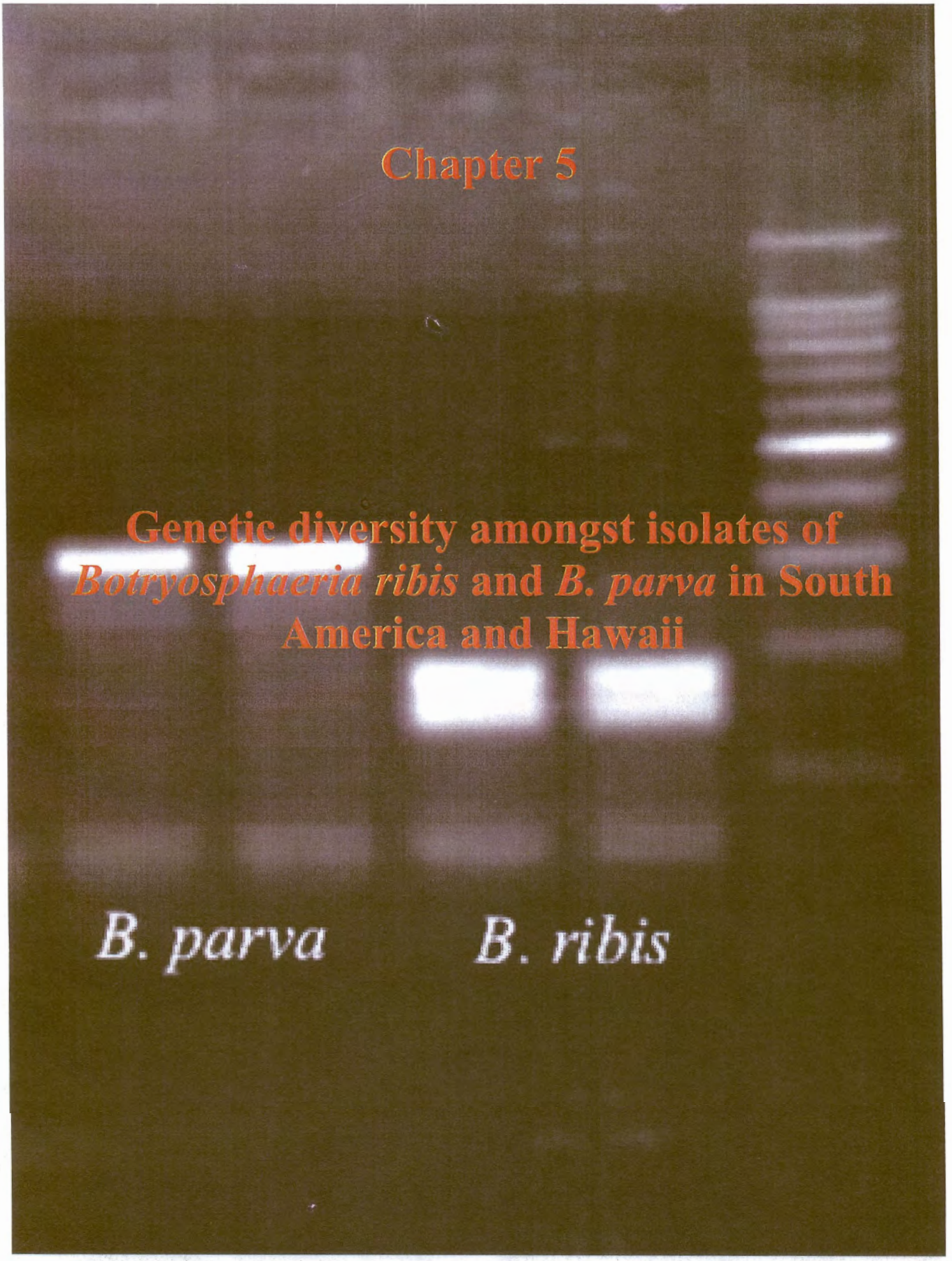
Chapter 5

Genetic diversity amongst isolates of *Botryosphaeria ribis* and *B. parva* in South America and Hawaii



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Botryosphaeria parva and *B. ribis* are common and pathogenic Ascomycete fungi on woody plants including *Eucalyptus* spp., in native forests and plantations around the world. These fungi form part of a complex of cryptic species for which the taxonomic boundaries are unclear. In this study, simple sequence repeat (SSR) markers were used to evaluate the genetic diversity within and between populations of *B. ribis* and *B. parva* from Venezuela, Colombia and Hawaii. Isolates were initially identified based on a PCR RFLP marker, reflecting a fixed single nucleotide polymorphism (SNP) between the species. Distance analyses of the SSR data generally separated these species consistent with separations based on the PCR RFLP marker. For the 59 isolates used (34 multilocus SSR genotypes), there were only three exceptions where isolates identified by PCR RFLP as *B. ribis*, grouped with *B. parva* based on SSR analyses. Whether this result reflects plasticity at the interface of contact and divergence between the two species, or hybridization events, the unfixed status of the SNP used for the initial identification or homoplasy in the SSR data, is not clear. There was no differentiation between the Venezuelan and Colombian populations of *B. ribis*. The data also indicate that these populations mainly reproduce asexually or are homothallic. In contrast, populations of *B. parva* from Colombia and Hawaii were significantly differentiated and alleles were randomly associated, suggesting random (most likely heterothallic) mating.

INTRODUCTION

The genus *Botryosphaeria* Ces. & De Not. occurs worldwide on a large number of mainly woody plants. These fungi are known to occur on dead or dying stems, branches and leaves of plants (saprophytes) or as endophytes within healthy plant tissue (von Arx, 1987; Barr, 1987; Smith *et al.*, 1996). Some species are also known to cause serious canker and dieback diseases when the plants are under stress (von Arx, 1987; Old, 2000; Old & Davison, 2000).

