9

Differentiation and phylogeography of cryptic species in the *B. parva* - *B. ribis* complex

Abstract: The identities of *B. parva* and *B. ribis* have been confused in the past because they share hosts, geographical distribution and have overlapping morphological features. A previous study using multiple gene genealogies and conidial morphology of representative ex-type and additional authentic isolates of each of these species has shown that they are distinct, but closely related. In this study, the boundaries between *B. ribis* and *B. parva* were investigated, using multiple gene sequence genealogies, together with data from highly polymorphic simple sequence repeat (SSR) and restriction fragment length polymorphism (RFLP) marker data. Analyses of these data sets illustrate inter-specific boundaries and intra-specific groups. The RFLP markers provided a rapid means of distinguishing between *B. ribis* and *B. parva.* Ex-type isolates of these two species grouped within well-defined clades in sequence and SSR marker analyses and are defined as the *sensu stricto* groups of these species. However, certain groups of isolates clustered closely with, but outside the groups defining the species. These isolates are designated here as *sensu lato* groups of *B. parva* and *B. ribis*. The results show clearly that *B. parva* has been moved to new environments around the world on hosts such as *Eucalyptus*. The same genotypes of this species occurred on three continents and on up to five hosts, including indigenous and exotic hosts in countries such as Australia. Results, furthermore, suggest that recombination occurs randomly between isolates representing a world-wide population of *B. parva sensu stricto*, but that clonal reproduction also plays an important role in structuring the population of this fungus.

INTRODUCTION

Species of *Botryosphaeria* are well known pathogens of a wide range of agricultural crops and forest trees (von Arx 1987). These fungi infect trees directly through stomata and other natural openings and can they exist as endophytes within healthy plant tissues (Fisher et al 1993, Smith et al 1996). When plants are subjected to environmental stress, endophytic infections can rapidly give rise to tissue colonization and disease symptoms. Disease symptoms include cankers and die-back, and these can lead to death of the trees (von Arx 1987, Sinclair et al 1987).

For many years, the taxonomy of *Botryosphaeria* spp. was based on spore morphology and host association. Recent studies have shown that both characters are unreliable, making the taxonomy of these fungi confused and many literature reports of species questionable or incorrect (Denman et al 2000, Slippers et al 2003). Some species of *Botryosphaeria,* identified based on spore morphology, have been found on more than one host (Punithalingam and Holliday 1973, Punithalingam and Waller 1973). Similarly, a single plant species can also be infected by several *Botryosphaeria* spp. (Denman et al 2003, Chapters 2, 5). The teleomorphs of *Botryosphaeria* spp. are rarely encountered in culture and where they are present on host tissue, ascospores of different species overlap in size ranges (Pennycook and Samuels 1985, Slippers et al 2003). Thus, identifications are commonly based on anamorph spore size and shape, but these also overlap between species (Pennycook and Samuels 1985, Phillips et al 2002, Slippers et al 2003).

Recent studies have introduced analysis of DNA sequence data to resolve taxonomic questions pertaining to *Botryosphaeria*. These studies have contributed substantially to our understanding of the phylogenetic relationships between species (Jacobs and Rehner 1998, Denman et al 2000, Zhou and Stanosz 2001). They have also assisted in the identification and description of previously unknown species (Smith et al 2001, Phillips et al 2002, Denman et al 2003). Multiple gene sequence genealogies have, however, revealed that some groups identified using single gene phylogenies harbor cryptic species (De Wet et al 2003, Slippers et al 2003).

Botryosphaeria parva Pennycook & Samuels and *B. ribis* Grossenb. & Duggar are species that have often been confused (Smith and Stanosz 2001, Slippers et al 2003). Subsequent to the description of *B. ribis* (Grossenbacher and Duggar 1911), fungi resembling this species and its anamorph, *F. ribis* Slippers, Crous & M.J. Wingf., have

been identified from many different hosts, worldwide (Punithalingam and Holliday 1973). *Botryosphaeria parva* was described for the first time in 1985 (Pennycook and Samuels 1985), and until recently was not known outside Australasia. Studies based on ITS rDNA sequence data have not been able to separate *B. ribis* and *B. parva*, but ISSR markers have shown that they reside in separate groups (Smith and Stanosz 2001, Zhou and Stanosz 2001, Zhou et al 2001). Slippers et al (2003) used sequence data of three gene regions to confirm that these are distinct species. Subsequent studies using the same multiple gene sequence approach have shown that isolates in the *B. parva* – *B. ribis* complex occur widely and on different hosts (Chapters 2, 4-6).

Multiple gene genealogies are increasingly being used to identify cryptic species in fungi (Geiser et al 1998, Taylor et al 2000, Koufopanou et al 2001, Steenkamp et al 2002). Recognition of cryptic species is, however, complicated by the difficulty involved in sampling these species prior to the availability of reliable techniques to identify them (Taylor et al 1999). Fisher et al (2001), however, showed that SSR markers, combined with multiple gene genealogies, can fulfill the dual purpose of indicating inter- and intra-specific variation in *Coccidioides immitis* G.W. Stills, thus alleviating the problem of identifying members of cryptic species, prior to further studies. Similarly, species boundaries and population level variation have been revealed by SSR markers for the cryptic species *Diplodia pinea* and *D. scrobiculata* De Wet, Slippers & M.J. Wingf. (Burgess et al 2001, 2003, De Wet et al 2003).

