no literature regarding *P. teres* fungicide sensitivities towards the other commercially used DMI fungicides in South Africa. Most fungicide studies on barley have focussed on the barley scald pathogen, *Rhynchosporium secalis* (Oudem.) Davis, that in various countries has been shown to be a more serious pathogen than net blotch (Scott et al., 1992). Robberse et al. (2001)

reported that *R. secalis* isolates also showed resistance towards triadimenol, but not to other DMI fungicides. The build-up of resistance by *P. teres* and *R. secalis* can be attributed to the use of triadimenol as a seed treatment since 1979. This treatment therefore exerted selection pressure on these two pathogens. However, because a wide range of DMI fungicides were available as foliar applications to control these pathogens, the specific DMI selection was limited. In the Western Cape province net blotch and barley scald are treated with the same DMI fungicides, namely triadimenol, tebuconazole, propiconazole, bromuconazole and flusilazole.

In the present study spot-type isolates of *P. teres* were found to be significantly more resistant to all the fungicides tested than net-type isolates. This could mean that spot-type isolates may have built up more resistance as a result of various evolutionary factors. Firstly, Scott (1988) and Louw *et al.* (1996) indicated that until recently the spot-type of *P. teres* was the predominant type (83%) in the Western Cape province. This was probably due to the introduction of the now dominant Australian cultivar Clipper, that is susceptible to spot-type but resistant to net-type. A higher proportion of the spot-type net blotch populations in the Western Cape province have thus been subjected to more intensive fungicide control programmes, and as a result have built up more resistance to them. Secondly, by crossing isolates of *P. teres*, Peever and Milgroom (1992a) were able to show that triadimenol resistance in the progeny was controlled by a major gene as well as three to five other minor genes. Shaw (1989) reported that sexual recombination and quantitative inheritance of resistance could change the distribution of resistant phenotypes. This would mean that progeny with various degrees of resistance would be produced. Peever and Milgroom (1992a) also stated that sexual reproduction occurring once a year in net blotch might be significant in increasing the genetic variation in resistance. This variation is then selected for in subsequent asexual generations. It is, therefore, feasible that sexual recombination increased the rate of resistance of spot-type isolates towards DMI fungicides, bearing in mind that the sexual stage of the spot-type has been collected from barley fields in the Western Cape province (Louw *et al.*, 1994). Thirdly, various studies have indicated that the spot-type of *P. teres* is more difficult to control than the net-type (Van den Berg & Rossnagel, 1990;

Scott *et al.* 1992). It was shown that two applications of propiconazole were required to reliably control spot-type, whereas only one application was required for net-type control (Scott *et al.* 1992).

Hollomon (1984) indicated that baseline sensitivity surveys provide a useful basis for comparison to determine whether resistance has developed among field isolates. In addition, the establishment of baseline sensitivity is important to monitor gradual shifts in sensitivity so that a build-up of resistance can be anticipated, and alternative fungicide programmes installed before the commercial application of a fungicide becomes ineffective. In the Western Cape province, however, it is not possible to set up a baseline sensitivity for *P. teres* populations towards DMI fungicides, as all barley production has been subjected to continual spray control programmes for the past two decades. Baseline sensitivity could only perhaps be determined for bromuconazole, due to its recent introduction into South Africa in 1996. However, there are reports that *P. teres* displays cross-resistance for some DMIs (Peever & Milgroom, 1992b), and therefore genes resistant to other DMIs may mask the actual sensitivity of net blotch isolates towards bromuconazole.

In conclusion, the findings of this study clearly indicate that despite the recent introduction of bromuconazole into South Africa as a foliar spray for fungal pathogens of barley, the most effective fungicide against net blotch is still flusilazole. In addition, triadimenol is clearly not beneficial in terms of net blotch resistance programmes, and an alternative seed treatment should be considered to combat the build-up of fungicide resistance.

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Table 1. Isolates of *Pyrenophora teres* used in this study

| Field | n | Variety | Cultivar | Sampling year |
|-------|----|---------|----------|---------------|
| A | 21 | Net | Stirling | 1997 |
| B | 25 | Net | Stirling | 1997 |
| C | 24 | Spot | Clipper | 1997 |
| D | 19 | Spot | Clipper | 1996 |

Table 2. Fungicide sensitivity ranges and mean IC₅₀ values of *P. teres* isolates from different fields to DMI fungicides

| Field | Fungicide | | | | | | | | | |
|-------|--------------------|-------------------|---------------|-------|-------------|-------|---------------|-------|--------------|-------|
| | Triadimenol | | Bromuconazole | | Flusilazole | | Propiconazole | | Tebuconazole | |
| | Range ^a | Mean ^b | Range | Mean | Range | Mean | Range | Mean | Range | Mean |
| A | 0.307-67.060 | 20.409 | 0.020-3.957 | 0.932 | 0.025-1.030 | 0.314 | 0.095-7.693 | 1.262 | 0.012-11.098 | 2.351 |
| B | 0.264-57.739 | 15.765 | 0.172-1.711 | 0.671 | 0.002-0.972 | 0.192 | 0.004-0.702 | 0.415 | 0.080-6.893 | 2.008 |
| C | 3.625-55.360 | 43.335 | 0.186-5.579 | 3.406 | 0.178-3.905 | 1.791 | 0.138-5.772 | 3.056 | 0.022-24.046 | 6.447 |
| D | 4.920-42.907 | 28.751 | 0.276-5.429 | 2.276 | 0.055-3.699 | 1.029 | 0.219-2.714 | 1.444 | 0.165-7.634 | 3.574 |

^{a,b} IC₅₀ values in µg/ml

Table 3. Analysis of variance of *in vitro* sensitivity of *P. teres* isolates towards DMI fungicides

| Source | df | Sum of squares | Mean squares | F | P |
|------------------|-----|----------------|--------------|-------|--------|
| Fungicides (F) | 4 | 300.50 | 75.13 | 69.48 | 0.0001 |
| Symptom type (S) | 1 | 60.10 | 60.10 | 55.59 | 0.0001 |
| F X S | 4 | 4.61 | 1.15 | 1.07 | 0.3778 |
| Error (a) | 88 | 95.14 | | | |
| Error (b) | 348 | 117.95 | | | |
| Corrected total | 445 | 578.31 | | | |

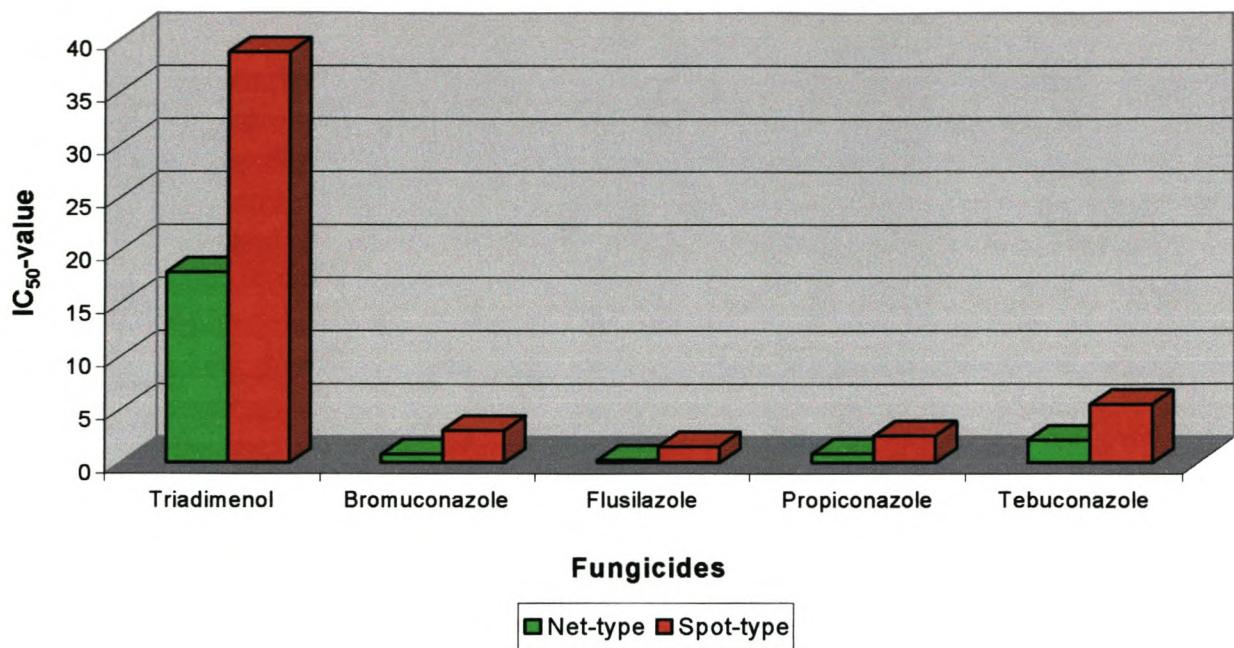


Fig. 1. Sensitivity distribution of DMI fungicides between net- and spot-type isolates of *P. teres*. IC_{50} values are in $\mu\text{g/ml}$.

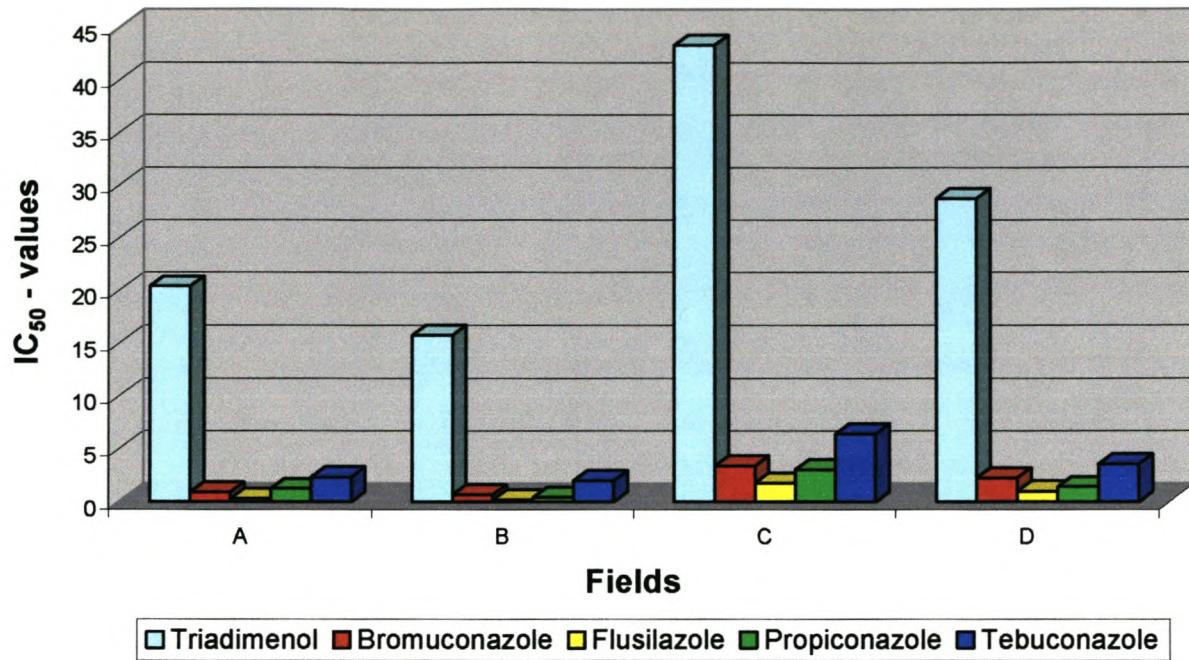


Fig. 2. Sensitivity distribution of *P. teres* to DMI fungicides between different fields. IC_{50} values are in $\mu\text{g/ml}$.