DNA based taxonomic tools have been successfully used to identify some *Botryosphaeria* species and to resolve their phylogenetic relationships, especially when combined with morphologic characters (Jacobs & Rehner, 1998; Crous & Palm, 1999; Denman *et al.*, 2000, 2003; Zhou & Stanosz, 2001; Phillips *et al.*, 2002; Slippers *et al.*, 2004b; Alves *et al.*, 2004). For example, analysis of sequence data for the ITS region of the nuclear rDNA has supported the separation of *B. ribis* Grossenb. & Duggar. from *B. dothidea* (Moug.:Fr.) Ces. & De Not., which was a controversial and important taxonomic problem for many years (Jacobs & Rehner, 1998; Smith & Stanosz, 2001; Smith *et al.*, 2001). Despite the fact that ITS sequence data have supported the separation of some *Botryosphaeria* species, this single DNA locus has not been sufficient to resolve boundaries for all species. Thus, closely related species such as *B. ribis* and *B. parva* Pennycook & Samuels have not been separated with confidence based on ITS, β-tubulin, translation elongation factor 1-α (EF 1-α) or mtSSU rDNA data alone (Smith & Stanosz, 2001; Zhou & Stanosz, 2001; Slippers *et al.*, 2004b).

Internal Simple Sequence Repeat (ISSR) markers have supported the differentiation of *B. parva* and *B. ribis*, but the phylogenetic significance of this finding was uncertain (Zhou *et al.*, 2001). The combination of sequence data from multiple gene regions (the ITS, β-tubulin and EF 1-α), however, confirmed the separation of these species, at least when considering

ex-type isolates (Slippers *et al.*, 2004b). Comparisons of sequence data for these regions has also been used to identify other cryptic *Botryosphaeria* spp. from trees in eastern Australia and South Africa (Slippers *et al.*, 2004c, d).

Botryosphaeria ribis and *B. parva* are well-known pathogens of forest tree species, including *Eucalyptus* spp. (Frezzi, 1952; Davison & Tay, 1983; Shearer *et al.*, 1987; Slippers *et al.*, 2004c). However, in a wide survey of *Botryosphaeria* spp. on *Eucalyptus* spp. growing in plantations and native environments in eastern Australia or as exotics in South Africa, the presence of *B. ribis* could not be confirmed (Slippers *et al.*, 2004b, c). A recent survey in Venezuela and Colombia, however, revealed that both *B. parva* and *B. ribis* occur on *Eucalyptus* in these countries (Chapter 3; Slippers unpublished data). Pathogenicity tests with these fungi on commercially propagated clones of *Eucalyptus* trees, indicated that *B. ribis* and *B. parva* were more pathogenic than other *Botryosphaeria* spp. (Rodas 2003; Chapter 6). Both species produced lesions and cracks in the bark, and black kino exudation from the points of inoculation. Furthermore, *B. parva* was more virulent giving rise to lesions almost double the length of those associated with *B. ribis* (Chapter 6).

SSR markers have been used to determine the gene and genotypic diversity, kinship, gene flow, reproductive mode and differentiation between populations, as well the differentiation between species morphotypes (Queller *et al.*, 1993; Taylor *et al.*, 1999). SSR markers also have recently been used to study populations of some *Botryosphaeria* species, such as *Diplodia pinea* (Desm.) J. Kickx., *D. scrobiculata* De Wet, Slippers & M. J. Wingf., and *Botryosphaeria rhodina* (Cooke) Arx (Burgess *et al.*, 2001a, 2003, 2004a, 2004b; De Wet *et al.*, 2003). These SSR markers that were developed for *Botryosphaeria* spp. with *Diplodia*-like anamorphs did not prove useful for population studies of *Botryosphaeria* spp. with *Fusicoccum*-like anamorphs (Slippers *et al.*, 2004a). Slippers *et al.* (2004a) thus

developed SSR markers that could be used in populations of *B. parva*, and these were also shown to be useful for nine other *Botryosphaeria* species with *Fusicoccum*-like anamorphs.

In this study, the SSR markers developed by Slippers *et al.* (2004a) are applied to isolates representing populations of *B. ribis* from Venezuela as well as *B. ribis* and *B. parva* from Colombia and Hawaii to determine their genetic diversity and distinction, and mode of reproduction. Additionally, the markers were applied to consider the possible spread of the populations or species and between these areas.

MATERIALS AND METHODS

Fungal isolates

Fifty-nine isolates of *Botryophaeria ribis* and *B. parva* from Venezuela, Colombia and Hawaii were choosen for this study. Sixteen isolates from *Eucalyptus urophylla* S.T. Blake x *E. grandis* W. Hill: Maiden hybrids growing in two locations of Venezuela (the states of Portuguesa and Cojedes) were made from asymptomatic plant tissue as well as from trees exhibiting blue stain and die-back, or entirely dead trees. Eighteen isolates originated from *E. grandis* in Hawaii and twenty-five isolates originated from *E. urophylla* in Cauca Valle and Andina zones, Colombia.

The fungi were isolated and single spore isolates were made as described by Slippers *et al.* (2004b). All isolates used in this study are maintained in the collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

DNA extraction, RFLP and SSR-PCR analysis

DNA was extracted from mycelium following the protocol of Raeder and Broda (1985) with some modification. These modifications were similar to those described by Slippers *et al.* (2004a). Nucleic acids were quantified using a spectrophotometer with an absorbance at 260 nm and 280 nm.

Restriction enzyme patterns for an unidentified DNA region-Locus *BotF15* (Slippers *et al.*, 2004a) were used to define the isolates belonging to either *B. ribis* or *B. parva* that are considered cryptic species residing in the *B. ribis-B. parva* complex. The DNA fragment was amplified using the primers BOT 15 and BOT 16 and the PCR reaction mixtures were made as described by Slippers *et al.* (2004a). The amplified fragments were digested with the RE *Cfo*I as described above. The RFLP reaction was incubated at 37 °C overnight and the restriction fragments separated on 2 % (w/v) agarose gels stained with ethidium bromide and visualized under UV light. The sizes of the PCR fragments were estimated as described above.

SSR-PCR was performed on all isolates with seven fluorescently-labelled markers, specifically designed to amplify polymorphic regions within isolates of the *B. parva-B. ribis* complex. The protocols used were as described previously by Slippers *et al.* (2004a). Fluorescently-labelled SSR-PCR products were diluted and one µl of the dilution was mixed with a LIZ^{TM} internal size standard (Perkin-Elmer Applied Biosystems, Foster City, CA) and formamide mix (1:14). The products were separated on an ABI PRISM 3100 automated sequencer (Perkin-Elmer Applied Biosystems). Allele size was estimated by comparing the mobility of the SSR products as determined by GeneScan 2.1 analysis software (Applied Biosystems) in conjunction with Genotyper 3.0 (Applied Biosystems). A reference sample was run on every gel to ensure reproducibility.

