Population structure of South African and Australian *Pyrenophora teres* isolates

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There are two recognised forms of the disease net blotch of barley. The net form of net blotch is caused by *P. teres* f. *teres* (PTT) while the spot form is caused by *P. teres* f. *maculata* (PTM). In this study, amplified fragment length polymorphism analysis was used to investigate the genetic diversity and population structure of 60 PTT and 64 PTM isolates collected across Australia (66 isolates) and in the south-western Cape of South Africa (58 isolates). For comparison, *Pyrenophora tritici-repentis*, *Exserohilum rostratum* and *Bipolaris sorokiniana* samples were also included in the analyses. Both distance- and model-based cluster analyses separated the PTT and PTM isolates into two strongly divergent genetic groups. Significant variation was observed both among the South African and Australian populations of PTT and PTM and among sampling locations for the PTT samples. Results suggest that South Africa and Australia harbour different biotypes for each form of *P. teres*. The isolates collected for this study will form the basis of an on-going collection available for future studies.

Keywords: AMOVA, STRUCTURE, NTSYS, *Hordeum vulgare*, form specific markers

Introduction

Net blotch, caused by the fungus *Pyrenophora teres* Dreschsler, is a serious production problem for the barley (*Hordeum vulgare* L.) industry in Australia, South Africa and elsewhere (Campbell & Crous, 2003; Gupta *et al*., 2003; Jonsson *et al*., 2000; Leisova *et al*., 2005a; Manninen *et al*., 2000; Steffenson *et al*., 1996). Two forms of net blotch exist: one is the net form (NFNB) caused by *P. teres* f. *teres* Dreschler (PTT) and the other is the spot form (SFNB) caused by *P. teres* f. *maculata* Smed.-Pet. (PTM) (Smedegård-Petersen, 1971). Lesions of NFNB are characterised by narrow, dark brown longitudinal streaks with transverse lines, giving the lesions a net-like appearance (Parry, 1990). Lesions may be surrounded by areas of chlorosis and large areas of dead tissue can be present. Lesions of SFNB are dark brown and elliptical in shape and may be surrounded by a chlorotic halo (Parry, 1990). As it can be difficult to distinguish between spot and net form lesions a number of polymerase chain reaction (PCR) based assays have been developed that differentiate spot form and net form isolates (Keiper *et al*., 2008; Leisova *et al*., 2005b; Williams *et al*., 2001). A real-time quantitative PCR assay to differentiate between the two forms of the disease and to quantify the pathogen load in infected barley leaves has also been produced (Leisova *et al*., 2006). It has previously been suggested that recombination between the two forms can occur under field conditions (Campbell & Crous, 2003; Campbell *et al*., 2002). However, a recent study of isolates collected mainly from Sardinia has tested the patterns of sequence divergence and haplotype structure at the mating-type (*MAT)* locus of *P. teres*. The results suggest long genetic isolation between the net and spot forms of *P. teres* and that hybridization is rare or absent under field conditions (Rau *et al*., 2007). This study concluded that the two forms should be considered as different species when studying host resistance.

Although the net blotches can cause high yield losses in South Africa and have recently been observed more frequently, there have only been a relative few published studies of the disease (Campbell *et al*., 2002; Campbell & Crous, 2003; Campbell *et al*., 1999; Louw *et al*., 1995; Louw *et al*., 1996; Scott, 1991). While work to date indicates that chemical control is not always effective and requires multiple applications (Campbell & Crous, 2002), limited breeding for resistance has been undertaken. To date, no one has determined the effectiveness of *P. teres* resistant sources in South Africa nor have virulence profiles of *P. teres* been studied.

In Australia thirteen different pathotypes of *P. teres* f. *teres* have been identified among 81 isolates (Platz *et al*., 2000), whereas four pathotypes were identified when Canadian-derived barley lines were tested with eight *P. teres* f. *maculata* isolates from five geographically distinct regions, including Australia (Wu *et al*., 2003). This variability, combined with the adoption of reduced or zero tillage practices have significantly increased the incidence of spot and net form of net blotch in recent years (McLean *et al*., 2009).

The genetic structure of fungal pathogen populations is a key indicator of how rapidly a pathogen is evolving and can be used to predict how long a control measure or resistance source is likely to be effective (Campbell *et al*., 2002; MacDonald & Linde, 2002; Serenius *et al*., 2007). Numbers of studies have used molecular markers, such as amplified fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD), to investigate the genetic variation of *P. teres* isolates (Bakonyi & Justesen, 2007; Campbell *et al*., 2002; Jonsson *et al*., 2000; Leisova *et al*., 2005a; Peltonen *et al*., 1996; Rau *et al*., 2003; Serenius *et al*., 2007). Most of these studies have used distance-based clustering methods to determine the degree of variation among accessions. This matrix is then used to construct a dendogram and

accession clusters are identified manually (Pritchard *et al*., 2000). Pritchard *et al*. (2000) developed a model-based cluster method that is implemented in the software package STRUCTURE. This program infers population structure and assigns individuals to populations. The advantages and disadvantages of distance- versus model-based clustering approaches have been widely discussed (Lu *et al*., 2005; Pritchard *et al*., 2000; Stajner *et al*., 2008).

In this study, we have assembled DNA samples from 120 *P. teres* isolates collected from across South African (SA) and Australian (AUS) barley growing regions. Following AFLP analysis, both the distance- and model-based clustering methods as well as AMOVA was employed to determine the genetic diversity and population structure of the collected isolates. Genetic variation was examined within and between pathogen populations from the two geographical regions, South Africa and Australia.

Materials and methods

Fungal isolates and single spore production

Fifty-eight SA isolates were obtained from barley leaf samples collected in 2007 from the major barley growing region in the south-western Cape in an area of about 520,000 ha (Table 1). The original field identifications based on lesion appearance are listed in the second column of Table 1. Some of these SA samples were tentatively identified as showing symptoms of spot blotch, caused by *Bipolaris sorokiniana* (SB; Column 2 Table 1). Once the field identifications had been confirmed (or otherwise) by diagnostic molecular markers (see below), the SA isolates were given the prefix PTT and PTM for net form and spot form, respectively. The 66 AUS *P. teres* isolates were obtained from the Department of Employment, Economic Development and Innovation (DEEDI), Hermitage Research Station (HRS) Queensland, from Dr Hugh

Wallwork at the South Australian Research and Development Institute (SARDI) and from Dr Sanjiv Gupta at Murdoch University, Western Australia. Information on the origin, year of collection and host source of each isolate used is listed in Table 2. For comparison six *B. sorokiniana* (Sacc.) Shoemaker isolates (including isolates that were used in the study by Knight *et al*., 2009), one *Pyrenophora tritici-repentis* (Died.) Drechsler isolate (PYSSc2) and one *Exserohilum rostratum* (previously known as *Drechslera rostrata;* DROSc3) isolate were included. The AUS isolates were given the prefix NB for net form, SNB for spot form and SB for spot blotch (*B. sorokiniana*) according to the diagnostic molecular marker classification (Table 2). Surface sterilized leaf samples or sections of leaf lesions were placed on water agar plates and incubated at room temperature and normal day/night light conditions for 2-3 days for conidia production. Single conidia were transferred to 39g/l Potato Dextrose Agar (Biolab Merck) (PDA) plates supplemented with streptomycin sulphate (0.3 ml/l Solustrep) and then subcultured onto new PDA plates.