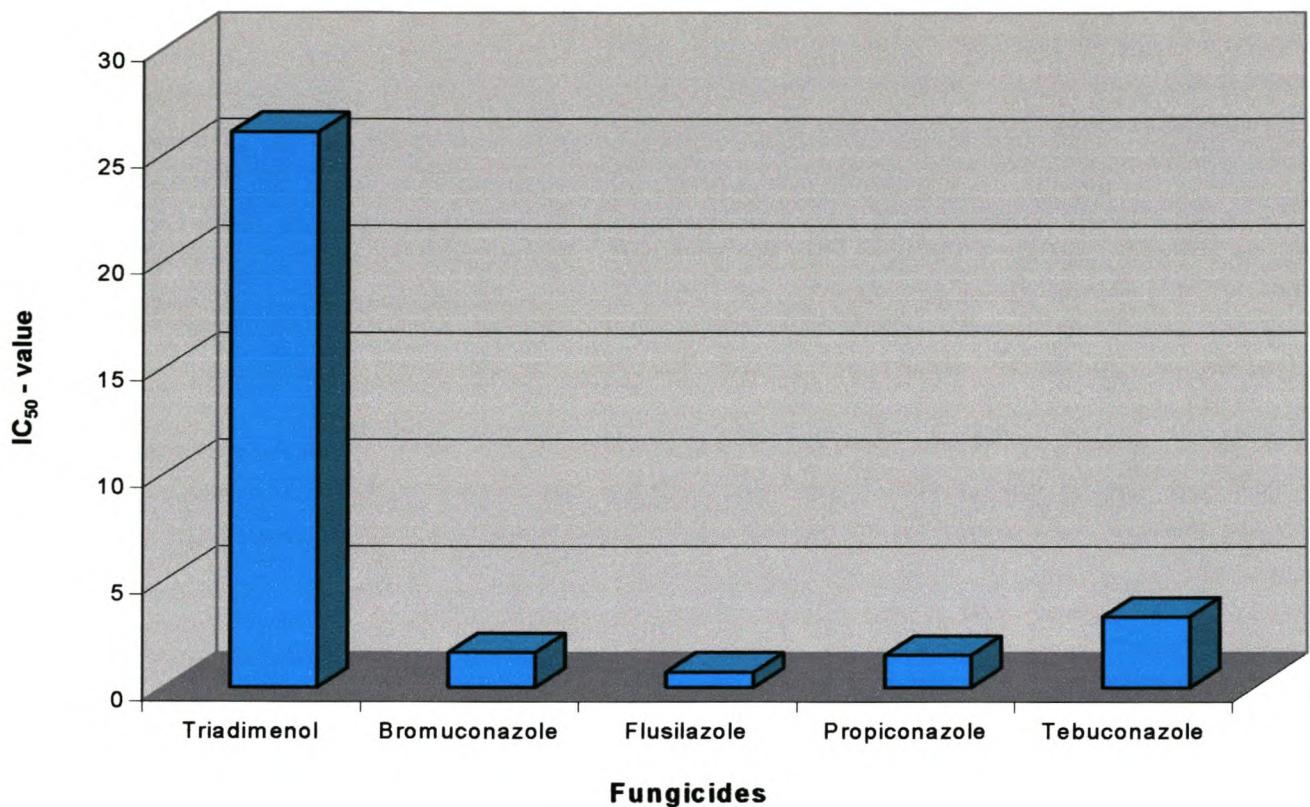


Fig. 3. Sensitivity distribution of different DMI fungicides towards isolates of *P. teres* (net- and spot-type). IC_{50} values are in $\mu\text{g/ml}$.

Appendix 1A-ITS rRNA gene

| | |
|------------|---|
| 4_3 | -GGATCATTAC-ACAA |
| 15_3 | -GGATCATTAC-ACAA |
| 16_3 | -GGATCATTAC-ACAA |
| 18 | -GGATCATTAC-ACAA |
| 19_3 | -GGATCATTAC-ACAA |
| Cal1 | -GGATCATTAC-ACAA |
| Swe1 | -GGATCATTAC-ACAA |
| Nap8 | -GGATCATTAC-ACAA |
| X78124_D.g | -AGGGATCATTAC-ACAA |
| Y08745_P.g | -C-ACAA |
| Y10859_P.g | -AGGGATCATTAC-ACAA |
| Y10858_P.g | -AGGGATCATTAC-ACAA |
| Y10857_P.g | -AGGGATCATTAC-ACAA |
| Y10748_P.g | -AGGGATCATTAC-ACAA |
| Y10923_P.g | -AGGGATCATTAC-ACAA |
| AF071347_P | -GGGATCATTAC-ACAA |
| Y08744_P.t | -C-ACAA |
| Y08746_P.t | -C-ACAA |
| AF163061_P | -ATTAC-ACAA |
| AY004778_B | -GTGAACCTGCGGAGGGATCATTAC-ACAA |
| AY004775_B | -GGTGAACCTGCGGAGGGATCATTAC-ACAA |
| AY004777_B | -GTGAACCTGCGGAGGGATCA-TTAC |
| AY004803_D | -CGTAGGTGAACCTGCGGAGGGATCATTAC-CCAA |
| AY004805_D | -CCGTAGGTGAACCTGCGGAGGGATCATTAC-ACAA |
| AY004796_D | -GGTGAACCTGCGGAGGGATCATTAC-CCAA |
| AY004788_D | -CCTCATTGGGCGTAACGTACGCC-GTGT |
| AF163057_D | -ATTACAATAT-GAAA |
| AF163058_D | -ATTACACAAATAT-GCAA |
| AY004781_D | -GTAGGTGAACCTGCGGAGGGATCATTACACAAA |
| AY004789_D | -ATATGAAAGCAGACCCTCACTGGGCGTAACCTACGCC-GTGT |
| AY004806_D | -GCGGAGGGATCATTAC-ACAA |
| AY004783_D | -CCGTAGGTGAACCTGCGGAGGGATCATTAC-CCAA |
| AY004791_D | TAGAAAAAGAAAAAGGGCAGTTCTGCTGCTTCCGCCGCATTT-GCGG |
| AY004794_D | -GCTGGGTTCCCCAGAGCCGGGTGTC-TTGT |
| AY004807_D | -CCGTAGGTGAACCTGCGGAGGGATCATTAC-ACAA |
| AY004801_D | -GTAGGTGAACCTGCGGAGGGATCATTAC-ACAA |
| AY004799_D | -GGAGGGATCATTAC-CCAA |
| AF163059_D | -CCCCAGAGGGCGACTTCTCGTGCC-GCGT |
| AF163060_D | -ATT |
| AY004809_P | -CCGTAGGTGAACCTGCGGAGGGATCATTACACAAA |
| AF081445_P | -GGGATCATTAC-CCAA |

AY004798_P -----CCGTAGGTGAACTGCGGAGGGATCATTAC-CCAA
AF081446_P -----GGGATCATTAC-ACAA
AY004808_P -----CCGTAGGTGAACTGCGGAGGGATCATTAC-ACAA
AF229479_P -----AGGGATCATTAC-ACAA

4_3 ATATGAAGG---CAGATTGGTAG--TCCC-----CGCTTTT--G
15_3 ATATGAAGG---CAGATTGGTAG--TCCC-----CGCTTTTT-G
16_3 ATATGAAGG---CAGATTGGTAG--TCCC-----CGCTTTT--G
18 ATATGAAGG---CAGATTGGTAG--TCCC-----CGCTTTT--G
19_3 ATATGAAGG---CAGATTGGTAG--TCCC-----CGCTTTT--G
Cal1 ATATGAAGG---CAGATTGGTAG--TCCC-----CGCTTTT--G
Swe1 ATATGAAGG---CAGATTGGTAG--TCCC-----CGCTTTT--G
Nap8 ATATGAAGG---CAGATTGGTAG--TCCC-----CGCTTTT--G
X78124_D.g ATATGAAGG---CAGATTGGTAG--TCCC-----CGCTTTG--G
Y08745_P.g ATATGAAGG---CAGATTGGTAG--TCCC-----CGCTTTT--G
Y10859_P.g ATATGAAGG---CAGATTGGTAG--TCCC-----CGCTTTG--G
Y10858_P.g ATATGAAGG---CAGATTGGTAG--TCCC-----CGCTTTG--G
Y10857_P.g ATATGAAGG---CAGATTGGTAG--TCCC-----CGCTTTG--G
Y10748_P.g ATATGAAGG---CAGATTGGTAG--TCCC-----CGCTTTT--G
Y10923_P.g ATATGAAGG---CAGATTGGTAG--TCCC-----CGCTTTG--G
AF071347_P ATATGAAGG---CAGATTGGTAN--TCCC-----CGCTTTT--G
Y08744_P.t ATATGAAGG---CAGATTGGTAG--TCCC-----CGCTTTT--G
Y08746_P.t ATATGAAGG---CAGATTGGTAG--TCCC-----CGCTTTT--G
AF163061_P ATATGAAGG---CAGATTGGTAG--TCCC-----CGCTTTT--G
AY004778_B --AGAATATGT--AGGCTGC--A---CGCGGCT-GT-GCCCTCTCTTTGG
AY004775_B CAAAATAGGA---AGGCC---TGGC-----TTCAC-----G
AY004777_B ACAATAAAATACGA-----AGGCCG-----TTCGC--
AY004803_D ATATGAAGG---GCAGATTGG--GCCGTCCC-----T-CTAGG--G
AY004805_D ATATGAAGC---CAGACTG-G-GC-----C--G
AY004796_D ATATGAAAC--GCAGACTGG--GCACCCCTCGAG-G---AGCGATTGTCG
AY004788_D CCGCAATAGGCCAT---TGCTGTTGTGCTGA----CGCGGCGG--GGG
AF163057_D CGCAGACT-GGGCACCTCGA--GGAGCGAT-----TCGTCGT--CC
AF163058_D GCCCAGGACTACCGCCCCCCC-CTACTTGA-----GCGGTA-GCCG
AY004781_D GTGAGAAT----GGCTGG--GCCTTCTAGGGCGG--CGGCTTAACG
AY004789_D CCGCAATGGTGCATT---CGCCGTTGTGCTGAC-G--C-----GGC
AY004806_D ATATGAAGC---CAGACTGG--GC-----CGTGG
AY004783_D ATATAACAGTT---G---GGCGCTCTCAAGGCG-GC-AGCTTCACGGCC-
AY004791_D TGCTGAAG-CGGCACGGGGC--CTACGTCT-----TGATTTT--TT
AY004794_D CCCCCGCTGCTTCCGC---CACATCTGTGGTGC----TGAAGCGGGGTGG
AY004807_D ATATGAAGC---CAGACTGG--G-----ATTG--G
AY004801_D ATATGA-AGCC--AGA-----TTGGGCC-----CGCCCCTTTG
AY004799_D AGATGAATGC---AGACTGG--GCA-CCGTAGA-GAGGAGCGATTGTCG
AF163059_D CCACTGCAGTCC-TG---TGCTGTTGTGCTGA----AGC-GGCAGGG-GG