Gene and genotypic diversity

For each isolate, a data matrix of multistate characters was compiled by assigning a different letter to each allele at each of the 7 polymorphic loci (eg. ABBCDEE). The frequency of each allele at each locus for complete and clone corrected populations was calculated, and allele diversity determined, using the program POPGENE (Yeh *et al.*, 1999) and the equation $H = 1-\sum x_k^2$, where x_k represents the frequency of the k^{th} allele (Nei, 1973).

Each genotype was assigned a number and the genotypic diversity (G) was estimated (Stoddart & Taylor, 1988) using the equation $G = 1/\sum p_i^2$ where p_i is the observed frequency of the i^{th} phenotype and compared to genotypic diversity expected for the null hypothesis of random mating. To compare G between populations, the maximum percentage of genotypic diversity was obtained using the equation $\hat{G} = G/N * 100$ where N is the population size (Chen *et al.*, 1994). Chi-square tests for differences in allele frequencies were calculated for each locus across clone corrected populations (Workman & Niswander, 1970).

Genetic distance

Genetic distance between populations based on microsatellite data was calculated using D_{AD}, a statistic based on absolute distance. Data were represented as total nucleotide length of the locus. The distance between isolates was calculated using the program MICROSAT (Minch *et al.*, 1995) and Neighbor-joining trees constructed in MEGA3 (Kumar *et al.*, 2004).

Mode of reproduction

The index of association (I_A) was used as a measure of the degree of association between loci or multilocus linkage disequilibrium for each population and for all populations combined (Maynard Smith *et al.*, 1993). I_A was calculated for the clone corrected population containing only one representative of each genotype. The tests were performed on a data matrix of seven

multistate characters using the program Multilocus (Agapow & Burt, 2001). The expected data for 1000 randomly recombining data sets was calculated and compared with the observed data. Where the observed data fall within the distribution range of the recombined data, then the hypothesis that the population was undergoing recombination cannot be rejected. If the observed data fall outside the distribution range with a significant P value ($P<0.05$), the population is most likely clonal.

RESULTS

Segregation of SSR alleles

The seven loci examined by the SSR markers produced a total of 44 alleles (Table 1). There were 17 alleles in the Venezuela population, 34 alleles for Colombia (20 alleles for *B. ribis* and 14 for *B. parva*) and 20 alleles for Hawaii isolates. Only one allele, (2.3 %) of the total of all alleles, was shared between populations (Table 1). Within each of these populations unique alleles were present. The Venezuelan population had four unique alleles (9.1 %); for the Colombia population, *B. ribis* had five unique alleles (11.4 %) and *B. parva* had three unique alleles (6.8 %); for the Hawaii population, *B. ribis* had two unique alleles (4.6 %) and *B. parva* 11 unique alleles (25 %) (Table 1).

Gene and genotype diversity

All populations showed a moderate genotypic diversity ($\hat{G}$ %); 32% for *B. ribis*, Venezuela population (eight genotypes), 39 % for Colombia (five genotypes); 36 % for *B. parva*, Colombia (nine genotypes) and 50 % for Hawaii (11 genotypes) (Table 2). There were no genotypes shared among the different populations studied for either *B. ribis* or *B. parva* (Table 2).

The gene diversity (H) for each of the clone corrected populations was 0.35 for Venezuela (*B. ribis* populations), 0.50 for Colombia (for both *B. ribis* and *B. parva* populations) and 0.37 for Hawaii (*B. parva* populations).

Contingency χ^2 test indicated no significant differences ($P<0.05$) in allele frequencies at any loci for the Venezuelan and Colombian populations of *B. ribis*, except the loci *BotF37* (Table 3). This is reflected in the lack of population differentiation, indicating a high gene flow between the different populations.

The Chi-square test indicated significant differences ($P<0.01$ and <0.001) in allele frequency between the Colombia and Hawaii populations of *B. parva* (Table 4). Four loci from seven polymorphic SSR were highly significant (*BotF17, 21, 24, 35*), showing differentiation between *B. parva* populations from Colombia and Hawaii, indicating low levels of gene flow.

Genetic distance

The cluster analysis with the neighbour-joining tree revealed the genetic diversity among 59 *B. ribis* and *B. parva* isolates and two *B. ribis* isolates from New York, (which were used to allow comparison to the type isolates for the species). The dendrogram was constructed based in the seven loci examined by the SSR markers (Fig. 1). The dendrogram separated isolates into two distinct clades, one containing *B. ribis* isolates and the other containing *B. parva* isolates. Two purported *B. ribis* isolates from Venezuela fell into the *B. parva* clade. The *B. parva* clade was further divided into two distinct groups, one for Colombia and one for Hawaii, thus reflecting observations of low gene flow between these populations.

Mode of reproduction

The I_A for the observed data differed significantly from the values obtained for the recombined data set for all the individual *B. ribis* and *B. parva* populations (Fig. 2A, B; Fig.

3). This indicates linkage of alleles between different loci and suggests a predominantly sexual mode of reproduction in *B. parva* from Colombia and Hawaii. This is in contrast to an apparently asexual (clonal) mode of reproduction for *B. ribis* from Venezuela and Colombia.

DISCUSSION

The primers develop by Slippers *et al.* (2004a) successfully amplified SSR containing markers in the populations of *B. parva* and *B. ribis* used in this study. These markers allowed the distinction between these two cryptic species, as well as for significant resolution of population diversity, population separation and reproductive mode in these fungi.

The total number of alleles shared between isolates identified as *B. parva* or *B. ribis* was very low (2.3 %). A neighbour-joining tree reflecting the genetic diversity among the 59 *B. ribis* and *B. parva* isolates from the three populations, separated the isolates into two well defined groups; one group corresponding to *B. parva* and the other one to *B. ribis*. These results support the separation of the taxa as independent species. This separation also has been confirmed in other studies using other techniques such as ISSR markers, combined gene genealogies of the ITS, EF 1-α and β-tubulin loci and PCR-RFLP fingerprints of part of the LSU and ITS regions (Smith & Stanosz, 2001; Slippers *et al.*, 2004b; Alves *et al.*, 2005).