DNA extractions

Fungal mycelium was harvested from the single-spore cultures grown on PDA plates at 25°C for one week. A cetyl trimethyl ammonium bromide (CTAB) DNA extraction method was used to extract the fungal DNA (Saghai-Maroof *et al*., 1984). Extracted DNA was quantified using an Implen NanoPhotometer (Integrated Sciences). For reasons of maintaining quarantine between South Africa and Australia, fungal DNA was extracted in South Africa and sent to Australia for analysis.

Form specific marker amplification

The forms of *P. teres* were verified using the two form specific PCR markers of Williams *et al*. (2001) and seven diagnostic markers (hSPT2_4tcac, hSPT2_24tcac, hSPT2_6tcac, hSPT2_13agtg, hSPT2_4agac, hSPT2_13tcac and hSPT2_3agtg) of Keiper et al. (2008). A standard PCR protocol was used to amplify these markers (Bovill *et al*., 2009).

AFLP analysis

The AFLP procedure described by (Vos *et al*., 1995) was carried out using an AFLP Core Reagent kit (Invitrogen). The protocol provided by the supplier was followed. The *EcoR*I and *Mse*I restriction enzymes were used to restrict approximately 150 ng of DNA. After adaptor ligation a 1:10 dilution was performed using TE buffer. The dilutions were used in the pre-selective amplification with *EcoR*I (E-A or E-G) and *Mse*I (M-A or M-G) primers with one extra base. Each pre-selective amplification reaction contained 5 µl of diluted restricted-ligated DNA, 0.5 U of GoTaq® Flexi DNA Polymerase (Promega Corporation), 4 µl of 5X reaction buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, and 0.25 uM of *EcoR*I and *Mse*I primers with one selective nucleotide, in a total volume of 20 µl. The following pre-selective amplification cycling conditions were used: 20 cycles of 94°C for 30 seconds, 56°C for 1 minute and 72°C for 1 minute. These amplified fragments were then subjected to selective amplification using primers which had two extra bases (E-AA with M-AA, M-AG, M-AT and M-AC and E-GC with M-GC, M-GA, M-GT and M-GG). The *EcoR*I primers were HEX-labelled. The selective PCR contained 2 µl of pre-selective amplified DNA, 0.5 U of GoTaq® Flexi DNA Polymerase, 3 µl of 5X reaction buffer, 1.5 mM $MgCl_2$, 200µM dNTPs, and 0.25uM of *EcoR*I and *Mse*I primers with two

selective nucleotides, in a total volume of 15 µl. The selective amplification cycling conditions were: 12 cycles of 94°C for 30 seconds, 65°C for 30 seconds and 72°C for 1 minute, followed by 23 cycles of 94°C for 30 seconds, 56°C for 30 seconds and 72°C for 1 minute. Four µl of 100% formamide loading buffer was added to the amplified samples. The samples were denatured for 4 minutes at 95°C and were visualized on 6% polyacrylamide gels using a Gel-Scan 2000™ DNA fragment analyser (Corbett Life Sciences). Gels were run for 90 minutes at 2500V.

Scoring and data analysis

Both monomorphic and polymorphic bands were scored and used in the data analysis. Bands were scored independently by two people and bands which showed large differences in intensities or could not be scored accurately by both people were removed from further analysis. Amplicons produced by each primer combination were scored as binary data.

Distance-based clustering analyses

A similarity matrix was constructed using the DICE coefficient (Dice, 1945) in the Qualitative data program of the NTSYS-pc version 2.20f software package. Cluster analysis of the matrix values was performed by employing the Unweighted Pair-Group Method with Arithmetic Mean (UPGMA; Sneath & Sokal, 1973) provided in the SAHN program of NTSYS-pc and a dendrogram was produced using Tree plot. The clade support was assessed through a 300 replicate bootstrap test in WINBOOT (http://www.irri.org/science/software/winboot.asp) to define confidence intervals (Felsenstein, 1985). Nodes were considered as unsupported when bootstrap values were less than 70%.

Analysis of molecular variance (AMOVA)

Transformer-3 (Caujapé-Castells & Baccarani-Rosas, 2005) was used to convert the format of the AFLP data from the Microsoft Excel file format to the Arlequin file format. Analysis of molecular variance was computed using the software ARLEQUIN version 2.0 (Excoffier *et al*., 1992; Schneider *et al*., 2000) with 1,000 permutations. Genotypic data was partitioned into groups in order to test for genetic variation between PTT and PTM isolates overall and among (between) countries. Subsequent AMOVA was performed for each *P.teres* form to test for genetic variation among and within sampling locations. As the isolate sampling area in South Africa was much smaller compared to the isolate sampling area in Australia, the towns listed in Table 1 were used as the sampling locations for the SA samples, whereas the regions indicated in Table 2 were used as the sampling locations for the AUS samples.

Model-based clustering analyses

STRUCTURE version 2.2 (Pritchard *et al*., 2000) was used to infer the genetic structure and the number of clusters or populations (*K*) in the dataset. Initially, the analysis was carried out on the whole AFLP dataset, consisting of ten independent runs performed for each value of *K*, *K* varying from one to eleven. The default settings of the program were used, i.e. admixture ancestry model, uncorrelated allele frequencies between populations and the degree of admixture alpha inferred from the data (Pritchard & Wen 2003; Falush *et al*., 2007). Each run was set to a burn-in period of 10,000 iterations followed by 100,000 Monte Carlo Markov Chain (MCMC) iterations. As the outlier samples (*B. sorokiniana*, *P. tritici-repentis* and *E. rostratum*) interfered with the resolution of the analyses, they were omitted from further analysis.

Final STRUCTURE analysis was performed on the separate PTT and PTM AFLP data sets (PTM and PTT; as established by the form specific markers and UPGMA cluster analysis). Ten independent runs of *K*, set between one and six, were performed for each data set and to determine the consistency of the results the burn-in was increased to 50,000 followed by 500,000 MCMC iterations.