AF163060_D ACACCAATATGAAGC---CGGACTGGGATAGG-----GC
 AY004809_P ATAGGAAG---CCGGACGG-GGG-----ACC----A
 AF081445_P ATATGAAACG--CAGACTGG--GCACCCCTCGAG-GAGC---GATTCTCG
 AY004798_P ATATG-AACGC--AGACTGG--GCACCCCTAGAG-A-GGAGCGATTCTCG
 AF081446_P GTATGCAAGCCCAGGACTACC-GCCGTCCCCTA-CTTGAGCGGTA-GCCG
 AY004808_P ATATG-AAGCCGGAC--TGGG-ATA-----GGGCCTCGCTGCC---
 AF229479_P -TATGAAAGC---GGGTTGG--GACCTCACCA-----TCGGTGAGGG

| | |
|------------|---|
| 4_3 | GGGG-----TTTGC-CCATT-CTGGCG---CCATATTACCCAT |
| 15_3 | GGGGG-----TTTGC-CCATT-CTGGCG---CCATATTACCCAT |
| 16_3 | GGGGG-----TTTGC-CCATT-CTGGCG---CCATATTACCCAT |
| 18 | GGG-----TTTGC-CCATT-CTGGCG---CCATATTACCCAT |
| 19_3 | GGGGG-----TTTGC-CCATT-CTGGCG---CCATATTACCCAT |
| Cal1 | GGGG-----TTTGC-CCATT-CTGGCG---CCATATTACCCAT |
| Swe1 | GGGG-----TTTGC-CCATT-CTGGCG---CCATATTACCCAT |
| Nap8 | GGGG-----TTTGC-CCATT-CTGGCG---CCATATTACCCAT |
| X78124_D.g | GGGG-----TTTGC-CCATT-CTGGCG---CCATATTACCCAT |
| Y08745_P.g | GGGGG-----TTTGC-CCATT-CTGGCG---CCATATTACCCAT |
| Y10859_P.g | GGGG-----TTTGC-CCATT-CTGGCG---CCATATTACCCAT |
| Y10858_P.g | GGGG-----TTTGC-CCATT-CTGGCG---CCATATTACCCAT |
| Y10857_P.g | GGGG-----TTTGC-CCATT-CTGGCG---CCATATTACCCAT |
| Y10748_P.g | GGGG-----TTTGC-CCATT-CTGGCG---CCATATTACCCAT |
| Y10923_P.g | GGGG-----TTTGC-CCATT-CTGGCG---CCATATTACCCAT |
| AF071347_P | GGGG-----TTTGC-CCATT-CTGGCG---CCATATTACCCAT |
| Y08744_P.t | GGG-----TTTGC-CCATT-CTGGCG---CCATATTACCCAT |
| Y08746_P.t | GGGG-----TTTGC-CCATT-CTGGCG---CCATATTACCCAT |
| AF163061_P | GGGG-----TTTGC-CCATT-CTGGCG---CCATATTACCCAT |
| AY004778_B | GGGGGCC-A----GTGGC-CGAGG-CTGAAA---TATTTTCACCCAT |
| AY004775_B | GCCG-----GCTGA---AAT-AT----TTTTTTCCACCCAT |
| AY004777_B | -----GGCTG--G----AC---T----ATTATT-ACCCCT |
| AY004803_D | GAGA-----CGCGCCCGT-CTGCCG---CCATATTACCCAT |
| AY004805_D | TGCCCTT---GTTGCCACGCCGT--TTGGTG---CCATATTACCCAT |
| AY004796_D | TCCC-----CCTCCTGGGCCGT-CTGACG---CCATATTACCCAT |
| AY004788_D | GTAA---ACGCCCTTGGCCAGT-CTGACT---CCATATTACCCAT |
| AF163057_D | CCC-----TCCTGGGCCGT-CTGACG---CCATATTACCCAT |
| AF163058_D | CTAG-----GGGAGGGTGGTGGC-CTGGGAT--CCATCTTCACCCAT |
| AY004781_D | GCCGC-----TGTGTTGGTTGGC-CTAGCT-CTCTCTATTACCCGT |
| AY004789_D | GGCGGGGTACGTTCTTGAGCCAGT-CTGACT---CCATATTACCCAT |
| AY004806_D | CCTT-----GTTGCCACGCCGT--TTGGTG---CCATATTACCCAT |
| AY004783_D | GCCGC-----GTTGGGGTTGTCTGG-CT-----CTATATTACCCGT |
| AY004791_D | TTA-----TTAGGGGCCAGC-CTGACT---ACATATTACCCAT |
| AY004794_D | GGTG--GGCGTCTTCTTGCCAGC-CTGACT---ACATATTACCCAT |
| AY004807_D | GGCCTT----GTTGCCACACCGT--CTGGCG---CCATATTACCCAT |

AY004801_D GG-----GAAAGGCCAT-CTGACG---CCATATTCAACCAT
AY004799_D CCCC-----CCCTCCTGGGCCGGT-CTGACG---CCATATTCAACCAT
AF163059_D GAGA---CGCCTTGTTGGG-CCAGT-CTGACT---CCATATTCAACCAT
AF163060_D CTCGCTG-----CCTGC-CCGGT-CTGGCG---CCATATTCAACCAT
AY004809_P GGGCCTC----GCTGCCCTGCCGT-CTGGCG---CCATATTCAACCAT
AF081445_P TCCC-----CCTCCTGGG-CCGGT-CTGACG---CCATATTCAACCAT
AY004798_P CCCC-----CCCTCCTGGGCCGGT-CTGACG---CCATATTCAACCAT
AF081446_P CTAG-----GGGAGGGTAGCCTGGGAT---CCATATTCAACCAT
AY004808_P -----TTG-CCCGT-CTGGCG---CCATATTCAACCAT
AF229479_P CTCC-----AGCTT----GT-CTGAA-----TTATTCAACCAT

| | |
|------------|---|
| 4_3 | GTCTTT-GCGTACTACTTGTTC-C-TTGG--CGGGCTCG-CCC GCCAAT |
| 15_3 | GTCTTTGGCGTACTACTTGTTC-C-TTGG--GGGGCTCG-CCCCCCAAT |
| 16_3 | GTCTTT-GCGTACTACTTGTTC-C-TTGG--CGGGCTCG-CCC GCCAAT |
| 18 | GTCTTT-GCGTACTACTTGTTC-C-TTGG--CGGGCTCGGGCCC GCCAAT |
| 19_3 | GTTTTT-GCGTACTACTTGTTC-C-TTGG--CGGGCTCG-CCC GCCAAT |
| Cal1 | GTCTTT-GCGTACTACTTGTTC-C-TTGG--GGGGCTCG-CCC GCCAAT |
| Swe1 | GTCTTT-GCGTACTACTTGTTC-C-TTGG--GGGGCTCG-CCC GCCAAT |
| Nap8 | GTCTTT-GCGTACTACTTGTTC-C-TTGG--GGGGCTCG-CCC GCCAAT |
| X78124_D.g | GTCTTT-GCGTACTACTTGTTC-C-TTGG--GGGGCTCG-CCC GCCAAT |
| Y08745_P.g | GTCTTT-GCGTACTACTTGTTC-C-TTGG--GGGGCTCG-CCC GCCAAT |
| Y10859_P.g | GTCTTT-GCGTACTACTTGTTC-C-TTGG--GGGGCTCG-CCC GCCAAT |
| Y10858_P.g | GTCTTT-GCGTACTACTTGTTC-C-TTGG--GGGGCTCG-CCC GCCAAT |
| Y10857_P.g | GTCTTT-GCGTACTACTTGTTC-C-TTGG--GGGGCTCG-CCC GCCAAT |
| Y10748_P.g | GTCTTT-GCGTACTACTTGTTC-C-TTGG--GGGGCTCG-CCC GCCAAT |
| Y10923_P.g | GTCTTT-GCGTACTACTTGTTC-C-TTGG--GGGGCTCG-CCC GCCAAT |
| AF071347_P | GTCTTT-GCGTACTACTTGTTC-C-TTGG--GGGGCTCG-CCC GCCAAT |
| Y08744_P.t | GTCTTT-GCGTACTACTTGTTC-C-TTGG--GGGGCTCG-CCC GCCAAT |
| Y08746_P.t | GTCTTT-GCGTACTACTTGTTC-C-TTGG--GGGGCTCG-CCC GCCAAT |
| AF163061_P | GTCTTT-GCGTACTACTTGTTC-C-TTGG--GGGGCTCG-CCC GCCAAT |
| AY004778_B | GTCTTT-GCGCACTTGT---TCC-TGGG--GGGTTCG-CCC GCCACC |
| AY004775_B | GTCTTT-GCGCACTTGTGTTC-C-TGGG--GGGCTCG-CCC GCCACC |
| AY004777_B | GTCTTT-GCGCACTTGTGTTC-C-TGGG--GGGTTCG-CTGCCACC |
| AY004803_D | GTCTTT-GCGTACTACTTGTTC-C-TTGG--GGGTTCG-CCC GCCAAC |
| AY004805_D | GTCTTTGCGTACTACTTGTTC-C-TTGG--GGGTCG-CCC GCCAAT |
| AY004796_D | GTCTTT-GCGTACTACTTGTTC-C-TTGG--GGGTTCG-CCC GCCATT |
| AY004788_D | GTCTTT-GCGTACTACTTGTTC-C-TTGG--GGGTTCG-CCC GCCAAT |
| AF163057_D | GTCTTT-GCGTACTACTTGTCTG-CCGGGTTCCGCCG-GCCAATT |
| AF163058_D | GTCTTTGCGTACTACCTGTTC-C-TTGG--CGAGCTCG-CTGCCAAT |
| AY004781_D | GTCTTTGCGTACCAATTGTTC-C-TTGG--GGGTTCG-CCC GCCATT |
| AY004789_D | GTCTTT-GCGTACTACTTGTTC-C-TTGG--GGGTTCG-CCC GCCAAT |
| AY004806_D | GTCTTTGCGTACTACTTGTTC-C-TTGG--GGGTCG-CCC GCCAAT |
| AY004783_D | GTCTTT-GCGTACTACCTGTTC-C-TTGG--GGGTTCG-CCC GCCAAT |

AY004791_D GTCTTTT-GCGTACTACTTGTTC-C-TGG--TGGGCTCG-CCC GCC AAA
AY004794_D GTCTTTT-GCGTACTACTTGTTC-C-TGG--TGGGCTCG-CCC ACC GAT
AY004807_D GTCTTTTGC GTACTACTTGTTC-C-TGG--C-GGGTCCG-CCC GCC AA
AY004801_D GTCTTTT-GCGTACTACTTGTTC-C-TGG--C-GGGTCCG-CCC GCC AA
AY004799_D GTCTTTT-GCGTACTACTTGTTC-C-TGG--C-GGGTCCG-CCC GCC ATT
AF163059_D GTCTTTT-GCGTACTACTTGTTC-C-TGG--C-GGGTCCG-CCC GCC AA
AF163060_D GTCTTTTGC GTACTACTTGTTC-C-TGG--C-GGGTCCG-CCC GCC AA
AY004809_P GTCTTTTGC GTACTACTTGTTC-C-TGG--C-GGGTCCG-CCC GCC AA
AF081445_P GTCTTTT-GCGTACTACTTGTTC-C-TGG--C-GGGTCCG-CCC GCC ATT
AY004798_P GTCTTTT-GCGTACTACTTGTTC-C-TGG--C-GGGTCCG-CCC GCC ATT
AF081446_P GTCTTTTGC GTACTAC CTGTTC-C-TGG--C-GAGCTCG-CTCGCCA AT
AY004808_P GTCTTTTGC GTACTACTTGTTC-C-TGG--C-GGGTCCG-CCC GCC AA
AF229479_P GTCTTTT-GCGCACTCTTGTTC-C-TGG--C-GGGTCCG-CCC GCC ACC