The *B. ribis* and *B. parva* groups identified in the neighbour-joining tree reflect the initial identification of isolates using the PCR RFLP fingerprint of locus *BotF15*, with the exception of three isolates. This microsatellite containing region has a unique restriction site for *B. ribis* based on a single nucleotide polymorphism (Slippers *et al.*, 2004a). The three exceptions were for isolates identified by the PCR RFLP technique as *B. ribis* (one from Hawaii and two from Venezuela), but that grouped as *B. parva* using the multilocus SSR markers. Whether these exceptions reflect plasticity at the interface of contact and divergence

between the two species, hybridisations events across the species boundary, the unfixed status of the SNP used for the initial identification, or homoplasy in the SSR data, is not clear and will be the subject of future investigations. It does, however, suggest that the *Cfo*I restriction profile for locus *BotF15* is relatively reliable for rapidly separating the cryptic species *B. ribis* and *B. parva*, which are otherwise, difficult to distinguish from each other.

The results emerging from the calculation of Index of Association (Taylor *et al.*, 1999) gave the appearance of sexual reproduction for *B. parva* populations (alleles randomly associated) and clonal reproduction for *B. ribis* populations (alleles non-randomly associated). No teleomorphs structures were observed for either *B. ribis* and *B. parva* collections used in this study. However, these fungi are known to exhibit both sexual and asexual reproduction (Grossenbacher & Duggar, 1911; Pennycook & Samuels, 1985; Slippers *et al* 2004a). This result could imply that *B. parva* is heterothallic and *B. ribis* homothallic although this seems unlikely for such closely related. Alternatively, is possible that *B. ribis* relies more strongly on asexual spores for dispersal and infection than *B. parva* on *Eucalyptus* in these environments. This could arise from the absence of one mating type if the fungus is heterothallic or from environmental factors that are not conducive to sexual reproduction. It is also possible that both fungi are homothallic, but that *B. parva* has also retained the ability to outcross. *Botryosphaeria parva* populations from Colombia and Hawaii had a higher genotypic diversity than *B. ribis* populations from Venezuela, Colombia or Hawaii. Both populations of *B. parva* from Colombia and Hawaii had a moderate level of diversity and sexual reproduction reflecting the behaviour of an outcrossing fungus. Slippers (2003) hypothesized that this fungus is homothallic, based on observations in cultures of *B. eucalyptorum* and a low level of diversity in populations of *B. parva* from South Africa despite the presence of sexual fruiting structures. If this fungus is homothallic, then it has retained the ability to occasionally outcross which would be similar to the situation in for

example *Cryphonectria parasitica* (Milgroom *et al.*, 1993; Marra & Milgroom, 2001). Alternatively, this could be a heterothallic fungus, of which only a limited number of genotypes occur on *Eucalyptus*, coupled to preferential reproduction by asexual spores on this host. These results reflect previous studies where a combination of clonal and sexual reproduction has been shown to significantly influence fungal population structure (Taylor *et al.*, 1999).

The *B. parva* populations showing recombination within each population and a high degree of differentiation, suggests that they have been separated geographically for some time (Burgess *et al.*, 2004a; Barnes, *et al.*, 2005). These populations might thus be native to each of the regions where they have been collected and that they have moved onto *Eucalyptus*. Alternatively, multiple introductions of *B. parva* into Hawaii and Colombia from different sources could also explain the result. Irrespective of the reason, the diversity and apparent sexual reproduction in these countries might aid the fungus in overcoming resistance in *Eucalyptus* clones. This adds to the importance of *B. parva* as one of the primary targets for disease screening, given its high virulence (see Chapter 6).

The *B. ribis* populations appeared to be predominantly clonal with a very similar structure in both Venezuela and Colombia. There was only one *B. ribis* isolate from Hawaii, so population statistics could not be done for that region. We found no geographic barriers separating populations from Venezuela and Colombia and the lack of allelic diversity in these populations could thus be due to natural gene flow between these countries (Burgess, *et al.*, 2001b; 2004b). The population differentiation is frequently related to the distance between populations. Thus, when two populations occur in areas closer to each other it is likely that they will have similar allelic frequencies (Slatkin, 1993; Linde *et al.*, 2002). However, the overlap could also be due to movement of *Eucalyptus* germplasm between Colombia and Venezuela. These results emphasize a ready exchange of pathogens between these

neighbouring countries of which forestry companies and researchers should be aware as more damaging diseases could also be exchanged.

Slippers (2003) observed some shared polymorphism between isolates of *B. ribis* and *B. parva* based on DNA sequence and SSR marker data, despite overall distinction between the species based on these data. This is similar to the results of the present study. Such shared polymorphism has been interpreted as: a) speciation is not completed and that some isolates retain the ability to interbreed, or b) not enough time has elapsed since speciation to allow full resolution of polymorphisms, or c) hybridisation between the distinct species (Taylor *et al.*, 2000; Slippers, 2003). This last possibility is important to consider. Hybridization is increased when closely related, but geographically isolated, pathogen populations come into contact (Brasier, 2000, 2001). This is possibly the case for various *Botryosphaeria* spp. (Slippers, 2003). Theoretically, this process presents an opportunity for rapid emergence of new or modified pathogens via gene flow, although it appears to be rare in nature (Brasier, 1995, 2001; Brasier *et al.*, 1998, 1999; Newcombe *et al.*, 2000; Garbelotto *et al.*, 2004). It is thus important to further test these hypotheses for *B. parva* and *B. ribis* as it will reflect on the importance of future quarantine measures to prevent pathogen movement. It might also help predict future emergence of new strains that could potentially be more pathogenic or infect new hosts.

This study was somewhat impaired by a relatively low number of isolates available. However, despite the somewhat limited number of isolates, results showed that *B. ribis* and *B. parva* appear to co-exist in South America and perhaps have different modes of reproduction. Their pathogenicity differs, but whether and how their roles in the ecosystem/plantations differ remains unclear. It will be important to continue these studies because this information can promote an improved understanding of the movement of pathogens between *Eucalyptus* growing regions, both within South America and between this

and other continents. Such knowledge could be important to help prevent pathogen movement, which might have unforeseen consequences. Furthermore it should also promote an understanding of and planning for breeding programs attempting to deal with these pathogens.