To estimate the most likely number of clusters the logarithmized probabilities of data [Pr(X|*K*] or L(K) for each value of *K* (Pritchard *et al*., 2000) was used. This was compared to the statistic delta *K* (Δ*K*), the second-order rate of change of the likelihood function with respect to *K* (Evanno *et al*., 2005). In brief, the mean difference between successive likelihood values, L'(*K*), was calculated after which the absolute values of the difference between successive values of L'(*K*), L''(*K*), were averaged over ten runs and divided by its standard deviation.

CLUMPP version 1.1.1 (Jakobsson & Rosenberg, 2007) was used to permute one output from the ten independent cluster outputs produced by STRUCTURE. Graphs were constructed in Microsoft Excel 2007.

Results

The classification of isolates as PTT or PTM according to their field identifications was verified prior to AFLP analysis by amplification with the PTT and PTM specific primers of Williams *et al*. (2001) and seven diagnostic microsatellite markers produced by Keiper *et al*. (2008). A further two diagnostic microsatellite markers from that study did not produce clear bands (hSPT2_24agac and hSPT2_5agac) and were discarded. Amplification with the selected markers indicated that four isolates (NB143, NB150, NB154 and NB160) originally identified as PTM were actually PTT (Table 2). Thirteen SA samples had been classified from leaf symptoms as *B.*

sorokiniana, but microscopic studies of spore morphology indicated that they were *Drechslera teres* (Sacc.) Shoem. (anamorph of *P. teres*) (Sivanesan, 1987). The PTM markers amplified on DNA from these accessions and they were therefore reassigned as PTM samples (Table 1). This was confirmed by AFLP analysis (see next section). The PTT and PTM specific markers did not amplify on samples SB230 and SB170 from Australia and AFLP analysis confirmed that these samples were not *P. teres* isolates as they clustered with the *B. sorokiniana* isolates (see next section).

AFLP analysis

AFLP analysis was conducted on DNA of 23 SA and 37 AUS PTT isolates, 37 SA (including two controls discussed below) and 29 AUS isolates identified as PTM in the field, six *B. sorokiniana* isolates, one *P. tritici-repentis* and one *E. rostratum* isolate (Table 1 and 2). Eight AFLP primer combinations were used and on average 50 loci were amplified with each primer combination. In total, 400 loci could be accurately scored across all samples and 168 of these loci (42%) were polymorphic in the *P. teres* samples. Independent DNA preparations (samples PTT37#1 and PTT37#2) and independent polymerase chain reaction samples (sample PTT21) produced the same banding patterns.

Distance-based clustering analysis

The distance-based clustering analysis subdivided the 134 isolates into seven groups (Figure 1). The two main groups (I and III) contained 60 PTT and 59 PTM isolates (plus two controls), respectively. Group II consisted of one PTM isolate, SNB172, which clustered away from the main PTM group and only showed 74% similarity with the other PTM isolates. Two SA isolates (PTM28#1 and PTM63#2) formed a

cluster on their own (group IV) and their identity could not be determined. The outliers, i.e. the *P. tritici-repentis* and *E. rostratum* isolates and the six *B. sorokiniana* isolates together with the two AUS isolates originally classified as PTM (SB170 and SB230) are presented in groups V, VI and VII, respectively. Based on the coefficient of similarity, the *P. teres* isolates were only 14, 12 and 9% similar to the outliers. Within the PTT and PTM groups the similarity between individuals was very high with a minimum similarity of 90% (Figure 1). However, distinct clusters containing only SA and AUS isolates were present. All but two of the SA PTM isolates clustered together in group III. PTM32 clustered with the AUS PTM isolates and PTM21 formed a cluster on its own. The AUS PTM isolates clustered together in group III with the exception of two isolates (SNB167 and SNB222), which formed a separate cluster. The separation of the SA and AUS isolates within the PTT group was not as clear with a number of SA isolates clustering amongst the AUS isolates.

Analysis of molecular variance (AMOVA)

AMOVA revealed highly significant differences among the two forms of *P. teres*, PTT and PTM, contributing 36.0% ($P<0.0001$) of the total genetic variation (Table 3). The smallest proportion, 17.72% ($P<0.0001$), was ascribed to the country of origin (SA or AUS) while 46.28% ($P<0.0001$) variation existed within the individual *P. teres* populations.

In the inter-population AMOVA, highly significant variation (32.81%; $P<0.0001$) was observed among SA PTT samples depending on the sampling locations, whereas no significant variation was observed with the SA PTM samples (Table 3). Although the variation observed among sampling locations with the AUS PTT samples was lower than with the SA PTT samples it was still significant (8.89%; $P<0.001$) while no

significant variation was observed with the AUS PTM samples among sampling locations. Variations within sampling locations were highly significant ($P<0.001$) for PTT and PTM isolates from both South Africa and Australia (Table 3).

Model-based clustering analysis

For the model-based clustering in STRUCTURE the AFLP data was analysed separately for the PTT and PTM groups. Averaged over replicates, the log-likelihood values (as described in Pritchard *et al*., 2000) divided both the PTT and PTM isolates into three clusters as the highest values [L(*K*)] were observed at *K*=3 (Figure 2A). When the rate of variation in likelihood values between successive *K's* (ΔK statistic of Evanno *et al*., 2005) was examined, the number of clusters was subsequently reduced to two for the PTT isolates while the ad-hoc statistic also indicated that the PTM isolates were assigned to three clusters (Figure 2B). Even when the burn-in iterations were increased to 100,000 followed by 1,000,000 MCMC iterations for the PTT isolates, the highest value for ΔK was observed at *K*=2. The ad-hoc statistic method was chosen to determine the final value of *K*, as this is the recommended method to determine the number of clusters (Basset *et al*., 2006; Evanno *et al*., 2005; Stajner *et al*., 2008).

Using the model-based cluster analysis, samples were then assigned into a specific group based upon the highest percentage of membership or co-ancestry (Figure 3). Most isolates could be assigned to a specific group as they shared more than 80% common ancestry. A minority of isolates shared less than 80% similarity and were considered to be of mixed origin, i.e. they were representative of more than one group. The first group of the PTT isolates consisted solely of 24 AUS isolates, whereas the second group consisted of seven AUS and 22 SA isolates. Six AUS

(NBHRS08119, NB085, NB330, NB308, NB327 and NB321) and one SA isolate (PTT51#1) were distinctly of mixed origin (Figure 3A). In the PTM group one population consisted only of AUS isolates, the second consisted only of SA isolates and the third group consisted of only one AUS isolate (SNB172). Five AUS isolates (SNB164, SNB264, SNBHRS07033, SNB167 and SNB222) and six SA isolates (PTM66#1, PTM25, PTM67#2, PTM39#2, PTM32 and PTM21) could not be assigned to a population as they were of mixed origin (Figure 3B).