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| 4_3 | TGGACTTTATTC--AACCTTTTTTT-----ATTGCAATCAGCGTC |
| 15_3 | TGGACTTTATTC--AACCTTTTTTT-----ATTGCAATCAGCGTC |
| 16_3 | TGGACTTTATTC--AACCTTTTTTT-----ATTGCAATCAGCGTC |
| 18 | TGGACTTTATTC--AACCTTTTTTT-----ATTGCAATCAGCGTC |
| 19_3 | TGGACTTTATTC--AACCTTTTTTT-----ATTGCAATCAGCGTC |
| Cal1 | TGGACTTTATTC--AACCTTTTTTT-----ATTGCAATCAGCGTC |
| Swe1 | TGGACTTTATTC--AACCTTTTTTT-----ATTGCAATCAGCGTC |
| Nap8 | TGGACTTTATTC--AACCTTTTTTT-----ATTGCAATCAGCGTC |
| X78124_D.g | TGGACTTTATTC--AACCTTTTTTT-----ATTGCAATCAGCGTC |
| Y08745_P.g | TGGACTTTATTC--AACCTTTTTTT-----ATTGCAATCAGCGTC |
| Y10859_P.g | TGGACTTTATTC--AACCTTTTTTT-----ATTGCAATCAGCGTC |
| Y10858_P.g | TGGACTTTATTC--AACCTTTTTTT-----ATTGCAATCAGCGTC |
| Y10857_P.g | TGGACTTTATTC--AACCTTTTTTT-----ATTGCAATCAGCGTC |
| Y10748_P.g | TGGACTTTATTC--AACCTTTTTTT-----ATTGCAATCAGCGTC |
| Y10923_P.g | TGGACTTTATTC--AACCTTTTTTT-----ATTGCAATCAGCGTC |
| AF071347_P | TGGACTTTATTC--AACCTTTTTTC-----ATTGCAATCAGCGTC |
| Y08744_P.t | TGGACTTTATTC--AACCTTTTTTT-----ATTGCAATCAGCGTC |
| Y08746_P.t | TGGACTTTATTC--AACCTTTTTTT-----ATTGCAATCAGCGTC |
| AF163061_P | TGGACTTTATTC--AACCTTTTTTT-----ATTGCAATCAGCGTC |
| AY004778_B | AGGACCACACCA-TAAACCTTTTT----ATGCAGTTGCAATCAGCGTC |
| AY004775_B | AGGACCAAAACCA-TAAACCTTTTTCTTATGCAGTTCCATCAGCGTC |
| AY004777_B | AGGACCACAAATA-TAAACCTTTTT----TATGCAGTTGCAATCAGCGTC |
| AY004803_D | TGGACCA-TTG-ATAAACCTTTTTTC--TTGTAGTTGCCGTAGCGTC |
| AY004805_D | TGGACC---TTATTGAACCCTTTTT----GCAATTGCAATCAGCGTC |
| AY004796_D | AGGACCAACCA-TAAA-CCTTTT-GT----AATTGCAATCAGCGTC |
| AY004788_D | TGGACCAA---TTAACCTTTTTGT----AATTGCAATCAGCGTC |
| AF163057_D | GGGACCAACCAATTAAACCTTTT-GT----AATTGCAATCAGCGTC |
| AF163058_D | TGGACCAACCA-TTAAACCTCTT-GT----AATTGCAATCAGCGTC |
| AY004781_D | AGGACCAACG--TTAACCTTTAATTTT---GCAGTTGCAGTCAGCGTC |

AY004789_D AGGACCCAA---TTAACCTTTTGT-----AATTGCAATCAGCGTC
AY004806_D TGGACCCTA---TTGAACCCTTTTGT-----GCAATTGCAATCAGCGTC
AY004783_D AGGACCAACA-TTAAACCTTTTCTT-----GTAATTGCAATCAGCGTC
AY004791_D AGGACCCCAA-TTAAACCTTTT-GT-----AATTGCAATCAGCGTC
AY004794_D AGGACAAAA-TTAAACCTTTGT-----AATTGTAATCAGCGTC
AY004807_D TGGACATTA-TTAAACCTTTCTT-----GCAATTGCAATCAGCGTC
AY004801_D TGGAC-AA-TATTAAC-CCTTTT-----GTAGTTGCAATCAGCGTC
AY004799_D AGGACCCAACTA-TAAA-CCTTTT-----GTAATTGCAATCAGCGTC
AF163059_D TGGACCACA-TTAAACCTTTTGT-----AATTGCAATCAGCGTC
AF163060_D TGGACCTTATTCAACCTTTTTC-----AGTTGCAATCAGCGTC
AY004809_P TGGACCTTGA-TTAAACCTTTTTC-----AGTTGCAATTAGCGTC
AF081445_P AGGACCCAACCA-TAAA-CCTTTT-----GTAATTGCAATCAGCGTC
AY004798_P AGGACCCAACTA-TAAA-CCTTTT-----GTAATTGCAATCAGCGTC
AF081446_P TGGACC-ACCAATTAAACCTTTT-----GTAATTGCAATCAGCGTC
AY004808_P TGGACCTTATTCAACCTTTTTC-----AGTTGCAATCAGCGTC
AF229479_P AGGACCAAACCA-TAAA-CCTTTT-----GTAATTGCAATCAGCGTC

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| 4_3 | AGC-AAAACAA-TGTAA-TCAATTACAACCTTCAACAAACGG-ATCTCTT |
| 15_3 | AGC-AAAACAA-TGTAA-TCAATTACAACCTTCAACAAACGGGATCTCTT |
| 16_3 | AGC-AAAACAA-TGTAA-TCAATTCAACCTTCAACAAACGG-ATCTCTT |
| 18 | AGC-AAAACAA-TGTAA-TCAATTACAACCTTCAACAAACGGGATCTCTT |
| 19_3 | AGC-AAAACAA-TGTAA-TCAATTACAACCTTCAACAAACGG-ATCTCTT |
| Cal1 | AGC-AAAACAA-TGTAA-TCAATTACAACCTTCAACAAACGG-ATCTCTT |
| Swe1 | AGC-AAAACAA-TGTAA-TCAATTACAACCTTCAACAAACGG-ATCTCTT |
| Nap8 | AGC-AAAACAA-TGTAA-TCAATTACAACCTTCAACAAACGG-ATCTCTT |
| X78124_D.g | AGC-AAAACAA-TGTAA-TCAATTACAACCTTCAACAAACGG-ATCTCTT |
| Y08745_P.g | AGC-AAAACAA-TGTAA-TCAATTACAACCTTCAACAAACGG-ATCTCTT |
| Y10859_P.g | AGC-AAAACAA-TGTAA-TCAATTACAACCTTCAACAAACGG-ATCTCTT |
| Y10858_P.g | AGC-AAAACAA-TGTAA-TCAATTACAACCTTCAACAAACGG-ATCTCTT |
| Y10857_P.g | AGC-AAAACAA-TGTAA-TCAATTACAACCTTCAACAAACGG-ATCTCTT |
| Y10748_P.g | AGC-AAAACAA-TGTAA-TCAATTACAACCTTCAACAAACGG-ATCTCTT |
| Y10923_P.g | AGC-AAAACAA-TGTAA-TCAATTACAACCTTCAACAAACGG-ATCTCTT |
| AF071347_P | AGC-AAAACAA-TGTAA-TCAATTACAACCTTCAACAAACGG-ATCTCTT |
| Y08744_P.t | AGC-AAAACAA-TGTAA-TCAATTACAACCTTCAACAAACGG-ATCTCTT |
| Y08746_P.t | AGC-AAAACAA-TGTAA-TCAATTACAACCTTCAACAAACGG-ATCTCTT |
| AF163061_P | AGC-AAAACAA-TGTAA-TCAATTACAACCTTCAACAAACGG-ATCTCTT |
| AY004778_B | AGT-ACAACATAATGTAAAATCATTTACAACCTTCAACAAACGG-ATCTCTT |
| AY004775_B | AGTAAAAACAA-TGTAA-TTA-TTACAACCTTCAACAAACGG-ATCTCTT |
| AY004777_B | AGT-ATAACAAATGTAAA-TCATTTACAACCTTCAACAAACGG-ATCTCTT |
| AY004803_D | AGCGAAAACAA-TGTAA-TCAATTACAACCTTCAACAAACGG-ATCTCTT |
| AY004805_D | AGC-AAAACAAATGTAA-TTAATTACAACCTTCAACAAACGG-ATCTCTT |
| AY004796_D | TG---AACAA-TGTAA-TAAATTACAACCTTCAACAAACGG-ATCTCTT |
| AY004788_D | AGC-AAAACAA-TGTAA-TAAATTACAACCTTCAACAAACGG-ATCTCTT |

AF163057_D TG----AACAA-TGTAA--TAAATTACAACCACTTCACAAACGG-ATCTCTT
 AF163058_D AGATAACACAAATGTAA--TAAATTACAACCACTTCACAAACGG-ATCTCTT
 AY004781_D AGC-ACAACAAATGTAATAACA-TTACAACCACTTCACAAACGG-ATCTCTT
 AY004789_D AGC-AAAACAA-TGTAA--TAAATTACAACCACTTCACAAACGG-ATCTCTT
 AY004806_D AGC-AAAACAAATGTAA--TCAATTACAACCACTTCACAAACGG-ATCTCTT
 AY004783_D AGC-AAAACAA-TGTAATAAAA-TTACAACCACTTCACAAACGG-ATCTCTT
 AY004791_D AGCAAAATAAAA-GTAATATAA-TTACAACCACTTCACAAACGG-ATCTCTT
 AY004794_D AGC-AAAACAA-TGTAA--TAAATTACAACCACTTCACAAACGG-ATCTCTT
 AY004807_D AGC-AAAACAAATGTAA--TCAATTACAACCACTTCACAAACGG-ATCTCTT
 AY004801_D AGC-AAAACAA-TGTAA--TAAATTACAACCACTTCACAAACGG-ATCTCTT
 AY004799_D TGAACAAACAA-TGTAA--TAAATTACAACCACTTCACAAACGG-ATCTCTT
 AF163059_D AGC-AAAACAA-TGTAA--TAAATTACAACCACTTCACAAACGG-ATCTCTT
 AF163060_D AGC-AAAACAAATGTAA--TAAATTACAACCACTTCACAAACGG-ATCTCTT
 AY004809_P AGCAAAACCAA-TGTAA--TGAATTACAACCACTTCACAAACGG-ATCTCTT
 AF081445_P TG----AACAA-TGTAA--TAAATTACAACCACTTCACAAACGG-ATCTCTT
 AY004798_P TGAACAAACAA-TGTAA--TAAATTACAACCACTTCACAAACGG-ATCTCTT
 AF081446_P AGATAACACAAATGTAA--TAAATTACAACCACTTCACAAACGG-ATCTCTT
 AY004808_P AGC-AAAACAAATGTAA--TCAATTACAACCACTTCACAAACGG-ATCTCTT
 AF229479_P AG-TAAA-CAA-TGTAA--TTA-TTACAACCACTTCACAAACGG-ATCTCTT

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| 4_3 | GGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAAT |
| 15_3 | GGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAAT |
| 16_3 | GGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAAT |
| 18 | GGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAAT |
| 19_3 | GGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAAT |
| Cal1 | GGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAAT |
| Swe1 | GGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAAT |
| Nap8 | GGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAAT |
| X78124_D.g | GGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAAT |
| Y08745_P.g | GGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAAT |
| Y10859_P.g | GGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAAT |
| Y10858_P.g | GGTTCTGGCATCGATGAAGAACGCAGTGAAATGCGATAAGTAGTGTGAAT |
| Y10857_P.g | GGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAAT |
| Y10748_P.g | GGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAAT |
| Y10923_P.g | GGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAAT |
| AF071347_P | GGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAAT |
| Y08744_P.t | GGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAAT |
| Y08746_P.t | GGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAAT |
| AF163061_P | GGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAAT |
| AY004778_B | GGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAACGTAGTGTGAAT |
| AY004775_B | GGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAACGTAGTGTGAAT |
| AY004777_B | GGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAACGTAGTGTGAAT |
| AY004803_D | GGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAAT |

AY004805_D GGTTCTGGCATCGATGAAGAACGCAGCGAAATCGATAAGTAGTGTGAAT
AY004796_D GGTTCTGGCATCGATGAAGAACGCAGCGAAATCGATAAGTAGTGTGAAT
AY004788_D GGTTCTGGCATCGATGAAGAACGCAGCGAAATCGATAAGTAGTGTGAAT
AF163057_D GGTTCTGGCATCGATGAAGAACGCAGCGAAATCGATAAGTAGTGTGAAT
AF163058_D GGTTCTGGCATCGATGAAGAACGCAGCGAAATCGATAAGTAGTGTGAAT
AY004781_D GGTTCTGGCATCGATGAAGAACGCAGCGAAATCGATAAGTAGTGTGAAT
AY004789_D GGTTCTGGCATCGATGAAGAACGCAGCGAAATCGATAAGTAGTGTGAAT
AY004806_D GGTTCTGGCATCGATGAAGAACGCAGCGAAATCGATAAGTAGTGTGAAT
AY004783_D GGTTCTGGCATCGATGAAGAACGCAGCGAAATCGATAAGTAGTGTGAAT
AY004791_D GGTTCTGGCATCGATGAAGAACGCAGCGAAATCGATAAGTAGTGTGAAT
AY004794_D GGTTCTGGCATCGATGAAGAACGCAGCGAAATCGATAAGTAGTGTGAAT
AY004807_D GGTTCTGGCATCGATGAAGAACGCAGCGAAATCGATAAGTAGTGTGAAT
AY004801_D GGTTCTGGCATCGATGAAGAACGCAGCGAAATCGATAAGTAGTGTGAAT
AY004799_D GGTTCTGGCATCGATGAAGAACGCAGCGAAATCGATAAGTAGTGTGAAT
AF163059_D GGTTCTGGCATCGATGAAGAACGCAGCGAAATCGATAAGTAGTGTGAAT
AF163060_D GGTTCTGGCATCGATGAAGAACGCAGCGAAATCGATAAGTAGTGTGAAT
AY004809_P GGTTCTGGCATCGATGAAGAACGCAGCGAAATCGATAAGTAGTGTGAAT
AF081445_P GGTTCTGGCATCGATGAAGAACGCAGCGAAATCGATAAGTAGTGTGAAT
AY004798_P GGTTCTGGCATCGATGAAGAACGCAGCGAAATCGATAAGTAGTGTGAAT
AF081446_P GGTTCTGGCATCGATGAAGAACGCAGCGAAATCGATAAGTAGTGTGAAT
AY004808_P GGTTCTGGCATCGATGAAGAACGCAGCGAAATCGATAAGTAGTGTGAAT
AF229479_P GGTTCTGGCATCGATGAAGAACGCAGCGAAATCGATAACGTAGTGTGAAT

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| 4_3 | TGCAGAATTCACTGAATCATCGAA-TCTTTGAACGCACATTGCGCCCTTT |
| 15_3 | TGCAGAATTCACTGAATCATCGAA-TCTTTGAACGCACATTGCGCCCTTT |
| 16_3 | TGCAGAATTCACTGAATCATCGAA-TCTTTGAACGCACATTGCGCCCTTT |
| 18 | TGCAGAATTCACTGAATCATCGAA-TCTTTGAACGCACATTGCGCCCTTT |
| 19_3 | TGCAGAATTCACTGAATCATCGAA-TCTTTGAACGCACATTGCGCCCTTT |
| Cal1 | TGCAGAATTCACTGAATCATCGAA-TCTTTGAACGCACATTGCGCCCTTT |
| Swe1 | TGCAGAATTCACTGAATCATCGAA-TCTTTGAACGCACATTGCGCCCTTT |
| Nap8 | TGCAGAATTCACTGAATCATCGAA-TCTTTGAACGCACATTGCGCCCTTT |
| X78124_D.g | TGCAGAATTCACTGAATCATCGAA-TCTTTGAACGCACATTGCGCCCTTT |
| Y08745_P.g | TGCAGAATTCACTGAATCATCGAAATCTTGAAACGCACATTGCGCCCTTT |
| Y10859_P.g | TGCAGAATTCACTGAATCATCGAA-TCTTTGAACGCACATTGCGCCCTTT |
| Y10858_P.g | TGCAGAATTCACTGAATCATCGAA-TCTTTGAACGCACATTGCGCCCTTT |
| Y10857_P.g | TGCAGAATTCACTGAATCATCGAA-TCTTTGAACGCACATTGCGCCCTTT |
| Y10748_P.g | TGCAGAATTCACTGAATCATCGAA-TCTTTGAACGCACATTGCGCCCTTT |
| Y10923_P.g | TGCAGAATTCACTGAATCATCGAA-TCTTTGAACGCACATTGCGCCCTTT |
| AF071347_P | TGCAGAATTCACTGAATCATCGAA-TCTTTGAACGCACATTGCGCCCTTT |
| Y08744_P.t | TGCAGAATTCACTGAATCATCGAAATCTTGAAACGCACATTGCGCCCTTT |
| Y08746_P.t | TGCAGAATTCACTGAATCATCGAA-TCTTTGAACGCACATTGCGCCCTTT |
| AF163061_P | TGCAGAATTCACTGAATCATCGAA-TCTTTGAACGCACATTGCGCCCTTT |
| AY004778_B | TGCAGAATTCACTGAATCATCGAA-TCTTTGAACGCACATTGCGCCCTTT |

AY004775_B TGCAGAATTCACTGAATCATCGAA-TCTTGAAACGCACATTGCGCCCTTT
AY004777_B TGCAGAATTCACTGAATCATCGAA-TCTTGAAACGCACATTGCGCCCTTT
AY004803_D TGCAGAATTCACTGAATCATCGAA-TCTTGAAACGCACATTGCGCCCTTT
AY004805_D TGCAGAATTCACTGAATCATCGAA-TCTTGAAACGCACATTGCGCCCTTT
AY004796_D TGCAGAATTCACTGAATCATCGAA-TCTTGAAACGCACATTGCGCCCTTT
AY004788_D TGCAGAATTCACTGAATCATCGAA-TCTTGAAACGCACATTGCGCCCTTT
AF163057_D TGCAGAATTCACTGAATCATCGAA-TCTTGAAACGCACATTGCGCCCTTT
AF163058_D TGCAGAATTCACTGAATCATCGAA-TCTTGAAACGCACATTGCGCCCTTT
AY004781_D TGCAGAATTCACTGAATCATCGAA-TCTTGAAACGCACATTGCGCCCTTT
AY004789_D TGCAGAATTCACTGAATCATCGAA-TCTTGAAACGCACATTGCGCCCTTT
AY004806_D TGCAGAATTCACTGAATCATCGAA-TCTTGAAACGCACATTGCGCCCTTT
AY004783_D TGCAGAATTCACTGAATCATCGAA-TCTTGAAACGCACATTGCGCCCTTT
AY004791_D TGCAGAATTCACTGAATCATCGAA-TCTTGAAACGCACATTGCGCCCTTT
AY004794_D TGCAGAATTCACTGAATCATCGAA-TCTTGAAACGCACATTGCGCCCTTT
AY004807_D TGCAGAATTCACTGAATCATCGAA-TCTTGAAACGCACATTGCGCCCTTT
AY004801_D TGCAGAATTCACTGAATCATCGAA-TCTTGAAACGCACATTGCGCCCTTT
AY004799_D TGCAGAATTCACTGAATCATCGAA-TCTTGAAACGCACATTGCGCCCTTT
AF163059_D TGCAGAATTCACTGAATCATCGAA-TCTTGAAACGCACATTGCGCCCTTT
AF163060_D TGCAGAATTCACTGAATCATCGAA-TCTTGAAACGCACATTGCGCCCTTT
AY004809_P TGCAGAATTCACTGAATCATCGAA-TCTTGAAACGCACATTGCGCCCTTT
AF081445_P TGCAGAATTCACTGAATCATCGAA-TCTTGAAACGCACATTGCGCCCTTT
AY004798_P TGCAGAATTCACTGAATCATCGAA-TCTTGAAACGCACATTGCGCCCTTT
AF081446_P TGCAGAATTCACTGAATCATCGAA-TCTTGAAACGCACATTGCGCCCTTT
AY004808_P TGCAGAATTCACTGAATCATCGAA-TCTTGAAACGCACATTGCGCCCTTT
AF229479_P TGCAGAATTCACTGAATCATCGAA-TCTTGAAACGCACATTGCGCCCTTT

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| 4_3 | GGTATTCAAAGGGCATGCCTGTTGAGCGTCATTGTACCCCTAAGCTT |
| 15_3 | GGTATTCAAAGGGCATGCCTGTTGAGCGTCATTGTACCCCTAAGCTT |
| 16_3 | GGTATTCAAAGGGCATGCCTGTTGAGCGTCATTGTACCCCTAAGCTT |
| 18 | GGTATTCAAAGGGCATGCCTGTTGAGCGTCATTGTACCCCTAAGCTT |
| 19_3 | GGTATTCAAAGGGCATGCCTGTTGAGCGTCATTGTACCCCTAAGCTT |
| Cal1 | GGTATTCAAAGGGCATGCCTGTTGAGCGTCATTGTACCCCTAAGCTT |
| Swe1 | GGTATTCAAAGGGCATGCCTGTTGAGCGTCATTGTACCCCTAAGCTT |
| Nap8 | GGTATTCAAAGGGCATGCCTGTTGAGCGTCATTGTACCCCTAAGCTT |
| X78124_D.g | GGTATTCAAAGGGCATGCCTGTTGAGCGTCATTGTACCCCTAAGCTT |
| Y08745_P.g | GGTATTCAAAGGGCATGCCTGTTGAGCGTCATTGTACCCCTAAGCTT |
| Y10859_P.g | GGTATTCAAAGGGCATGCCTGTTGAGCGTCATTGTACCCCTAAGCTT |
| Y10858_P.g | GGTATTCAAAGGGCATGCCTGTTGAGCGTCATTGTACCCCTAAGCTT |
| Y10857_P.g | GGTATTCAAAGGGCATGCCTGTTGAGCGTCATTGTACCCCTAAGCTT |
| Y10748_P.g | GGTATTCAAAGGGCATGCCTGTTGAGCGTCATTGTACCCCTAAGCTT |
| Y10923_P.g | GGTATTCAAAGGGCATGCCTGTTGAGCGTCATTGTACCCCTAAGCTT |
| AF071347_P | GGTATTCAAAGGGCATGCCTGTTGAGCGTCATTGTACCCCTAAGCTT |
| Y08744_P.t | GGTATTCAAAGGGCATGCCTGTTGAGCGTCATTGTACCCCTAAGCTT |