REFERENCES

Agapow, P.-M. & Burt, A. (2001) Multilocus 1.3. Department of Biology, Imperial College, Silwood Park, Ascot, UK.

Alves, A., Phillips, A. J. L., Henriques, I. & Correia, A. (2005) Evaluation of amplified ribosomal DNA restriction analysis as a method for the identification of *Botryosphaeria* species. *FEMS Microbiology Letters* 245: 221-229.

Alves, A., Correia, A., Luque, J. & Phillips, A. (2004) *Botryosphaeria corticola*, sp. nov. on *Quercus* species, with notes and description of *Botryosphaeria stevensii* and its anamorph, *Diplodia mutila*. *Mycologia* 96: 598-613.

Arx, J. A von. (1987) Plant pathogenic fungi. Beheifte zur Nova Hedwigia 87. Berlin, Germany: J. Cramer.

Barnes, I., Nakabonge, G., Roux, J., Wingfield, B. D. & Wingfield, M. J. (2005) Comparison of populations of the wilt pathogen *Ceratocystis albifundus* in South Africa and Uganda. *Plant Pathology* 54: 189-195.

Barr, M. E. (1987) Prodromus to class Loculoascomycetes. Amherst, Massachusetts: Hamilton I. Newell, Inc.

Brasier, C. M. (1995) Episodic selection as a force in fungal microevolution with special reference to clonal speciation and hybrid introgression. *Canadian Journal of Botany* 73: 1213-1221.

Brasier, C. M. (2000) The rise of the hybrid fungi. *Nature* 405: 134-135.

Brasier, C. M. (2001) Rapid evolution of introduced plant pathogens via interspecific hybridisation. *BioScience* 51: 123-133.

Brasier, C. M., Cooke, D. E. L. & Duncan, J. M. (1999) Origin of a new *Phytophthora* pathogen through interspecific hybridisation. *Proceeding of the National Academy of Science* 96: 5878-5883.

Brasier, C. M., Kirk, S. A., Pipe, N. D. & Buck, K. W. (1998) Rare interspecific hybrids in natural populations of the Dutch elm disease pathogens *Ophiostoma ulmi* and *O. novo-ulmi*. *Mycological Research* 102: 45-57.

Burgess, T. I., Gordon, T. R., Wingfield, M. J & Wingfield, B. D. (2004a) Geographic isolation of *Diplodia scrobiculata* and its association with native *Pinus radiata*. *Mycological Research* 108: 1399-1406.

Burgess, T., Wingfield, M. J & Wingfield, B. D. (2001a) Simple sequence repeat markers distinguish among morphotypes of *Sphaeropsis sapinea*. *Applied and Environmental Microbiology* 67: 354-362.

Burgess, T., Wingfield, B. D. & Wingfield, M. J. (2001b) Comparison of genotypic diversity in native and introduced populations of *Sphaeropsis sapinea* isolated from *Pinus radiata*. *Mycological Research* 105: 1331-1339.

Burgess, T., Wingfield, M. J & Wingfield, B. D. (2003) Development and characterization of microsatellite loci for the tropical tree pathogen *Botryosphaeria rhodina*. *Molecular Ecology Notes* 3: 91-94.

Burgess, T. I., Wingfield, M. J & Wingfield, B. D. (2004b) Global distribution of *Diplodia pinea* genotypes revealed using simple sequence repeat (SSR) markers. *Australasian Plant Pathology* 33: 513-519.

Chen, R. S., Boeger, J. M & McDonald, B. A. (1994) Genetic stability in a population of a plant pathogenic fungus over time. *Molecular Ecology* 3: 209-218.

Crous, P. W. & Palm, M. E. (1999) Reassessment of the genera *Botryodiplodia*, *Dothiorella* and *Fusicoccum*. *Sydowia* 51: 167-175.

Davison, E. M. & Tay, C. S. (1983) Twig, branch and upper trunk cankers of *Eucalyptus marginata*. *Plant Disease* 67: 1285-1287.

De Wet J., Burgess, T., Slippers, B., Preisig, O., Wingfield, B. D & Wingfield, M. J. (2003) Multiple gene genealogies and microsatellite markers reflect relationships between morphotypes of *Sphaeropsis sapinea* and distinguish a new species of *Diplodia*. *Mycological Research* 107: 557-566.

Denman, S., Crous, P. W., Groenewald, J. Z., Slippers, B., Wingfield, B. & Wingfield, M. J. (2003) Circumscription of *Botryosphaeria* species associated with Proteaceae based on morphology and DNA sequences data. *Mycologia* 95: 294-307.

Denman, S., Crous, P. W., Taylor, J. E., Kang, J., Pascoe, I. & Wingfield, M. J. (2000) An overview of the taxonomic history of *Botryosphaeria*, and a re-evaluation of its anamorphs based on morphology and ITS rDNA phylogeny. *Studies in Mycology* 45: 129-140.

Frezzi, M. J. (1952) Presencia de "*Botryosphaeria ribis*" en la Republica Argentina y su importancia economica. *Revista de Investigacion Agricola* 6: 247-262.

Garbelotto, M., Gonthier, P., Linzer, R., Nicolotti, G. & Otrosina, W. (2004) A shift in nuclear state as the result of natural interspecific hybridization between two North American taxa of the basidiomycete complex *Heterobasidion*. *Fungal Genetics and Biology* 41: 1046-1051.

Grossenbacher, J. G. & Duggar, B. M. (1911) A contribution to the life-history, parasitism, and biology of *Botryosphaeria ribis*. New York Agricutural Experimental Station. *Technical Bulletin* 18: 113-190.

Jacobs, K. A. & Rehner, S. A. (1998) Comparison of cultural and morphological characters and ITS sequences in anamorphs of *Botryosphaeria* and related taxa. *Mycologia* 90: 601-610.

Kumar, S. Tamura, K. & Nei, M. (2004) MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Briefings in Bioinformatics* 5: 150-163.

Linde, C., Zhan, J. & McDonald, B. A. (2002) Population structure of *Mycosphaerella graminicola*: from lesions to continents. *Phytopathology* 92: 946-955.

Marra, R. E. & Milgroom, M. G. (2001) The mating system of the fungus *Cryphonectria parasitica*: selfing and self-incompatibility. *Heredity* 86: 134-143.