The seven PTT isolates of mixed origin (NBHRS08119, NB085, NB330, NB308, NB327, NB321 and PTT51#1) observed with the model-based (STRUCTURE) analysis were clustered amongst the other samples in the distance-based (NTSYS) analysis and could therefore not be distinguished from the rest. Four of the eleven PTM isolates (PTM67#2, SNB167, SNB222, PTM21) of mixed origin also formed a cluster to one side of the main group in the distance-based analysis (Figure 3). The other seven PTM isolates of mixed origin (SNB164, SNB264, SNBHRS07033, PTM66#1, PTM25, PTM39#2 and PTM32) clustered amongst the main group in the dendogram.

Discussion

In this study 58 SA and 66 AUS *P. teres* monoconidial isolates were investigated through AFLP analysis to establish genetic differences among and within these fungal populations. Previous studies have investigated the genetic variation of *P. teres* isolates collected from different regions all over the world (Bakonyi & Justesen, 2007; Campbell *et al*., 2002; Jonsson *et al*., 2000; Leisova *et al*., 2005a; Peltonen *et al*., 1996; Rau *et al*., 2003; Serenius *et al*., 2007). However most of these studies employed RAPD markers and did not use a large number of molecular markers in the

cluster analysis. Our study however used a model-based cluster analysis to determine the distribution of *P. teres* isolates. To our knowledge only one other study has so far employed a model-based clustering approach to determine the population structure in a fungus (Bayon *et al*., 2009).

Diagnostic markers (Williams *et al*., 2001; Keiper *et al*., 2008) were used to verify that the initial field classification of the isolates into the PTT and PTM groups was correct. This indicated that four Australian isolates had been misclassified when sampled. Furthermore the diagnostic markers amplified on thirteen SA isolates which had been tentatively classified as spot blotch (*B. sorokiniana*) indicated these accessions were all PTM. The difficulty with field identification based on visible symptoms has been recognised in other studies (Leisova *et al*., 2005a; Rau *et al*., 2003). Furthermore the two forms of *P. teres* are difficult to discriminate based on spore morphology (Crous *et al*., 1995). This suggests that diagnostic markers should be used more frequently to classify barley foliar diseases, in particular NFNB, SFNB and SB which are difficult to distinguish and can easily be mistaken for other spot-like symptoms (e.g. boron toxicity and genetic necrosis) on the leaves (Campbell *et al*., 2002).

Distance-based cluster analysis using AFLP markers separated the SA and AUS *P. teres* isolates into two distinct groups consistently identified as PTT and PTM by the diagnostic markers. A clear differentiation was observed between these groups and the other leaf pathogens used as outliers (*P. tritici-repentis*, *E. rostratum* and *B. sorokiniana*). The 14% genetic similarity between the *P. teres* and *P. tritici-repentis* isolates is similar to the 19% observed by Singh & Hughes (2006) who conducted a cluster analysis on 33 *P. tritici-repentis* isolates using two *P. teres* isolates as outliers. The similarity observed between the PTT and PTM clusters was 65%, thus clearly

separating the two forms. Within each cluster the variation was very low (minimum similarity of about 90%). Similar results have been observed in a number of other studies. For example, minimum similarities of 88% and 91% were observed within the PTT and PTM groups, respectively, in a study using RAPD analysis to determine the genetic relationship between 32 *P. teres* isolates from geographically diverse areas (Bakonyi & Justesen, 2007). In this study, the similarity between the PTT and PTM groups was also high (84%). In another study, nineteen reproducible RAPD loci were used to determine the genetic diversity of two Swedish net blotch populations each consisting of 64 monoconidial isolates. A mean similarity of 90% based on the genetic distance coefficients among all subpopulations was observed (Jonsson *et al*., 2000). In contrast, a high level of variation was observed in a study using AFLPs and the UPGMA cluster method with 37 PTT and 30 PTM isolates collected mainly from the Czech and Slovak Republics (Leisova *et al*., 2005a). The clear distinction between the two forms of *P. teres* was further confirmed by AMOVA with highly significant differences observed among the PTT and PTM groups. This suggests that sexual crossing between the two forms does not occur in these two countries and is in agreement with other studies indicating that recombination is rare between the two forms (Rau et al. 2003; Serenius *et al*., 2007). It is however in disagreement with the findings by Campbell *et al*. (2002, 2003), which indicated that sexual reproduction between the two forms is likely within SA barley-growing regions. Campbell *et al*. (2002), using RAPD analysis, identified unique net- and spot-type DNA bands in one isolate and therefore concluded that sexual recombination may be occurring between the two forms.

Significant variation was observed among the SA and AUS populations within groups (PTT and PTM) indicating that South Africa and Australia harbour different biotypes

for each form of *P. teres*. However, it is unlikely that AFLP analyses has identified variation at the pathotype level as several groupings at or above the 94% level of similarity contain isolates of distinctly different virulence spectra (data not shown). Further studies are planned to compare SA and AUS isolates using a common set of differential lines to determine the pathotype variation in SA net blotch populations.

A significant difference was also observed amongst sampling locations in which SA PTT and AUS PTT samples had been collected. In contrast, in the SA and AUS PTM samples the percentage of variation within sampling locations was much greater than among sampling locations and no significant variation was observed among SA and AUS sampling locations. A similar partitioning of genetic variation was found by Serenius *et al*. (2007) who examined 116 AUS isolates of PTT and PTM using two AFLP primer combinations (87 unique genotypes). They also found the highest genetic variation within sampling locations (fields) compared to among sampling locations. Our findings also agree with Rau *et al*. (2003) who have concluded from their study of Sardinian *P. teres* isolates that genetic divergence among PTT populations is higher than among PTM populations.

Pairwise genetic similarity values are calculated as a proportion of loci with shared alleles in distance-based approaches used in programs such as NTSYS. With this approach the number of groups identified is based on a subjective cut-off made by the user (Lu *et al*., 2005, Pritchard *et al*., 2000). The model-based clustering algorithm used in programs such as STRUCTURE identifies subgroups of accessions with distinct allele frequencies within the samples tested (Maccaferri *et al*., 2005). Whereas samples are not overlapping in the distance-based analysis, in the model-based analysis each sample is allowed to have membership in several different subgroups, with membership coefficients totalling one (Maccaferri *et al*., 2005). It has been

indicated previously that the ability of STRUCTURE to converge to a robust solution is reduced when using systems with a complex structure (Kiær *et al*., 2009; Stajner *et al*., 2008). We also found that by excluding the outliers in the STRUCTURE analysis and by sub-dividing the *P. teres* isolates into the two forms, the ability of the program to distinguish differences within the PTT and PTM groups was increased. The distance-based model is therefore useful to first identify sub-groups, which subsequently can be analysed in STRUCTURE.