Y08746_P.t GGTATTCAAAGGGCATGCCTGTCGAGCGTCAAGCTT
 AF163061_P GGTATTCAAAGGGCATGCCTGTCGAGCGTCAAGCTT
 AY004778_B GGTATTCAAAGGGCATGCCTGTCGAGCGTCAAGCTT
 AY004775_B GGTATTCAAAGGGCATGCCTGTCGAGCGTCAAGCTT
 AY004777_B GGTATTCAAAGGGCATGCCTGTCGAGCGTCAAGCTT
 AY004803_D GGTATTCAAAGGGCATGCCTGTCGAGCGTCAAGCTT
 AY004805_D GGTATTCAAAGGGCATGCCTGTCGAGCGTCAAGCTT
 AY004796_D GGTATTCAAAGGGCATGCCTGTCGAGCGTCAAGCTT
 AY004788_D GGTATTCAAAGGGCATGCCTGTCGAGCGTCAAGCTT
 AF163057_D GGTATTCAAAGGGCATGCCTGTCGAGCGTCAAGCTT
 AF163058_D GGTATTCAAAGGGCATGCCTGTCGAGCGTCAAGCTT
 AY004781_D GGTATTCAAAGGGCATGCCTGTCGAGCGTCAAGCTT
 AY004789_D GGTATTCAAAGGGCATGCCTGTCGAGCGTCAAGCTT
 AY004806_D GGTATTCAAAGGGCATGCCTGTCGAGCGTCAAGCTT
 AY004783_D GGTATTCAAAGGGCATGCCTGTCGAGCGTCAAGCTT
 AY004791_D GGTATTCAAAGGGCATGCCTGTCGAGCGTCAAGCTT
 AY004794_D GGTATTCAAAGGGCATGCCTGTCGAGCGTCAAGCTT
 AY004807_D GGTATTCAAAGGGCATGCCTGTCGAGCGTCAAGCTT
 AY004801_D GGTATTCAAAGGGCATGCCTGTCGAGCGTCAAGCTT
 AY004799_D GGTATTCAAAGGGCATGCCTGTCGAGCGTCAAGCTT
 AF163059_D GGTATTCAAAGGGCATGCCTGTCGAGCGTCAAGCTT
 AF163060_D GGTATTCAAAGGGCATGCCTGTCGAGCGTCAAGCTT
 AY004809_P GGTATTCAAAGGGCATGCCTGTCGAGCGTCAAGCTT
 AF081445_P GGTATTCAAAGGGCATGCCTGTCGAGCGTCAAGCTT
 AY004798_P GGTATTCAAAGGGCATGCCTGTCGAGCGTCAAGCTT
 AF081446_P GGTATTCAAAGGGCATGCCTGTCGAGCGTCAAGCTT
 AY004808_P GGTATTCAAAGGGCATGCCTGTCGAGCGTCAAGCTT
 AF229479_P GGTATTCAAAGGGCATGCCTGTCGAGCGTCAAGCTT

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|------------|---|
| 4_3 | T-GCTTGGTGTGGCGTCTTT---GTCTCTCCCC-----G |
| 15_3 | T-GCTTGGTGTGGCGTCTTT---GTCTCTCCCC-----G |
| 16_3 | T-GCTTGGTGTGGCGTCTTT---GTCTCTCCCC-----G |
| 18 | T-GCTTGGTGTGGCGTCTTT---GTCTCTCCCC-----G |
| 19_3 | T-GCTTGGTGTGGCGTCTTT---GTCTCTCCCC-----G |
| Cal1 | T-GCTTGGTGTGGCGTCTTT---GTCTCTCCCC-----G |
| Swe1 | T-GCTTGGTGTGGCGTCTTT---GTCTCTCCCC-----G |
| Nap8 | T-GCTTGGTGTGGCGTCTTT---GTCTCTCCCC-----G |
| X78124_D.g | T-GCTTGGTGTGGCGTCTTT---GTCTCTCCCC-----G |
| Y08745_P.g | T-GCTTGGTGTGGCGTCTTT---GTCTCTCCCC-----G |
| Y10859_P.g | T-GCTTGGTGTGGCGTCTTT---GTCTCTCCCC-----G |
| Y10858_P.g | T-GCTTGGTGTGGCGTCTTT---GTCTCTCCCC-----G |
| Y10857_P.g | T-GCTTGGTGTGGCGTCTTT---GTCTCTCCCC-----G |
| Y10748_P.g | T-GCTTGGTGTGGCGTCTTT---GTCTCTCCCC-----G |

Y10923_P.g T-GCTTGGTGTGGCGTCTTT---GTCTCTCCCC-----G
 AF071347_P T-GCTTGGTGTGGCGTCTTT---GTCTCTCCCC-----G
 Y08744_P.t T-GCTTGGTGTGGCGTCTTT---GTCTCTCCCC-----G
 Y08746_P.t T-GCTTGGTGTGGCGTCTTT---GTCTCTCCCC-----G
 AF163061_P T-GCTTGGTGTGGCGTCTTT---GTCTCTCCCC-----G
 AY004778_B T-GCTTGGTGTGGCGTTGTT---GTCT---TGC---TAT---CCGCA
 AY004775_B T-GCTTGGTGTGGCGTCTTT---GTCTCTCC-----TTT---GCGGG
 AY004777_B T-GCTTGGTGTGGCGTTTTT---GTCT--TTGG-----CCGCCAA
 AY004803_D T-GCTTGGTGTGGCGTCTT---GTGTTGGGGG-GCTTTACCCCCGA
 AY004805_D T-GCTTGGTGTGGCGTCTT---GTCTTGGGGC-----TCCTCTCA
 AY004796_D T-GCTTGGTGTGGCGTTTTT---GTCTT--GGGT-----CCGCCCG
 AY004788_D T-GCTTGGTGTGGCGTTTTT---GTCT---CGGGT-----CCGCCCG
 AF163057_D T-GCTTGGTGTGGCGTTTTT---GTCT--CGGGT-----CCGCCCG
 AF163058_D TTGCTTGGTGTGGCGTTTTT---GTCGT--GGG-----TCTGCCA
 AY004781_D T-GCTTGGTGTGGCGTTTTT---GTCTTGGGGTATTGCCCATGA
 AY004789_D T-GCTTGGTGTGGCGTTTTT---GTCT---CGGGT---CCGCCCG
 AY004806_D T-GCTTGGTGTGGCGTCTT---GTCTTGGGGC-----TTCCCTCTCA
 AY004783_D T-GCTTGGTGTGGCGTTTTTTTGTCTT---GGGT---CCGCCCCG
 AY004791_D T-GCTTGGTGTGGCGTTTTT---GTCC--CTGGT-----TCGCCA-G
 AY004794_D T-GCTTGGTGTGGCGTGT---T---GTCT--CTGG---TTT---ACCAG
 AY004807_D T-GCTTGGTGTGGCGTCTT---GTCTTGGGGG-TCCATCCCCA
 AY004801_D T-GCTTGGTGTGGCGTTTTT---GTCTT--GGGT-----CCGCCCG
 AY004799_D T-GCTTGGTGTGGCGTCTT---GTCTT--GGGT-----CCGCCCG
 AF163059_D T-GCTTGGTGTGGCGT-TTT---GTCT--CGGG-----CCGCCCG
 AF163060_D T-GCTTGGTGTGGG-----GTCTT--GTCT---CTCTCCG
 AY004809_P T-GCTTGGTGTGGCGTCTT---GTCTC-TGG-----G
 AF081445_P T-GCTTGGTGTGGCGTTTTT---GTCTT--GGGT-----CCGCCCG
 AY004798_P T-GCTTGGTGTGGCGTTTTT---GTCTT--GGGT-----CCGCCCG
 AF081446_P TTGCTTGGTGTGGCGTTTTT---GTACG-TGGG-----TGTGCCA
 AY004808_P T-GCTTGGTGTGGG-----GTCTT--GTCT---CTCTCCG
 AF229479_P T-GCTTGGTGTGGCGTCTT---GTCTC-----TCACG

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| 4_3 | AGACTCGCCTTAAAAACATTGGCAGCCGGCTACTGGTTCGGAGCGCAG |
| 15_3 | AGACTCGCCTTAAAAACATTGGCAGCCGGCTACTGGTTCGGAGCGCAG |
| 16_3 | AGACTCGCCTTAAAAACATTGGCAGCCGGCTACTGGTTCGGAGCGCAG |
| 18 | AGACTCGCCTTAAAAACATTGGCAGCCGGCTACTGGTTCGGAGCGCAG |
| 19_3 | AGACTCGCCTTAAAAACATTGGCAGCCGGCTACTGGTTCGGAGCGCAG |
| Cal1 | AGACTCGCCTTAAAAACATTGGCAGCCGGCTACTGGTTCGGAGCGCAG |
| Swe1 | AGACTCGCCTTAAAAACATTGGCAGCCGGCTACTGGTTCGGAGCGCAG |
| Nap8 | AGACTCGCCTTAAAAACATTGGCAGCCGGCTACTGGTTCGGAGCGCAG |
| X78124_D.g | AGACTCGCCTTAAAAACATTGGCAGCCGGCTACTGGTTCGGAGCGCAG |
| Y08745_P.g | AGACTCGCCTTAAAAACATTGGCAGCCGGCTACTGGTTCGGAGCGCAG |
| Y10859_P.g | AGACTCGCCTTAAAAACATTGGCAGCCGGCTACTGGTTCGGAGCGCAG |

Y10858_P.g AGACTCGCCTTAAAAACATTGGCAGCCGGCCTACTGGTTCGGAGCGCAG
 Y10857_P.g AGACTCGCCTTAAAAACATTGGCAGCCGGCCTACTGGTTCGGAGCGCAG
 Y10748_P.g AGACTCGCCTTAAAAACATTGGCAGCCGGCCTACTGGTTCGGAGCGCAG
 Y10923_P.g AGACTCGCCTTAAAAACATTGGCAGCCGGCCTACTGGTTCGGAGCGCAG
 AF071347_P AGACTCGCCTTAAAAACATTGGCAGCCGGCCTACTGGTTCGGAGCGCAG
 Y08744_P.t AGACTCGCCTTAAAAACATTGGCAGCCGGCCTACTGGTTCGGAGCGCAG
 Y08746_P.t AGACTCGCCTTAAAAACATTGGCAGCCGGCCTACTGGTTCGGAGCGCAG
 AF163061_P AGACTCGCCTTAAAAACATTGGCAGCCGGCCTACTGGTTCGGAGCGCAG
 AY004778_B AGACTCGCCTTAAAACGATTGGCAGCCGGCCTACTGGTTCGGAGCGCAG
 AY004775_B AGACTCGCCTTAAAACGATTGGCAGCCGGCCTACTGGTTCGGAGCGCAG
 AY004777_B AGACTCGCCTTAAAATGATTGGCAGCCGGCCTACTGGTTCGCAGCGCAG
 AY004803_D T-ACTCGCCTTAAAATCATTGGCAGCCGGCCTACTGGTTCCGGAGCGCAG
 AY004805_D AGACTCGCCTTAAAATCATTGGCAGCCGGCCTACTGGTTCGGAGCGCAG
 AY004796_D AGACTCGCCTTAAAATCATTGGCAGCCGGCCTACTGGTTCGGAGCGCAG
 AY004788_D AGACTCGCCTTAAAATCATTGGCAGCCGGCCTACTGGTTCGGAGCGCAG
 AF163057_D AGACTCGCCTTAAAATCATTGGCAGCCGGCCTACTGGTTCGGAGCGCAG
 AF163058_D CGACTCGCCTTAAAATCATTGGCAGCCGGCCTACTGGTTCGGAGCGCAG
 AY004781_D AGACTCGCCTTAAAGTCATTGGCAGCCGGCCTACTGGTTCGGAGCGCAG
 AY004789_D AGACTCGCCTTAAAATCATTGGCAGCCGGCCTACTGGTTCGGAGCGCAG
 AY004806_D AGACTCGCCTTAAAATCATTGGCAGCCGGCCTACTGGTTACGGAGCGCAG
 AY004783_D AGACTCGCCTTAAAATCATTGGCAGCCGGCCTACTGGTTCGGAGCGCAG
 AY004791_D AGACTCGCCTTAAAATCATTGGCAGCCGGCCTACTGGTTCGGAGCGCAG
 AY004794_D AGACTCGCCTTAAAATCATTGGCAGCCGGCCTACTGGTTCGGAGCGCAG
 AY004807_D CGACTCGCCTTAAAATCATTGGCAGCCGGCCTACTGGTTCGGAGCGCAG
 AY004801_D AGACTCGCCTTAAAATCATTGGCAGCCGGCCTACTGGTTCGGAGCGCAG
 AY004799_D AGAATCGCCTTAAAATCATTGGCAGCCGGCCTACTGGTTCGGAGCGCAG
 AF163059_D AGACTCGCCTTAAAATCATTGGCAGCCGGCCTACTGGTTCGGAGCGCAG
 AF163060_D AGACTCGCCTTAAAATCATTGGCAGCCGGCCTACTGGTTCGGAGCGCAG
 AY004809_P AGACTCGCCTTAAAATCATTGGCAGCCGGCCTACTGGTTCGGAGCGCAG
 AF081445_P AGACTCGCCTTAAAATCATTGGCAGCCGGCCTACTGGTTCGGAGCGCAG
 AY004798_P AGACTCGCCTTAAAATCATTGGCAGCCGGCCTACTGGTTCGGAGCGCAG
 AF081446_P CGACTCGCCTTAAAATCATTGGCAGCCGGCCTACTGGTTCGGAGCGCAG
 AY004808_P AGACTCGCCTTAAAATCATTGGCAGCCGGCCTACTGGTTCGGAGCGCAG
 AF229479_P AGACTCGCCTTAAAATGATTGGCAGCCGACCTACTGGTTCGGAGCGCAG