Maynard Smith, J., Smith, N. H., O' Rourke, M. & Spratt, B. G. (1993) How clonal are Bacteria?. *Proceeding of the National Academic of Sciences, USA* 90: 4384-4388.

Milgroom, M. G., Lipari, S. E., Ennos, R. A. & Liu, Y.-C. (1993) Estimation of the outcrossing rate in the chestnut blight fungus, *Cryphonectria parasitica*. *Heredity* 70: 385-392.

Minch, E., Ruiz-Linares, A., Goldstein, D., Feldman, M. & Cavalli-Sforza, L. L. (1995) *Microsat: a computer program for calculating various statistics on microsatellite data.* Stanford University, Stanford, California.

Nei, M. (1973) Analysis of gene diversity in subdivided populations. *Proceeding of the National Academy of Sciences, USA* 70: 3321-3323.

Newcombe, G., Stirling, B., McDonald, S. & Bradshaw jr, H. D. (2000) *Melampsora Xcolumbiana*, a natural hybrid of *M. medusae* and *M. occidentalis*. *Mycological Research* 104: 261-274.

Old, K. M. (2000) Eucalypt diseases of complex etiology. In: Diseases and pathogens of eucalypts, by Keane, P. J., Kile, G. A., Podger, F. D. & Brown, B. N, 411-425, CSIRO Publishing, Collingwood, Australia.

Old, K. M. & Davison, E. M. (2000) Canker Diseases of *Eucalyptus*. In: Diseases and pathogens of eucalypts, by Keane, P. J., Kile, G. A., Podger, F. D. & Brown, B. N. 241-257, CSIRO Publishing, Collingwood, Australia.

Pennycook, S. R. & Samuels, G. J. (1985) *Botryosphaeria* and *Fusicoccum* species associated with ripe fruit rot of *Actinidia deliciosa* (Kiwifruit) in New Zealand. *Mycotaxon* 24: 445-458.

Phillips, A. J. L., Fonseca, F., Povoa, V., Castilho, R. & Nolasco, G. (2002) A reassessment of the anamorphic fungus *Fusicoccum luteum* and description of its teleomorph *Botryosphaeria lutea* sp. nov. *Sydowia* 54: 59-77.

Queller, D. C., Strassmann, J. E. & Hughes, C. R. (1993) Microsatellites and kinship. *Tree* 8: 285-288.

Raeder, U. & Broda, P. (1985) Rapid preparation of DNA from filamentous fungi. *Letters in Applied Microbiology* 1: 17-20.

Rodas, C. (2003) Diseases of *Eucalyptus* in Colombia. M.Sc. thesis. University of Pretoria, Pretoria, South Africa.

Shearer, B. L., Tippett, J. T. & Bartle, J. R. (1987) *Botryosphaeria ribis* infection associated with death of *Eucalyptus radiata* in species selection trials. *Plant Disease* 71: 140-145.

Slatkin, M. (1993) Isolation by distance in equilibrium and non-equilibrium populations. *Evolution* 47: 264-279.

Slippers B. (2003) Taxonomy, phylogeny and ecology of Botryosphaeriaceous fungi occurring on various woody hosts. Ph.D. thesis. University of Pretoria, Pretoria, South Africa.

Slippers, B; Burgess, T; Wingfield, B. D; Crous, P. W; Coutinho, T. A. & Wingfield, M. J. (2004a) Development of simple sequence repeats markers for *Botryosphaeria* spp. with *Fusicoccum* anamorphs. *Molecular Ecology Notes* 4: 675-677.

Slippers, B., Crous P. W., Denman, S., Coutinho, T. A., Wingfield, B. D. & Wingfield M. J. (2004b) Combined multiple gene genealogies and phenotypic characters differentiate several species previously identified as *Botryosphaeria dothidea*. *Mycologia* 96: 83-101.

Slippers, B., Fourie, G., Crous, P. W., Coutinho, T. A., Wingfield, B. D., Carnegie, A. J. & Wingfield, M. J. (2004c) Speciation and distribution of *Botryosphaeria* spp. on native and introduced *Eucalyptus* trees in Australia and South Africa. *Studies in Mycology* 50: 343-358.

Slippers, B., Fourie, G., Crous, P. W., Coutinho, T. A., Wingfield, B. D., & Wingfield, M. J. (2004d) Multiple gene sequences delimit *Botryosphaeria australis* sp. nov. from *B. lutea*. *Mycologia* 96: 1030-1041.

Smith, D. R., Michailides, T. J. & Stanosz, G. R. (2001) Differentiation of a *Fusicoccum* sp. causing panicle and shoot blight on California pistachio trees from *Botryosphaeria dothidea*. *Plant Disease* 85: 1235-1240.

Smith, D. R. & Stanosz, G. R. (2001) Molecular and morphological differentiation of *Botryosphaeria dothidea* (anamorphs *Fusicoccum aesculi*) from some other fungi with *Fusicoccum* anamorphs. *Mycologia* 93: 505-515.

Smith, H., Wingfield, M. J., Crous, P. W. & Coutinho, T. A. (1996) *Sphaeropsis sapinea* and *Botryosphaeria dothidea* endophytic in *Pinus* spp. and *Eucalyptus* spp. in South Africa. *South African Journal of Botany* 62: 86-88.

Stoddart J. A. & Taylor, J. F. (1988) Genotypic diversity: estimation and prediction in samples. *Genetics* 118: 705-711.

Taylor, J. W., Jacobson, D. J. & Fisher, M. C. (1999) The evolution of asexual fungi: reproduction, speciation and classification. *Annual Review of Phytopathology* 37: 197-246.

Taylor, J. W., Jacobson, D. J., Kroken, S., Kasuga, T., Gieser, D. M., Hibbett, D. S. & Fisher, M. C. (2000) Phylogenetic species recognition and species concepts in fungi. *Fungal Genetics and Biology* 31: 21-32.

Workman, P. L. & Niswander, J. D. (1970) Population studies in South-western Indian tribes. II. Local genetic variation in the Papago. *American Journal of Human Genetics* 22: 24-49.

Yeh, F.C., Yang, R.-C. & Boyle, T. (1999) POPGENE. Version 1.31. *Microsoft windows based freeware for population genetic analysis*. Alberta.