The high level of genetic relatedness observed within the PTT and PTM groups in the model-based cluster analyses suggests that reproduction in these fungi is, in the main, asexual. A number of samples of mixed origin were however also identified for both forms and therefore sexual reproduction cannot be entirely excluded. Of the SA samples six PTM were of mixed origin versus only one PTT sample. This suggests that in South Africa, sexual crosses between PTM isolates may be more frequent than for PTT. The occurrence of sexual recombination in *P. teres* has been suggested by several studies in different environments (Campbell *et al*., 2002; Peever & Milgroom, 1994; Wu *et al*., 2003), while Rau *et al.* (2003) have proposed that the relative contribution of sexual and asexual reproduction varies among different environments. The AUS isolate, SNB172 was distinctly different from the other PTM isolates both in the distance- and model-based cluster analyses and needs to be further investigated. Unfortunately *P. teres* samples collected in South Africa in the past (Campbell *et al*., 2002) have not been preserved (P. W. Crous, Centraalbureau voor Schimmelcultures, Netherlands, personal communication). Samples collected for this study will form the basis of an on-going collection available for future studies.

In conclusion, the AFLP analysis of PTT and PTM isolates indicated high genetic variation among the two forms of *P. teres* as well as among the SA and AUS isolates.

AMOVA analysis indicated that genetic variation was considerably higher among sampling locations for PTT compared to PTM isolates while genetic variation was high within sampling locations for both. Overall, these results suggest that sexual reproduction/recombination between the two forms is unlikely; isolates are most probably specific to a geographical region they occur in; and reproduction within the PTT and PTM groups occurs mainly asexually.

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Table 1 South African *P. teres* isolates collected in 2007 in the south-western Cape

Table 2 Sources of Australian isolates

Table 3 Analysis of molecular variance (AMOVA) of *P. teres* populations

Figure 1 Dendogram produced using the UPGMA cluster analysis. The bootstrap values of the subgroups are indicated

Figure 2 STRUCTURE analyses. The most likely number of clusters (*K*) for PTT and PTM according to A) the mean ln probability values [L(*K*) estimated over ten independent runs for each value of *K* and B) values of Δ*K* calculated for each *K*

Figure 3 Population structure and UPGMA clustering of 120 South African and Australian PTT (A) and PTM (B) isolates. The estimated population structure is indicated on the left. Each individual is represented by a horizontal line while colours are representative of the inferred populations. Clusters produced by UPGMA are indicated on the right. Samples of mixed origin are boxed

Table 1.

Isolate	Symptoms[a]	Town/Sampling location
PTT02	PTT	Waenshuiskrans
PTT04	PTT	Waenshuiskrans
PTT07	PTT	Waenshuiskrans
PTT09	PTT	Waenshuiskrans
PTT10	PTT	Waenshuiskrans
PTT17	PTT	Waenshuiskrans
PTT50#2	PTT	Bredasdorp
PTT51#1	PTT	Bredasdorp
PTT52#1	PTT	Bredasdorp
PTT53#2	PTT	Bredasdorp
PTT54#2	PTT	Bredasdorp
PTT75	PTT	Riviersonderend
PTT77	PTT	Riviersonderend
PTT78	PTT	Riviersonderend
PTT82#2	PTT	Heidelberg
PTT83#2	PTT	Heidelberg
PTT84#2	PTT	Heidelberg
PTT86#1	PTT	Heidelberg
PTT87#2	PTT	Heidelberg
PTT88#2	PTT	Heidelberg
PTT89#2	PTT	Caledon
PTT90#2	PTT	Caledon
PTT92#1	PTT	Caledon
PTM12#2	SB	Waenshuiskrans
PTM14	SB	Waenshuiskrans
PTM15#1	SB	Waenshuiskrans
PTM18	SB	Waenshuiskrans
PTM19	SB	Waenshuiskrans
PTM21	SB	Waenshuiskrans
PTM22	SB	Waenshuiskrans
PTM23	SB	Waenshuiskrans
PTM24	SB	Waenshuiskrans
PTM25	SB	Waenshuiskrans
PTM26	SB	Waenshuiskrans
PTM27#1	SB	Waenshuiskrans
PTM28#1	SB	Waenshuiskrans
PTM32	PTM	Riviersonderend
PTM37#1	PTM	Swellendam
PTM37#2	PTM	Swellendam
PTM38	PTM	Swellendam
PTM39#2	PTM	Bredasdorp
PTM41#1	PTM	Bredasdorp
PTM55#2	PTM	Waenshuiskrans
PTM56#2	PTM	Waenshuiskrans
PTM57#1	PTM	Waenshuiskrans
PTM58#2	PTM	Waenshuiskrans
PTM59#2	PTM	Waenshuiskrans
PTM62#2	PTM	Waenshuiskrans
PTM63#2	PTM	Waenshuiskrans
PTM64#1	PTM	Waenshuiskrans
PTM65#2	PTM	Waenshuiskrans
PTM66#1	PTM	Waenshuiskrans
PTM67#2	PTM	Waenshuiskrans
PTM68#1	PTM	Waenshuiskrans
PTM69#2	PTM	Waenshuiskrans
PTM70	PTM	Waenshuiskrans
PTM71	PTM	Waenshuiskrans
PTM72	PTM	Waenshuiskrans
PTM73	PTM	Waenshuiskrans

[a]Symptoms observed on barley leaves

Table 2.