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|------|--|
| 4_3 | CACATTATTT-GCGCTCTTGTCCAGCCGC----GGTCGCGCGTCCATGAA |
| 15_3 | CACATTATTT-GCGCTCTTGTCCAGCCGC----GGTCGCGCGTCCATGAA |
| 16_3 | CACATTATTT-GCGCTCTTGTCCAGCCGC----GGTCGCGCGTCCATGAA |
| 18 | CACATTATTT-GCGCTCTTGTCCAGCCGC----GGTCGCGCGTCCATGAA |
| 19_3 | CACATTATTT-GCGCTCTTGTCCAGCCGC----GGTCGCGCGTCCATGAA |
| Cal1 | CACATTATTT-GCGCTCTTGTCCAGCCGC----GGTCGCGCGTCCATGAA |
| Swe1 | CACATTATTT-GCGCTCTTGTCCAGCCGC----GGTCGCGCGTCCATGAA |
| Nap8 | CAAATTATTT-GCGCTCTTGTCCAGCCGC----GGTCGCGCGTCCATGAA |

X78124_D.g CACATTATTT-GCGCTCTTGTCCAG-CGC----GGTCGCGCGTCCATGAA
Y08745_P.g CACATTATTT-GCGCTCTTGTCCAGCCGC----GGTCGCGCGTCCATGAA
Y10859_P.g CACATTATTT-GCGCTCTTGTCCAGC-GC----GGTCGCGCGTCCATGAA
Y10858_P.g CACATTATTT-GCGCTCTTGTCCAGC-GC----GGTCGCGCGTCCATGAA
Y10857_P.g CACATTATTT-GCGCTCTTGTCCAGC-GC----GGTCGCGCGTCCATGAA
Y10748_P.g CACATTATTT-GCGCTCTTGTCCAGC-GC----GGTCGCGCGTCCATGAA
Y10923_P.g CACATTATTT-GCGCTCTTGTCCAGC-GC----GGTCGCGCGTCCATGAA
AF071347_P CACATTATTT-GCGCTCTTGTCCAGCCGC----GGTCGCGCGTCCATGAA
Y08744_P.t CACATTATTT-GCGCTCTTGTCCAGCCGC----GGTCGCGCGTCCATGAA
Y08746_P.t CACATTATTT-GCGCTCTTGTCCAGCCGC----GGTCGCGCGTCCATGAA
AF163061_P CACATTATTT-GCGCTCTTGTCCAGCCGC----GGTCGCGCGTCCATGAA
AY004778_B CACATATTTT-GCGCT-TGCAACCAGAAAAGAGGTTGGCGATCCAGCAA
AY004775_B CACATTTTTT-GCGCTTTGTATCAGGAGAA-AAGGACGGTACTCCATCAA
AY004777_B CACATTTTTT-GCGCTTGCAATCAGAAAA-GAGGACGGCAATCCATCAA
AY004803_D CACATTTTTT-GCGCTCTTGTCCCGCCGC----GGTCGCGCGTCCAGCAA
AY004805_D CACATTATTT-GCGCTCTTGTCCAGCCGC----GGTCGCGCGTCCATGAA
AY004796_D CACATTTTTT-GCGCTTTGGTCTAGT-----GGTCCAGCGTCCATGAA
AY004788_D CACATT-TTT-GCGCT-TTGTCCAGTCGT---GGTCTTGCCTCATGAA
AF163057_D CACATT-TTT-GCGCTCTGTCCAGTT-GT---GGTT-TGCGTCCATGAA
AF163058_D CACATT-TTT-GCGCTCAGTCCAGCA-GC----GGTCACCGCGTCCACGAA
AY004781_D CACATT-TTT-GCGCTCT-GTCCAGTTGT---GGTTCTGCCTCATGAA
AY004789_D CACATT-TTT-GCGCT-TTGTCCAGTCGT---GGTCTTGCCTCATGAA
AY004806_D CACATTATTT-GCGTTCTTGTCCAGCCGC----GGTCGCGCGTCCATGAA
AY004783_D CACATT-TTT-GCGCT-TTGTCCAGTTGC----GGTC-CGCGTCCATGAA
AY004791_D CACAAT-TTT-GCGCTTCTTCCAGCT-GT---GGTTGTGCCTCATGAA
AY004794_D CACAAT-TTT-GCGCCTCT-TCCAGCAGT---GGTC-TGCGTCCAT-AA
AY004807_D CACATTCTTT-GCGCTCTTGTCCAGCCGC----GGTCGCGCATCCATGAA
AY004801_D CACATT-TTT-GCGCT-TTGTCCAGTTGC----GGTC-CGCGTCCATGAA
AY004799_D CACATTTTTT-GCGCTTTGGTCTAGT-----GGTCCCGCGTCCATGAA
AF163059_D CACATT-TTT-GCGCT-TTGTCCAGTTGT---GGTC-CGCGTCCATGAA
AF163060_D CACATTATTT-GCGCTCTTGTCCAGCCGC----GGTCGCGCGTCCATGAA
AY004809_P CACATTCTTTGCGCTCTTGTCCAGCCGC----GGTCGCGCGTCCATGAA
AF081445_P CACATTTTTT-GCGCTTTGGTCTA---GT---GGTCCAGCGTCCATGAA
AY004798_P CACATTTTTT-GCGCTTTGGTCTAGT-----GGTCCCGCGTCCATGAA
AF081446_P CACATT-TTT-GCGCTCAGTCCAGCAGC-----GGTCACCGCGTCCACGAA
AY004808_P CACATTATTT-GCGCTCTTGTCCAGCCGC----GGTCGCGCGTCCATGAA
AF229479_P CACAATTCTT-GCACTTGAATCAGCCTT---GGTTGAGCATCCATCAA

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|------|---|
| 4_3 | -G-CC----TTTTTTTTTC-AACCTTTGACCTCGGATCAGGTAGGGATA |
| 15_3 | -G-CC----TTTTTTTTTC-AACCTTTGACCTCGGATCAGGTNGGGATA |
| 16_3 | -G-CC--TTTTTTTTTC-AACCTTTGACCTCGGATCAGGTAGGGATA |
| 18 | -G-CC----TTTTTTTTTC-AACCTTTGACCTCGGATCAGGTAGGGATA |
| 19_3 | -G-CC----TTTTTTTTTC-AACCTTTGACCTCGGATCAGGTAGGGATA |

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|------------|--|
| Cal1 | -G-C---TTTTTTTTT--AACCTTTGACCTCGGATCAGGTAGGGATA |
| Swe1 | -G-CC---TTTTTTTTT--AACCTTTGACCTCGGATCAGGTAGGGATA |
| Nap8 | -G-CC-----TTTTTTT--AACCTTTGACCTCGGATCAGGTAGGGATA |
| X78124_D.g | -G-CC-----TTTTTTTC-AACCTTTGACCTCGGATCAGGTAGGGATA |
| Y08745_P.g | -G-CC-----TTTTTTTC-AACCTTTGACCTCGGATCAGGTAGGGATA |
| Y10859_P.g | -G-CC-----TTTTTTTC-AACCTTTGACCTCGGATCAGGTAGGGATA |
| Y10858_P.g | -G-CC---TTTTTTTTTC-AACCTTTGACCTCGGATCAGGTAGGGATA |
| Y10857_P.g | -G-CC-----TTTTTTTC-AACCTTTGACCTCGGATCAGGTAGGGATA |
| Y10748_P.g | -G-CC-----TTTTTTTC-AACCTTTGACCTCGGATCAGGTAGGGATA |
| Y10923_P.g | -G-CC-----TTTTTTTC-AACCTTTGACCTCGGATCAGGTAGGGATA |
| AF071347_P | -G-CC--TTTTTTTTTC--AC-TTTTGA----- |
| Y08744_P.t | -G-CC-----TTTTTTTC-AACCTTTGACCTCGGATCAGGTAGGGATA |
| Y08746_P.t | -G-CC-----TTTTTTTC-AACCTTTGACCTCGGATCAGGTAGGGATA |
| AF163061_P | -G-CC-----TTTTTTTC-AACCTTTGACCTCGGA----- |
| AY004778_B | -G-TACA----TCTTCTC--AC-TTTTGACCTCGGATCAGGTAGGGATA |
| AY004775_B | -GACGTTTACA-TTTGTTT--AC-TTTTGACCTCGGATCAGGTAGGGATA |
| AY004777_B | -G---A---CTCCTTCTC--ACG-TTTGACCTCGGATCAGGTAGGGATA |
| AY004803_D | -G-CCACACCTTTTTT--AC--TTTGACCTCGGATCAGGTAGGGATA |
| AY004805_D | -G-----CTGTCTTC-AACCTTTGACCTCGGATCAGGTAGGGATA |
| AY004796_D | -G-CGAATA----TTTTC-AA---TTTGACCTCGGATCAGGTAGGGATA |
| AY004788_D | -G-CGAATA----TTTTC-AACG-TTTGACCTCGGATCAGGTAGGGATA |
| AF163057_D | -G-CGAATA----TTTTC-AACG-TTTGACCTCGGA----- |
| AF163058_D | -G-CGAA----TTTTTTTC-AACG-TTTGACCTCGGA----- |
| AY004781_D | -G-CTGAAATA----TTC-AACG-TTTGACCTCGGATCAGGTAGGGATA |
| AY004789_D | -G-CGAATA----TTTTTC-AACG-TTTGACCTCGGATCAGGTAGGGATA |
| AY004806_D | -G-----CTTCTTTC-AACCTTTGACCTCGGATCAGGTAGGGATA |
| AY004783_D | -G-CAAA----TAGTCAAACG-TTTGACCTCGGATCAGGTAGGGATA |
| AY004791_D | AG-CCAT----TTTTC-AACG-TTTGACCTCGGATCAGGTAGGGATA |
| AY004794_D | -G-CCA----TTTTTTTC-AACC-TTTGACCTCGGATCAGGTAGGGATA |
| AY004807_D | -G-----CTTCTTTC-AACCTTTGACCTCGGATCAGGTAGGGATA |
| AY004801_D | -G-CGAA-TA----TTTTC-AACG-TTTGACCTCGGATCAGGTAGGGATA |
| AY004799_D | -G-CGAAATACATTTTTC-AA---TTTGACCTCGGATCAGGTAGGGATA |
| AF163059_D | -G-CGAA----TTTTC-AACG-TTTGACCTCGGA----- |
| AF163060_D | -G-----CTTC-TTTC-AACCTTTGACCTCGGA----- |
| AY004809_P | -G-CA----TTTTTTTCAAACCTTTGACCTCGGATCAGGTAGGGATA |
| AF081445_P | -G-CG---AA-TATTTTC-AA---TTTGACCTC----- |
| AY004798_P | -G-CGAATACA-TTTTTTC-AA---TTTGACCTCCGATCAGGTAGGGATA |
| AF081446_P | -G-CGAA----TTTTTTTC-AACG-TTTGACCTC----- |
| AY004808_P | -G-----CTTCTTTC-AACCTTTGACCTCGGATCAGGTAGGGATA |
| AF229479_P | -G---ACCACATTTTTC-AAC-TTTGACCTCGGATCAGGTAGGGATA |