Zhou, S., Smith, D. R. & Stanosz, G. R. (2001) Differentiation of *Botryosphaeria* species and related anamorphic fungi using inter simple or short sequence repeat (ISSR) fingerprinting. *Mycological Research* 105: 919-926.

Zhou, S. & Stanosz, G. R. (2001) Relationships among *Botryosphaeria* species and associated anamorphic fungi inferred from the analyses of ITS and 5.8S rDNA sequences. *Mycologia* 93: 516-527.

Table 1. Allele size (bp) and frequency at 7 loci for *Botryosphaeria ribis* (R) and *B. parva* (P) isolates collected from Venezuela (VEN), Colombia (COL) and Hawaii (HAW).

Locus	Allele	COL(R)	VEN(R)	HAW(R)	COL(P)	HAW(P)
BOT11	421	-	-	-	-	0.14
	428	0.11	0.06	-	0.75	0.72
	430	0.44	-	-	0.25	0.14
	431	0.33	0.88	-	-	-
	434	0.11	0.06	-	-	-
	438	-	-	1.00	-	-
BOT15	377	0.56	1.00	1.00	1.00	1.00
	389	0.44	-	-	-	-
BOT17	228	0.89	0.88	-	-	-
	230	-	0.06	-	-	-
	232	-	0.06	-	0.87	-
	234	-	-	1.00	-	-
	240	-	-	-	-	0.57
	242	-	-	-	-	0.07
	248	-	-	-	-	0.21
	252	-	-	-	-	0.14
	null	0.11	-	-	0.13	-
BOT21	196	-	-	1.00	-	0.15
	207	0.11	0.25	-	1.00	0.21
	208	0.44	0.56	-	-	-
	217	0.44	0.19	-	-	-
	219	-	-	-	-	0.07
	231	-	-	-	-	0.21
	null	-	-	-	-	0.36
BOT24	422	0.44	-	1.00	0.44	0.86
	423	-	-	-	0.50	-
	425	0.56	1.00	-	0.06	0.14
BOT35	221	-	-	-	-	0.50
	224	0.44	-	-	-	0.29
	227	-	-	-	-	0.14
	230	-	-	-	-	0.07
	237	-	0.25	1.00	0.06	-
	241	0.11	0.69	-	0.75	-
	245	-	-	-	0.13	-
	247	0.44	-	-	-	-
	258	-	0.06	-	-	-
	null	-	-	-	0.06	-
BOT37	305	0.11	-	-	-	-
	312	-	0.06	1.00	1.00	1.00
	320	0.67	-	-	-	-
	321	0.11	-	-	-	-
	323	-	0.13	-	-	-
	324	-	0.81	-	-	-
	null	0.11	-	-	-	-
No. of isolates		9	16	4	16	14
No. of alleles		20	17	7	14	20
No. of unique alleles		6	4	2	3	11

Table 2. *Botryosphaeria ribis* (R) and *B. parva* (P) genotypes as estimated from multilocus profiles generated from the 7 polymorphic SSR loci. Genotypes were distributed among populations collected Colombia (COL), Venezuela (VEN) and Hawaii (HAW).

Code	Genotype	RFLP profile	COL (P)	COL (R)	VEN (R)	HAW (P)	HAW (R)
COL1	ECCDDGF	P	3				
COL2	ECCDEEF	P	1				
COL3	ECCDEGF	P	5				
COL4	ECCDEOF	P	1				
COL5	ECNDFHF	P	1				
COL6	ECNDDGF	P	1				
COL7	FCCDDGF	P	2				
COL8	FCCDDHF	P	1				
COL9	FCCDEGF	P	1				
COL10	ECNDEIM	R		1			
COL11	FEAECBH	R		4			
COL12	GCACEIC	R		1			
COL13	GCADEIH	R		2			
COL14	HCADEGI	R		1			
VEN1	ECCEELF	R			1		
VEN2	GCADEGK	R			4		
VEN3	GCAEEFJ	R			1		
VEN4	GCAEEGK	R			5		
VEN5	GCAEEGJ	R			1		
VEN6	GCAFEEK	R			2		
VEN7	GCAFEGK	R			1		
VEN8	HCBEEEK	R			1		
HAW1	BCFIEBF	P				2	
HAW2	ECFGCAF	P				1	
HAW3	ECFKCCF	P				1	
HAW4	ECFACCF	P				1	
HAW5	ECFICDF	P				1	
HAW6	ECGKCAF	P				1	
HAW7	ECJKCAF	P				2	
HAW8	ECJDCAF	P				1	
HAW9	ECKDCAF	P				2	
HAW10	FCFKCBF	P				1	
HAW11	FCFACBF	P				1	
HAW12	KCDADEF	R					4
N			16	9	16	14	4
N(g)			9	5	8	11	1
G			5.83	3.52	5.12	8.99	1
G%			36	39	32	50	25

N = number of isolates
N(g) = number of genotypes
G = Genotypic diversity (Stoddart and Taylor, 1988)
$\hat{G}$ = G/N% = percent maximum diversity.

Table 3. Gene diversity (H) and contingency χ^2 tests for differences in allele frequencies for the 7 polymorphic SSR loci across clone corrected populations of *Botryosphaeria ribis* from Venezuela (VEN) and Colombia (COL). For χ^2 values, stars indicate level of significance (*P<0.05), no stars indicate no significant differentiation between populations.

	Gene Diversity (H)			
Locus	VEN	COL	χ^2	df
BotF11	0.40	0.72	1.8	2
BotF15	0.00	0.31	1.7	1
BotF17	0.40	0.32	2.9	3
BotF21	0.53	0.56	6.3	3
BotF24	0.00	0.32	1.7	1
BotF35	0.59	0.56	9.4	4
BotF37	0.53	0.72	13.0*	6
N	8	5		
MEAN	0.35	0.50		

Table 4. Gene diversity (H) and contingency χ^2 tests for differences in allele frequencies for the 7 polymorphic SSR loci across clone corrected populations of *Botryosphaeria parva* from Hawaii (HAW) and Colombia (COL). For χ^2 values stars indicate level of significance (**P<0.01, ***P<0.001), no stars indicate no significant differentiation between populations.

