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Evidence of recombination between net- and spot-type populations of Pyrenophora teres as determined by RAPD analysis

Graham F. CAMPBELL[1], John A. LUCAS[2] and Pedro W. CROUS[1]*

Department of Plant Pathology, University of Stellenbosch, P. Bag X1, Matieland 7602, South Africa.
Plant Pathology Division, IACR-Rothamsted, Harpenden, Herts, AL5 2JQ, UK.
E-mail: pwc maties.sun.ac.za

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The genetic structure of Pyrenophora teres, the causal agent of net blotch of barley, was examined in two fields 30 km apart in the south-western Cape 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 quadrants) and among population components using Nei s G_{ST}. A coefficient of genetic differentiation (G_S) of 0·0149 was obtained between quadrants 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 of the two populations together with six progeny from a 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 (Meng, Shoemaker & Yang 1999, Nyasse et al. 1999, Morris, Connolly & St Clair 2000).

Pyrenophora teres (anamorph Drechslera teres), the causal agent of net blotch disease of barley (Hordeum vulgare) is an economically important disease of this crop in South Africa and throughout most other barley production regions in the world (Steffenson, Webster & Jackson 1991). Yield losses attributed to net blotch, ranging between 26–77 %, have been reported in various countries (Deadman & Cooke 1987, Delserone & Cole 1987, Steffenson et al. 1991). Two types of leaf

* Corresponding author.

symptoms are associated with net blotch disease, namely net and spot blotch (Smedegard-Petersen 1971). P. japonica [anamorph Drechslera tuberosa] was originally described as the pathogen causing spot-type symptoms, whereas P. teres was associated with net-type lesions (Shoemaker 1962). After successful mating between net- and spot-type isolates by McDonald (1967) and Smedegard-Petersen (1971) it was concluded that the two types were forms 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 of South Africa (Louw, Crous & Holz 1996).

Under laboratory conditions, mating has been induced between net- and spot-type isolates of P. teres (Smedegard-Petersen 1976, Crous et al. 1995, Campbell, Crous & Lucas 1999). However, to date no reports have been made of net 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. 1996, Jonsson, Sall & Bryngelsson 2000). However, only populations comprising net-type isolates were evaluated in these studies. In the Western Cape 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 (Louw, Crous & Holz 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 south-western Cape 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. The Stirling field (net-type) was treated as population A, while the Clipper field (spot-type) was treated as population B. These two fields were located approximately 30 km apart in the south-western Cape of South Africa, the major barley production region. 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 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. Additional isolates included in the molecular analysis were obtained as progeny from a net- spot-type mating conducted in a previous study (Campbell et al. 1999).

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 that were then incubated at 4 C for 3–4 d. Single conidia were subsequently inoculated onto 2% potato dextrose agar (PDA) and incubated for 7 d. Cultures were maintained on PDA slants at 4 .

DNA isolation

Mycelial plugs from 7-d-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 rev min at 25 for 2 d. Further procedures were conducted as described by Campbell et al. (1999).

RAPD analysis

Amplification reactions were performed in a final volume of 25 l of reaction mixture. The reaction mixture contained 2·5 l of 10 NH_4 buffer [160 mm $(NH_4)_2SO_4$, 670 mm Tris-HCl (pH 8·8), 0·1% Tween-20) [Bioline, London; Whitehead Scientific, South Africa]; 200 m of each dNTP; 10 pmol of oligonucleotide primer, 50 ng genomic DNA and 1·0 U of BIOTAQ DNA polymerase. The MgCl 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 (Peever & Milgroom 1994), OPE 15: 5 ACGCACAACC 3 , OPE 18: 5 GGACTGCAGA 3 , OPM 10: 5 TCTGGCGCAC 3 (Operon Technologies Inc. Alameda, California, 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).

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 spot mating were excluded, as these were not 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 where $H_i = 2p_i(1-p_i)$ where p_i is the allele frequency at locus i (Crow 1986). For l loci the mean genetic diversity is given by $H = \frac{1}{l}\sum_{i=1}^{l} H_i$ (Weir 1996). Estimation of H_i was done by using the n (n−1) correction for small samples of size n (Nei 1978).