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|------|----------------|
| 4_3 | CCCGCTGAA----- |
| 15_3 | CCCGCTGAA----- |

16_3 CCCGCTGAA-----
18 CCCGCTGAA-----
19_3 CCCGCTGAA-----
Cal1 CCCGCTGAACCTTAA-
Swe1 CCCGCTGAACCTTAA-
Nap8 CCCGCTGAACCTTAA-
X78124_D.g CCCGCTGAACCTTAA-
Y08745_P.g CCCGCTGAACCTTAA-
Y10859_P.g CCCGCTGAACCTTAA-
Y10858_P.g CCCGCTGAACCTTAA-
Y10857_P.g CCCGCTGAACCTTAA-
Y10748_P.g CCCGCTGAACCTTAA-
Y10923_P.g CCCGCTGAACCTTAA-
AF071347_P -----
Y08744_P.t CCCGCTGAACCTTAA-
Y08746_P.t CCCGCTGAACCTTAA-
AF163061_P -----
AY004778_B CCC-----
AY004775_B CCC-----
AY004777_B CCC-----
AY004803_D CCC-----
AY004805_D CCC-----
AY004796_D CCC-----
AY004788_D CCC-----
AF163057_D -----
AF163058_D -----
AY004781_D CCC-----
AY004789_D CCC-----
AY004806_D CCC-----
AY004783_D CCC-----
AY004791_D CCC-----
AY004794_D CCC-----
AY004807_D CCC-----
AY004801_D CCC-----
AY004799_D CCC-----
AF163059_D -----
AF163060_D -----
AY004809_P CCC-----
AF081445_P -----
AY004798_P CCC-----
AF081446_P -----
AY004808_P CC-----
AF229479_P CCCGCTGAACTTACA

Appendix 1B-Histone H3 gene

6_3 -----TGGTGGCAAAGCCCCCGCAAGCAGTAAGCCTTGGCCTCGCA
 Swe_1 -----TGGTGGCAAAGCCCCCGCAAGCAGTAAGCCTTGGCCTCGCA
 Nap_2 -----TGGTGGCAAAGCCCCCGCAAGCAGTAAGCCTTGGCCTCGCA
 23 -----TGGTGGCAAAGCCCCCGCAAGCAGTAAGCCTTGGCCTCGCA
 12 -----TGGTGGCAAAGCCCCCGCAAGCAGTAAGCCTTGGCCTCGCA
 17 -----TGGTGGCAAAGCCCCCGCAAGCAGTAAGCCTTGGCCTCGCA
 16 -----TGGTGGCAAAGCCCCCGCAAGCAGTAAGCCTTGGCCTCGCA
 4_3 -----TGGTGGCAAAGCCCCCGCAAGCAGTAAGCCTTGGCCTCGCA
 7_3 -----TGGTGGCAAAGCCCCCGCAAGCAGTAAGCCTTGGCCTCGCA
 14 -----TGGTGGCAAAGCCCCCGCAAGCAGTAAGCCTTGGCCTCGCA
 16_3 -----TGGTGGCAAAGCCCCCGCAAGCAGTAAGCCTTGGCCTCGCA
 Cal_1 -----TGGTGGCAAAGCCCCCGCAAGCAGTAAGCCTTGGCCTCGCA
 2_3 AGGTCCACTGGTGGCAAAGCCCCCGCAAGCAGTAAGCCTTGGCCTCGCA
 3_3 AGGTCCACTGGTGGCAAAGCCCCCGCAAGCAGTAAGCCTTGGCCTCGCA
 Riv_8 AGGTCCACTGGTGGCAAAGCCCCCGCAAGCAGTAAGCCTTGGCCTCGCA
 Nap_8 AGGTCCACTGGTGGCAAAGCCCCCGCAAGCAGTAAGCCTTGGCCTCGCA
 PH_21 AGGTCCACTGGTGGCAAAGCCCCCGCAAGCAGTAAGCCTTGGCCTCGCA

6_3 CCTCGTCTTCTCATCGCATCGTCGCTAACATCCTCCCAGGGCTGCCCTCCA
 Swe_1 CCTCGTCTTCTCATCGCATCGTCGCTAACATCCTCCCAGGGCTGCCCTCCA
 Nap_2 CCTCGTCTTCTCATCGCATCGTCGCTAACATCCTCCCAGGGCTGCCCTCCA
 23 CCTCGTCTTCTCATCGCATCGTCGCTAACATCCTCCCAGGGCTGCCCTCCA
 12 CCTCGTCTTCTCATCGCATCGTCGCTAACATCCTCCCAGGGCTGCCCTCCA
 17 CCTCGTCTTCTCATCGCATCGTCGCTAACATCCTCCCAGGGCTGCCCTCCA
 16 CCTCGTCTTCTCATCGCATCGTCGCTAACATCCTCCCAGGGCTGCCCTCCA
 4_3 CCTCGTCTTCTCATCGCATCGTCGCTAACATCCTCCCAGGGCTGCCCTCCA
 7_3 CCTCGTCTTCTCATCGCATCGTCGCTAACATCCTCCCAGGGCTGCCCTCCA
 14 CCTCGTCTTCTCATCGCATCGTCGCTAACATCCTCCCAGGGCTGCCCTCCA
 16_3 CCTCGTCTTCTCATCGCATCGTCGCTAACATCCTCCCAGGGCTGCCCTCCA
 Cal_1 CCTCGTCTTCTCATCGCATCGTCGCTAACATCCTCCCAGGGCTGCCCTCCA
 2_3 CCTCGTCTTCTCATCGCATCGTCGCTAACATCCTCCCAGGGCTGCCCTCCA
 3_3 CCTCGTCTTCTCATCGCATCGTCGCTAACATCCTCCCAGGGCTGCCCTCCA
 Riv_8 CCTCGTCTTCTCATCGCATCGTCGCTAACATCCTCCCAGGGCTGCCCTCCA
 Nap_8 CCTCGTCTTCTCATCGCATCGTCGCTAACATCCTCCCAGGGCTGCCCTCCA
 PH_21 CCTCGTCTTCTCATCGCATCGTCGCTAACATCCTCCCAGGGCTGCCCTCCA

6_3 AGGCTGCTCGCAAGTCTGCACCATCAACCGGTGGTGTCAAGAAGCCTCAC
 Swe_1 AGGCTGCTCGCAAGTCTGCACCATCAACCGGTGGTGTCAAGAAGCCTCAC
 Nap_2 AGGCTGCTCGCAAGTCTGCACCATCAACCGGTGGTGTCAAGAAGCCTCAC
 23 AGGCTGCTCGCAAGTCTGCACCATCAACCGGTGGTGTCAAGAAGCCTCAC
 12 AGGCTGCTCGCAAGTCTGCACCATCAACCGGTGGTGTCAAGAAGCCTCAC

17 AGGCTGCTCGCAAGTCTGCACCATCAACCCTGGTGTCAAGAAGCCTCAC
 16 AGGCTGCTCGCAAGTCTGCACCATCAACCCTGGTGTCAAGAAGCCTCAC
 4_3 AGGCTGCTCGCAAGTCTGCACCATCAACCCTGGTGTCAAGAAGCCTCAC
 7_3 AGGCTGCTCGCAAGTCTGCACCATCAACCCTGGTGTCAAGAAGCCTCAC
 14 AGGCTGCTCGCAAGTCTGCACCATCAACCCTGGTGTCAAGAAGCCTCAC
 16_3 AGGCTGCTCGCAAGTCTGCACCATCAACCCTGGTGTCAAGAAGCCTCAC
 Cal_1 AGGCTGCTCGCAAGTCTGCACCATCAACCCTGGTGTCAAGAAGCCTCAC
 2_3 AGGCTGCTCGCAAGTCTGCACCATCAACCCTGGTGTCAAGAAGCCTCAC
 3_3 AGGCTGCTCGCAAGTCTGCACCATCAACCCTGGTGTCAAGAAGCCTCAC
 Riv_8 AGGCTGCTCGCAAGTCTGCACCATCAACCCTGGTGTCAAGAAGCCTCAC
 Nap_8 AGGCTGCTCGCAAGTCTGCACCATCAACCCTGGTGTCAAGAAGCCTCAC
 PH_21 AGGCTGCTCGCAAGTCTGCACCATCAACCCTGGTGTCAAGAAGCCTCAC

 6_3 CGCTACAAGCCTGGTACCGTCGCTCCGTGAGATCCGTGCTACCAGAA
 Swe_1 CGCTACAAGCCTGGTACCGTCGCTCCGTGAGATCCGTGCTACCAGAA
 Nap_2 CGCTACAAGCCTGGTACCGTCGCTCCGTGAGATCCGTGCTACCAGAA
 23 CGCTACAAGCCTGGTACCGTCGCTCCGTGAGATCCGTGCTACCAGAA
 12 CGCTACAAGCCTGGTACCGTCGCTCCGTGAGATCCGTGCTACCAGAA
 17 CGCTACAAGCCTGGTACCGTCGCTCCGTGAGATCCGTGCTACCAGAA
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 3_3 CGCTACAAGCCTGGTACCGTCGCTCCGTGAGATCCGTGCTACCAGAA
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 Nap_8 CGCTACAAGCCTGGTACCGTCGCTCCGTGAGATCCGTGCTACCAGAA
 PH_21 CGCTACAAGCCTGGTACCGTCGCTCCGTGAGATCCGTGCTACCAGAA

 6_3 GTCGACCGAGCTCCTCATCCGCAAGCTGCCCTTCCAGCGTCTTGTTCGTG
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 Nap_2 GTCGACCGAGCTCCTCATCCGCAAGCTGCCCTTCCAGCGTCTTGTTCGTG
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 Nap_8 AGATTGCTCAGGACTTCAAGTCCGACTTGCCTCCAGTCGTCCGCCATC
 PH_21 AGATTGCTCAGGACTTCAAGTCCGACTTGCCTCCAGTCGTCCGCCATC

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16 ACATCCAGCTGCCCGCAA-----
4_3 -----
7_3 -----
14 -----
16_3 -----
Cal_1 -----
2_3 -----
3_3 -----
Riv_8 -----
Nap_8 -----
PH_21 -----