Isolate	Symptoms[a]	Region/Sampling location	State	Town	Year	Host
NB022	PTT	North	QLD	Allora	1977	Barley
NB032	PTT	North	QLD	Kingsthorpe	1984	Barley
NB034	PTT	North	QLD	Boodua	1989	Barley
NB050	PTT	North	QLD	Gatton	1994	Barley
NB073	PTT	North	QLD	Tansey	1995	Barley
NB077	PTT	North	QLD	Chinchilla	1995	Barley
NB085	PTT	North	QLD	Gatton	1995	Barley
NB321	PTT	North	NSW	Moree	1999	Barley
NB330	PTT	North	NSW	Moree	2003	Barley
NB07067	PTT	North	NSW	Bithramere	2007	Barley
NB052B	PTT	South	SA	Rendelsham	1994	Barley
NB053	PTT	South	SA	Narracoorte	1994	Barley
NB223	PTT	South	SA	Pinery	1996	Barley
NB308	PTT	South	SA	Warooka	1998	Barley
NB323	PTT	South	SA	Freeling	2000	Barley
NBSA21/08	PTT	South	SA	Halbury	2008	Barley
NBSA25/08	PTT	South	SA	Balaklava	2008	Barley
NBSA32/98	PTT	South	SA	Mallala	1998	Barley
NBSA49/07	PTT	South	SA	York Peninsula	2007	Barley
NBSA55/07	PTT	South	SA	Freeling	2007	Barley
NBHRS08119	PTT	South	SA	York Peninsula	2007	Barley
NB127	PTT	South	VIC	Woomelong	1996	Barley
NB188	PTT	South	VIC	Charlton	1996	Barley
NB327	PTT	South	VIC	Horsham	2001	Barley
NB029	PTT	West	WA	Wogan Hills	1985	Barley
NB023	PTT	West	WA	Badgingarra	1976	Barley
NB026	PTT	West	WA	New Norcia	1978	Barley
NB063	PTT	West	WA	15km N of Williams	1994	Barley
NB090	PTT	West	WA	Wongan Hills	1995	Barley
NB130	PTT	West	WA	Toodyay	1995	Barley
NB132	PTT	West	WA	Wongan Hills	1995	Barley
NB335	PTT	West	WA	Wongan Hills	2008	Barley
NB336	PTT	West	WA	South Perth	2008	Barley
NB143	PTM	West	WA	Merridin	1995	Barley
NB150	PTM	West	WA	33 km E of Lake Grace	1995	Barley
NB154	PTM	West	WA	22 km N of Nyabing	1995	Barley
NB160	PTM	West	WA	25 km N of Katanning	1995	Barley
SNB06022	PTM	North	QLD	Jambin	2006	Barley
SNB247	PTM	North	QLD	Brookstead	1996	Barley
SNB74S	PTM	North	QLD	Millmerran	1995	Barley
SNBHRS07033	PTM	North	QLD	Comet	2007	Barley
SNB05064	PTM	North	NSW	Caroona	2005	Barley
SNB264	PTM	North	NSW	Boggabilla	1996	Barley
SNB331	PTM	North	NSW	Moree	2003	Barley
SNB104	PTM	North	NSW	Brocklesby	1995	Barley
SNB175	PTM	South	SA	Arno Bay	1996	Barley
SNB222	PTM	South	SA	Pinery	1996	Barley
SNB233	PTM	South	SA	Yeelanna	1996	Barley
SNB258	PTM	South	SA	Arno Bay	1996	Barley
SNBSA2/8	PTM	South	SA	Yeelanna	1998	Barley
SNBSA5/03	PTM	South	SA	Myponga	2003	Barley
SNBSA10/97	PTM	South	SA	Maitland	1997	Barley
SNBSA24/08	PTM	South	SA	Jamestown	2008	Barley
SNBSA61/07	PTM	South	SA	Turretfield	2007	Barley
SNB049	PTM	South	VIC	Swan Hill	1993	Barley
SNB202	PTM	South	VIC	Echuca	1996	Barley
SNB131	PTM	West	WA	Goomalling	1995	Barley
SNB164	PTM	West	WA	Badgingarra	1995	Barley
SNB167	PTM	West	WA	Mt Ridley	1995	Barley
SNB171	PTM	West	WA	Palinup River	1995	Barley
SNB172	PTM	West	WA	Mt Barker	1995	Barley
SNB340	PTM	West	WA	Shenton Park	2007	Barley
SNB341	PTM	West	WA	Badgingarra	2008	Barley
SNB344	PTM	West	WA	Dumbleyung	2007	Barley

SB170	PTM	West	WA	Gairdner	1995	Barley
SB230	PTM	South	SA	Cummins	1996	Barley
SB08014	SB	North	QLD	Acacia Plateau	2008	Triticale
SB05050	SB	North	QLD	Pilton	2005	Barley
SB60	SB	North	QLD	Hermitage	1999	Barley
SB20004	SB	North	NSW	Casino	2004	Prairie Grass
SB96#14	CRR	North	QLD	Nindigully	1996	Barley
SBA01#36	CRR	North	NSW	Bullarah	2001	Wheat
PYSSc2	PYS	North	QLD	Unknown	2008	Wheat
DROSc3	DRO	North	QLD	Unknown	2008	Barley

SB=spot blotch; CRR=common root rot; PYS=*Pyrenophora tritici-repentis*; DRO=*Exserohilum rostratum*
[a]Symptoms observed on leaves and roots (*Bipolaris* only)

Table 3.

Source of variation	Degrees of freedom	Sum of squares	Variance components	Variation (%)
Among groups (PTT vs PTM)	1	683.82	8.79	36.00**
Among populations within groups (SA vs AUS)	2	277.12	4.33	17.72**
Within populations	118	1332.91	11.29	46.28**
Among sampling locations (towns) for SA PTT	4	44.84	1.79	32.81**
Within sampling locations (towns) for SA PTT	17	61.30	3.61	67.19*
Among sampling locations (regions) for AUS PTT	2	22.85	0.51	8.89*
Within sampling locations (regions) for AUS PTT	34	177.23	5.21	91.11**
Among sampling locations (towns) for SA PTM	3	19.86	0.53	9.45ns
Within sampling locations (towns) for SA PTM	29	146.32	5.06	90.55*
Among sampling locations (regions) for AUS PTM	2	15.49	0.14	2.15ns
Within sampling locations (regions) for AUS PTM	24	155.95	6.50	97.85*