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 $G_S = (H_S - H) / H_S$ and the proportion between populations was determined using $G_T = (H_T - H) / H_T$ (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 o (np 3 8) (n 3 4) 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 & 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 dissimilarity 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. Independent DNA preparations from the same isolate used in separate PCR amplifications produced the same banding patterns.

The two populations were each represented by two quadrants within populations with 11–26 monoconidial isolates per quadrant. The proportion of polymorphic RAPD loci at each of these two quadrants 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).

The mean gene diversities (H) were 0·0625 and 0·082 in populations A and B respectively. Nei s diversity

Table 1. Polymorphism and genetic diversity (H_S^G)[a] for 65 RAPD loci between and within populations and sites of Pyrenophora teres.

	Sites				
	Population A		Population B		
	A1	A2	B1	B2	Total
Isolates	11	26	14	15	
Polymorphic loci	20 (30·8%)	9 (13·8%)	38 (58·8%)	11 (16·9%)	
H_S^G	0·1007	0·0240	0·1332	0·0302	0·0614
St. error[d]	0·0209	0·0102	0·0181	0·0101	0·0080
H_S^G[b]	0·0489		0·0794		0·0623
St. error	0·0112		0·0119		0·0081
H_T^G[c]					0·1659
St. error					0·0247

[a] H_S^G 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] H_S^G is expected gene diversity within populations calculated from mean allele frequencies averaged over sites, and then averaged over all loci.

[c] H_T^G 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 2. Analysis of variance for effects of populations, sites within populations and loci as inferred by differences in gene diversity in Pyrenophora teres.

Effect	df	Mean square	F
Populations	1	0·1394	6·6726[b]
Sites within populations	2	0·7845	37·5561[c]
Loci	64	0·0333	1·5945[b]
Loci × Populations	64	0·0401	1·9174[a]
Loci × Sites within populations (error)	128	0·0209	

[a] $P < 0.05$.

[b] $P < 0.01$.

[c] $P < 0.001$.

statistics are summarised in Table 1. 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 2) of the gene diversities indicated significant differences between the two populations and loci ($P < 0.01$) while highly significant differences were obtained for sites within populations ($P < 0.001$).

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.

A dendrogram based on simple matching coefficients amongst the field isolates from the two populations and the progeny from the net × spot mating (Campbell et al. 1999) is presented in Fig. 1. 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 (Campbell et al. 1999) 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 Pyrenophora teres populations have to date only involved net-type populations (Peever & Milgroom 1994, Jonsson et al. 2000). However, in the Western Cape of South Africa 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). In the present study monomorphic as well as polymorphic loci were taken into consideration. The gene diversities obtained by Peever & 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 & 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 pop-