	Gene Diversity (H)			
Locus	HAW	COL	χ^2	df
BotF11	0.43	0.44	1.3	2
BotF15	0.00	0.00	-	-
BotF17	0.54	0.35	20.0**	5
BotF21	0.76	0.00	13.4**	4
BotF24	0.16	0.59	16.8***	3
BotF35	0.68	0.61	20.0**	7
BotF37	0.00	0.00	-	-
N	11	5		
MEAN	0.37	0.50		

Fig. 1. Neighbour-joining tree for the distance DAD based on total nucleotide length at each of the 7 SSR-loci, showing the relationship between genotypes of *Botryosphaeria ribis* (R) and *B. parva* (P) from Venezuela (VEN), Colombia (COL) and Hawaii (HAW). Also included are two isolates of the ex-type of *B. ribis* from New York.

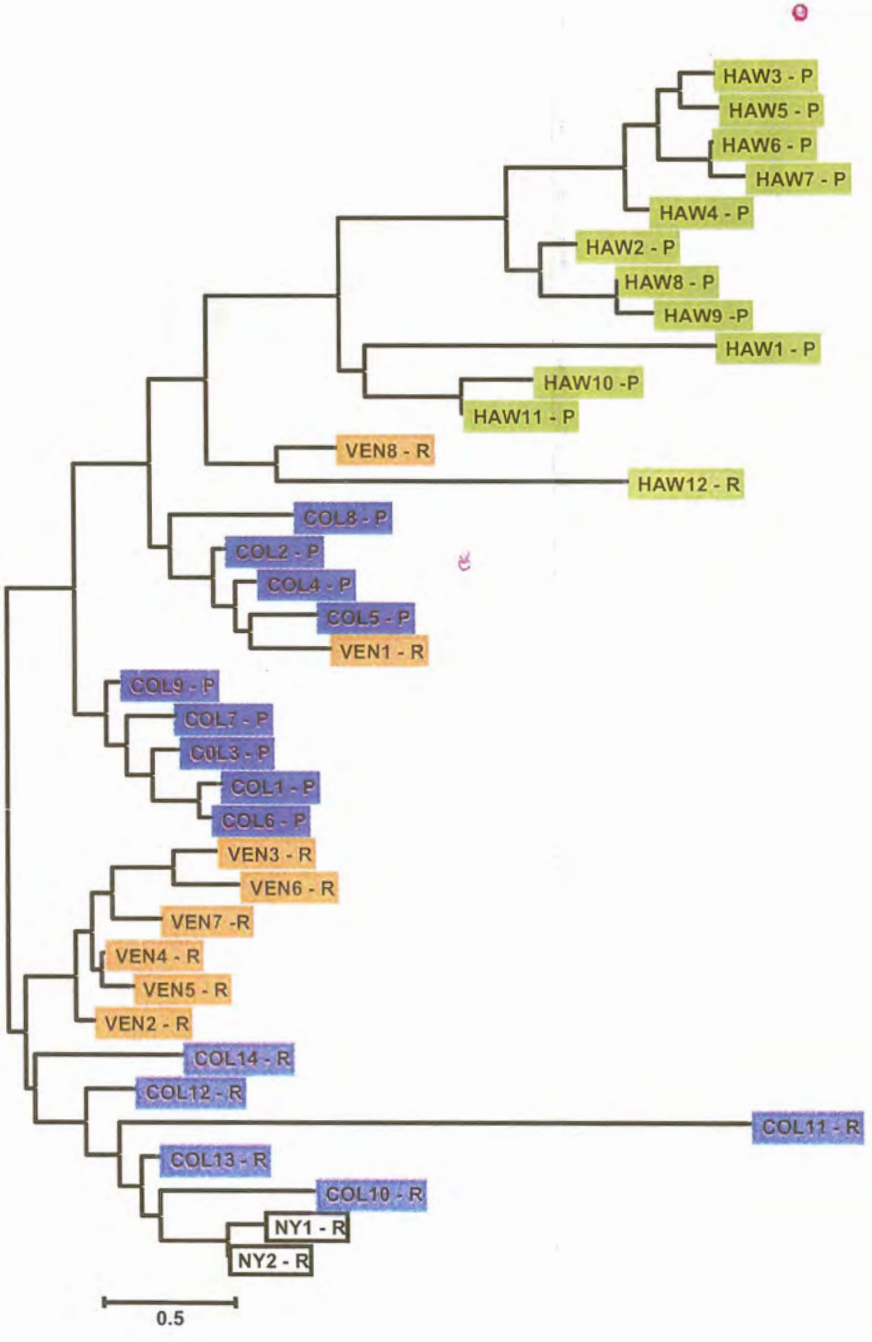

Fig. 2. Histograms of the frequency distribution representing multilocus disequilibrium estimate I_A for 1000 randomized datasets of *Botryosphaeria parva*. (A) Colombia, (B) Hawaii Results were compared with the observed dataset (arrows).

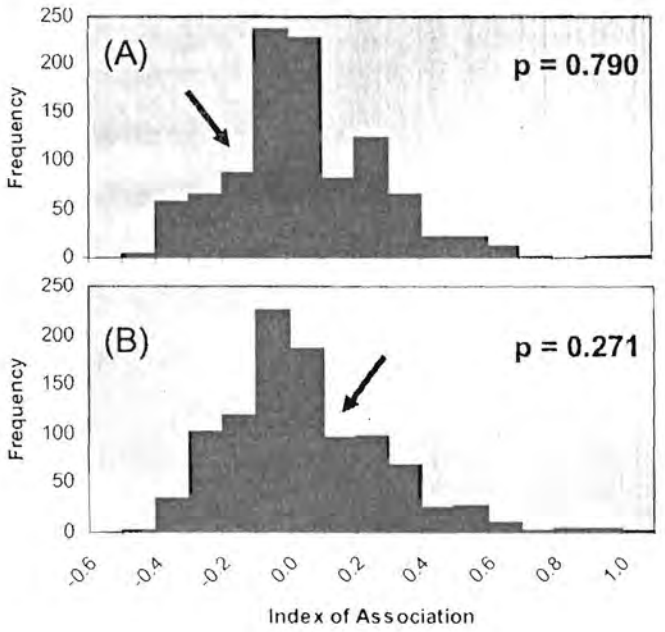

Fig. 3. Histograms of the frequency distribution representing multilocus disequilibrium estimate I_A for 1000 randomised datasets of *Botryosphaeria ribis* (Venezuela and Colombia combined). Results were compared with the observed dataset (arrows).