*P < 0.001; **P < 0.0001; ns = not significant

Figure 1

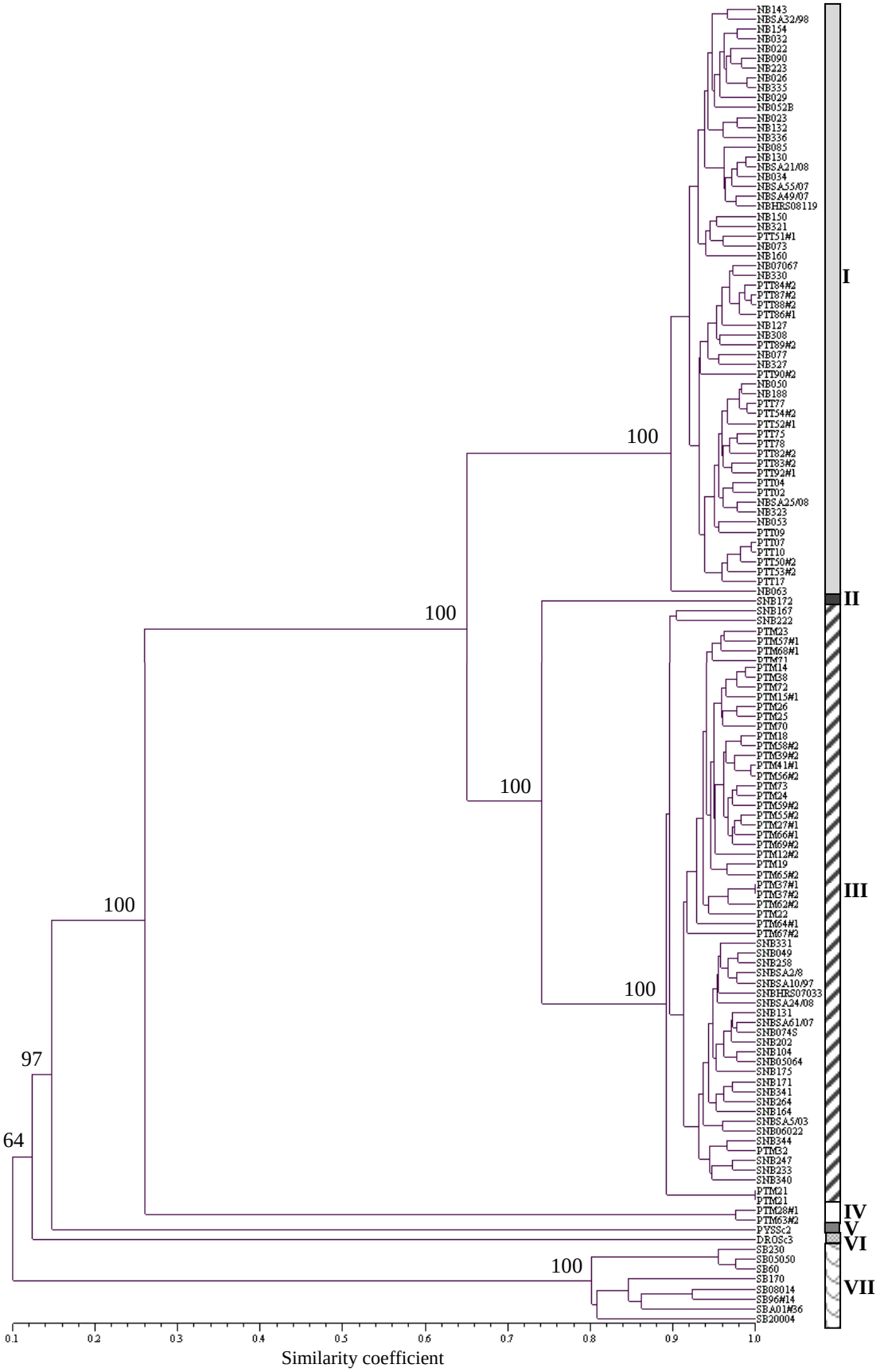
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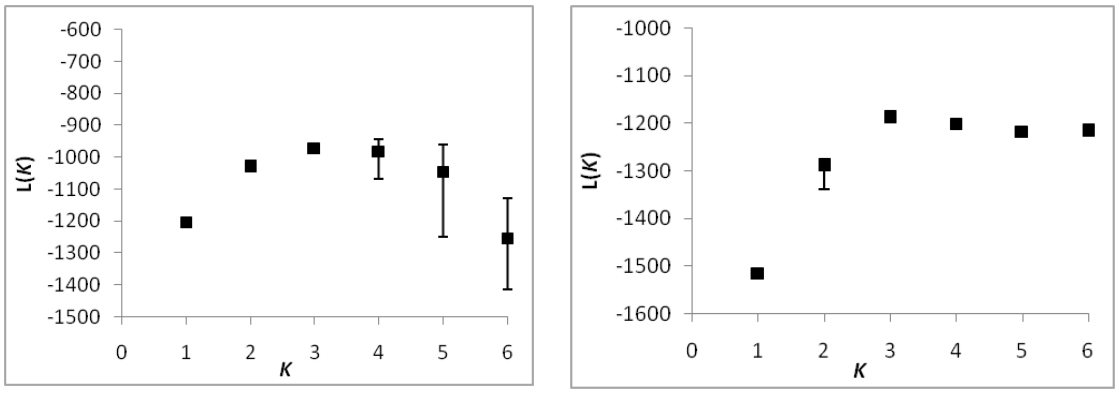
Figure 2

A) PTT PTM



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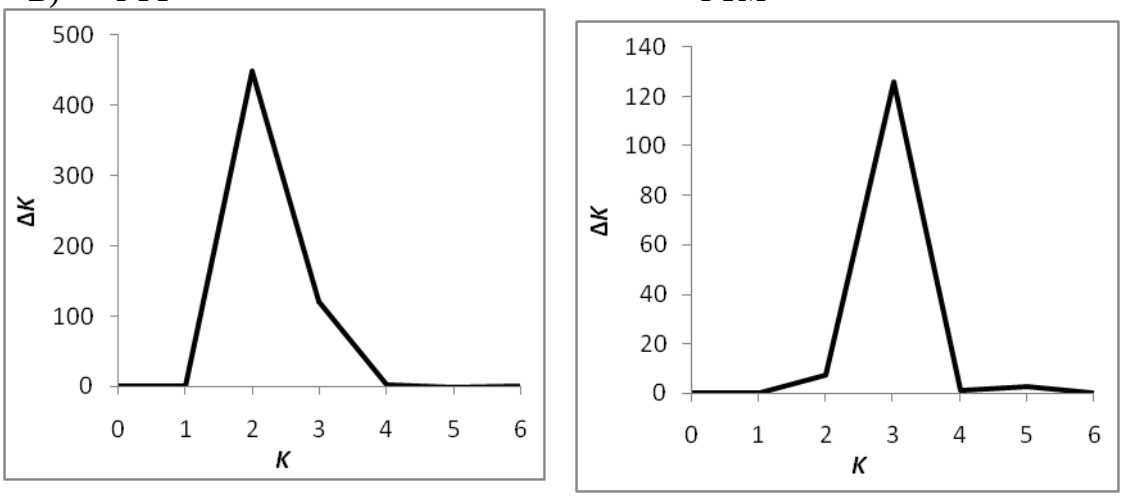
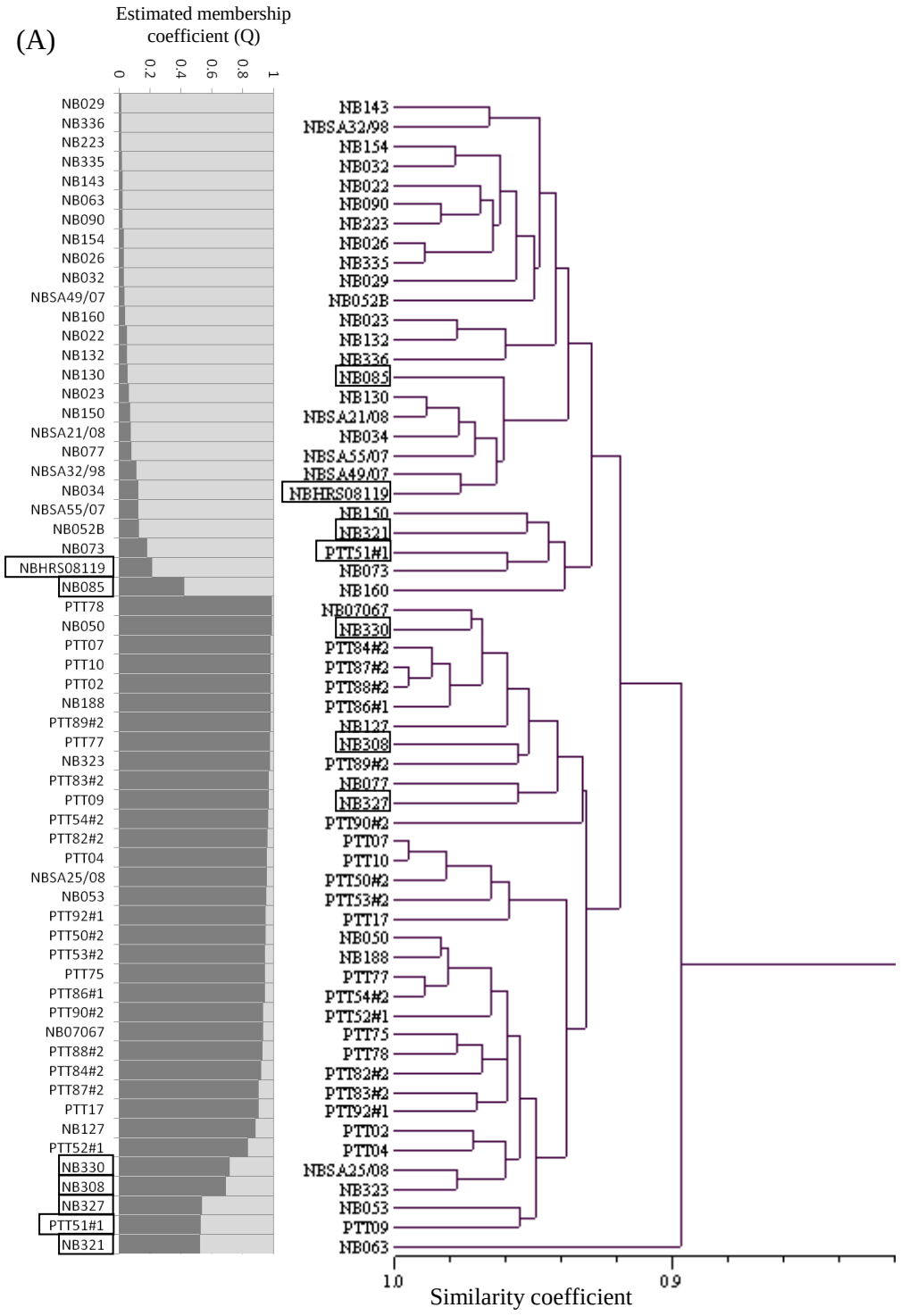