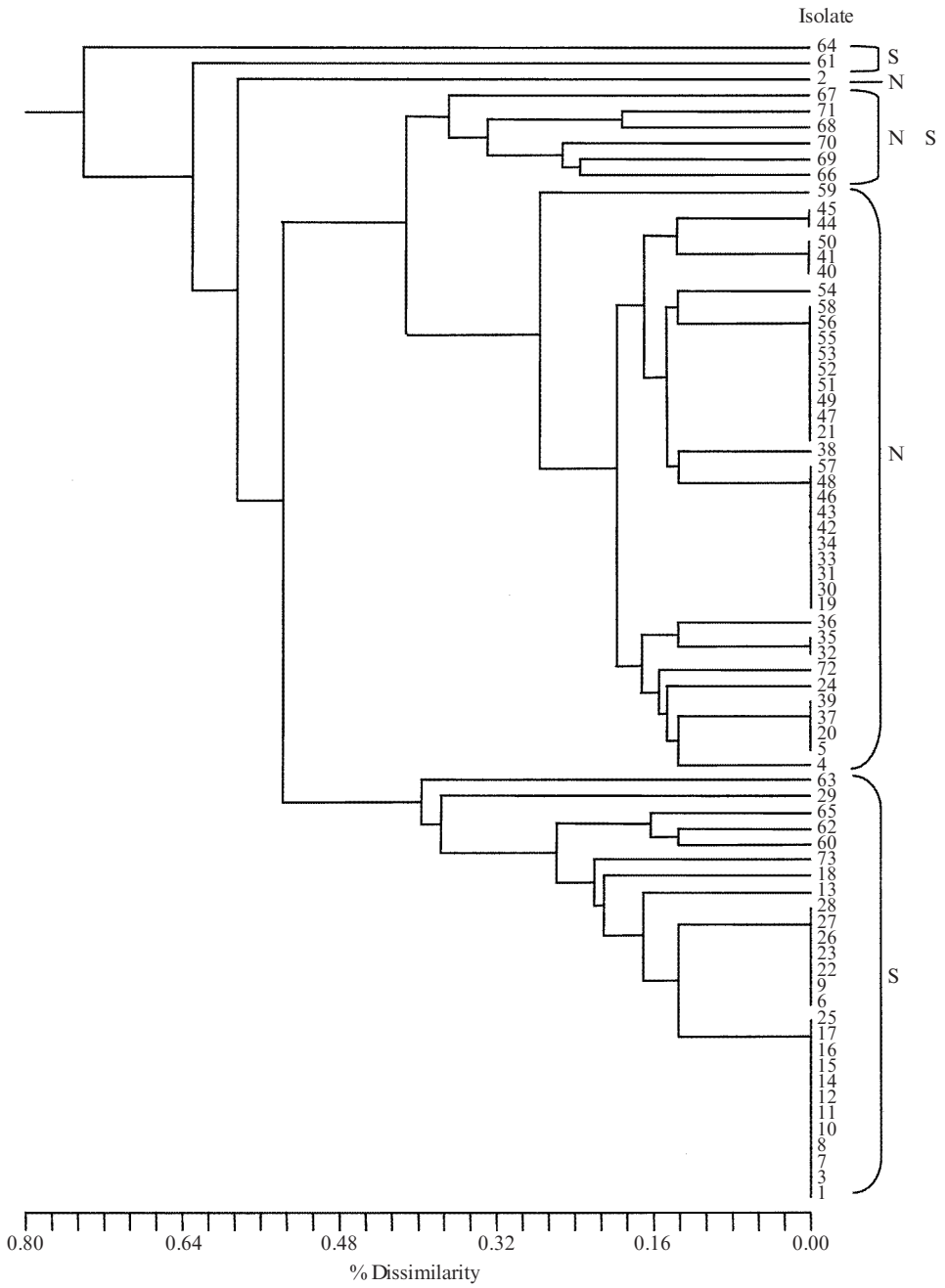

Fig. 1. Dendogram showing relationships among 73 isolates of Pyrenophora teres based on RAPD banding patterns. The dendogram was constructed from dissimilarity coefficients using UPGMA. Brackets with N or S represent net- and spot-type clusters. The N S cluster represents hybrid progeny from a net spot mating (Campbell et al. 1999).

ulations was 0·63. Peever & 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 that were only 20 km apart a value of 0·05 was obtained. This 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 from one another. Further evidence that the two types are genetically distinct is provided by conventional markers such as symptom expression on differentially susceptible cultivars (Smedegard-Petersen 1971).

The genotypic variation observed in the present study requires clarification. At one sampling site where 26 isolates were collected 10 multilocus genotype haplotypes were obtained. This is very similar to results obtained by Peever & 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 slerotiorum 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 (Smedegard-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.

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 between the two types 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 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 (Smedegard-Petersen 1976, Campbell et al. 1999), variation within spot-type symptoms incited by P. teres f. maculata (Tekauz & Mills 1974, Skou & Haahr 1987, Scott 1991), 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.

The sexual stage of the spot-type of P. teres has been isolated from barley fields in the western Cape of South Africa (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 population B had a distinct multilocus haplotype. 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 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 (Campbell et al. 1999), 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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