Figure 3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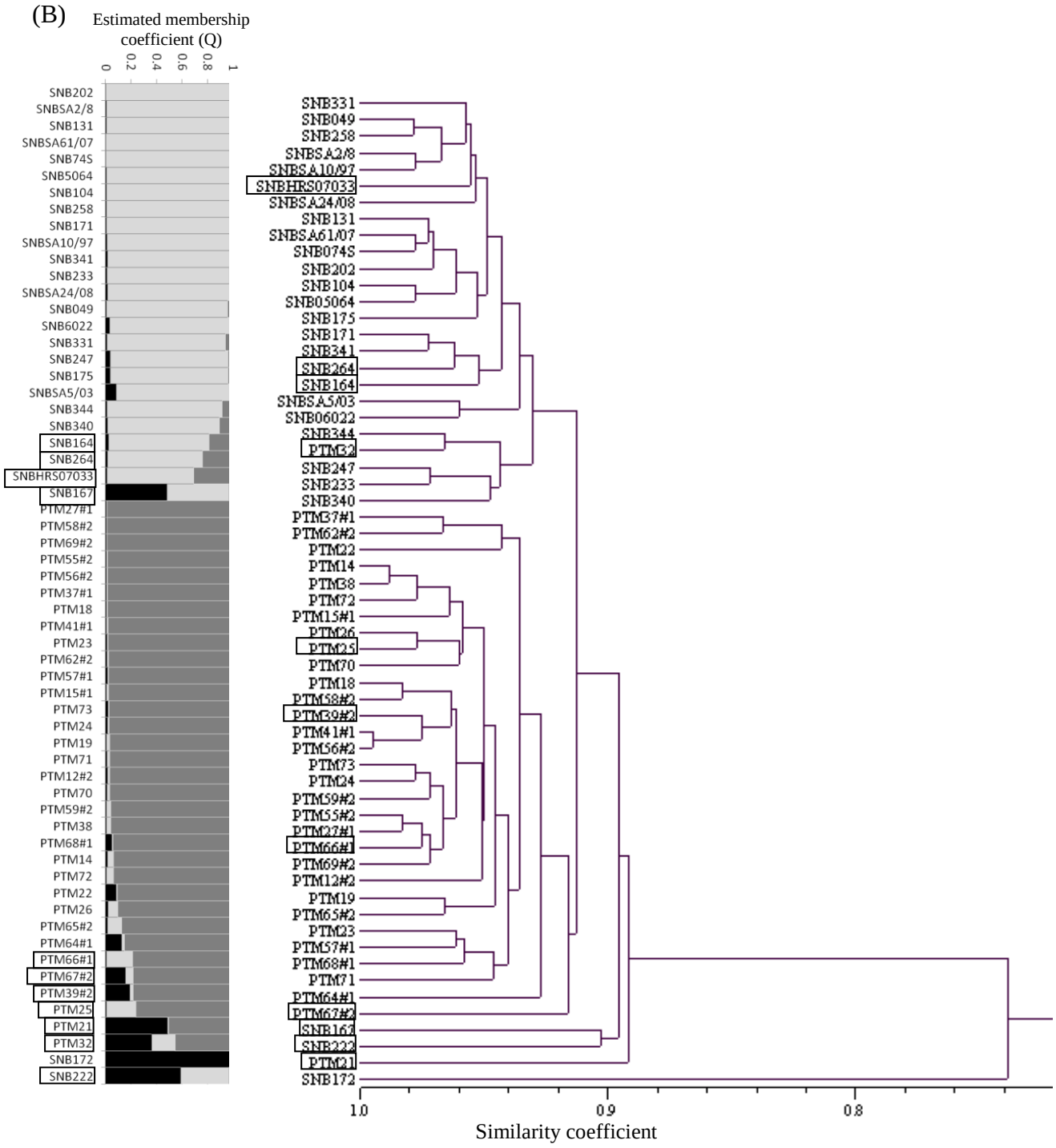
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Similarity coefficient