In this study, data from SSR markers and multiple gene DNA sequences are used to consider genetic boundaries between *B. parva* and *B. ribis*. Furthermore, PCR RFLP fingerprints are considered as a potential means of rapidly distinguishing these species. In the case of *B. parva,* where a reasonably large collection of isolates was available, the mode of reproduction in the fungus was also considered. Considering the distribution of genotypes also made it possible to determine movement of this species between native and exotic plants, as well as between different geographical regions.

MATERIALS AND METHODS

Origin and identity of isolates used.--One hundred isolates from Australia, Colombia, Hawaii, New Zealand and South Africa, primarily collected by the authors or their collaborators in prior studies, were used in this study (TABLE I). All of these isolates have been characterized previously using morphological characteristics, PCR RFLP and

sequence data, and thus shown to belong in the *B. parva* – *B. ribis* complex (Jacobs 2002, Rodas 2003, Slippers et al 2003, Chapters 5, 6). Isolates were obtained from diseased and asymptomatic tissue from a wide range of native and exotic hosts in five different geographic regions (TABLE I), although the majority were from *Eucalyptus* trees. Each isolate originated from a single conidium or hyphal tip to ensure that only single genotypes were considered. All cultures are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa.

Phylogenetic analysis.--ITS rDNA, β-tubulin and elongation factor 1-α (EF1-α) sequence data for 22 isolates were obtained from previous studies (Slippers et al 2003, Jacobs 2002, Rodas 2003, Chapters 5, 6) (TABLE II). These data were analyzed in PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b8 (Swofford 1999). The sequence data were manually aligned by inserting gaps. Gaps were treated as a fifth character and all characters were unordered and of equal weight.

Repetitive minisatellite regions in the intron of the EF1-α were coded to represent a single, rather than multiple evolutionary events. Data sets from the three gene regions were analyzed separately and in combination. Statistical congruence between the data sets was tested using partition homogeneity tests (PHT) (Farris et al 1995, Huelsenbeck et al 1996) in PAUP.

Most parsimonious trees were found using heuristic searches with stepwise (random) additions and tree bisection and reconstruction (TBR) as the branch swapping algorithm. Maxtrees were unlimited, branches of zero length were collapsed and all multiple equally parsimonious trees were saved. Branch supports, using 1000 bootstrap replicates (Felsenstein 1985), and estimated levels of homoplasy and phylogenetic signal (retention and consistency indices and g1-value) (Hillis and Huelsenbeck 1992) were also determined in PAUP.

RFLP analysis.--Three loci with known restriction site polymorphisms, for which primers have previously been developed (Chapter 8), were used to characterize the isolates. Loci amplified with primer pairs BOT1 and BOT2, BOT11 and BOT12, BOT15 and 16 were digested with the restriction endonucleases (RE) *Hae*III, *Sau*3A and *Cfo*I, respectively. Amplicons of primer pairs BOT11 and BOT12, and BOT15 and

BOT16 also contain SSR regions and were subsequently used as size polymorphic markers. Digestions were done as previously described (Chapter 8), and fragments separated on 1.5 % agarose gels stained with ethidium bromide, and visualized under UV light.

SSR amplification and scoring.--Eight loci that are known to contain SSR sequences were amplified for all 109 isolates using previously developed primer pairs (Chapter 8). The primers were fluorescently labeled (TABLE III). PCR reaction mixtures and conditions were the same as those described previously (Chapter 8). PCR products were subjected to electrophoresis and visualized under UV light on agarose gels that were stained with Ethidium Bromide, and their concentrations estimated against a λ DNA standard. Amplified products were diluted 1:50 to 1:200, depending on concentration, and one μl of the dilution mixed with a LIZ^{TM} internal size standard (Perkin-Elmer Applied Biosystems, Foster City, CA): formamide mix (1:14). The products were then separated on an ABI PRISM 3100 autosequencer (Perkin-Elmer Applied BioSystems). Amplicons of all loci were multiplexed in one lane, as the fluorescent labels and sizes of the fragments did not overlap in most cases. Where they did overlap in the case of BOT17&18 and BOT35&36, these amplicons were analyzed on separate gels. Results were analyzed using the Genescan® 2.1 (Perkin Elmer) and Genotyper® 3.0 (Perkin Elmer) software.

For each isolate, a data matrix of multistate characters was compiled by assigning a different letter to each allele size at each of the eight loci (e.g. BBFEADB) (TABLE IV). Each genotype was then assigned a unique number (TABLE IV). Missing data were treated as distinct characters when assigning numbers to genotypes. All further analyses were performed on clone corrected populations, where only one representative of each genotype was included.

Distance analyses.--The genetic structure among the isolates was determined using distance analysis. A clone corrected (only one representative of each genotype) data matrix was compiled from the multistate characters for SSR and RFLP markers (TABLE IV). The relationship between genotypes was determined in PAUP using Unweighted Pair-Group Method with Arithmetic mean (UPGMA) and Neighbor Joining (NJ).

A distance matrix was also calculated based on absolute distance (D_{AD}) using the program MICROSAT (Erich Minch; http://hpgl.stanford.edu/projects/microsat/). The total nucleotide length of alleles was used for these analyses, because the variation was due to both changes in microsatellite repeats and the flanking sequence. The distance matrix was used to construct UPGMA (Unweighted Pair-Group Method with Arithmetic mean) trees in MEGA v.2.1 (Kumar et al 2001).

Mode of reproduction.--Multilocus linkage disequilibrium was calculated for a world-wide group of isolates representing *B. parva sensu stricto*, using the index of association (I_A) (Brown et al 1980, Maynard Smith et al 1993). These isolates were identified using the various techniques described above and they represented the only population for which sufficient isolates were available to justify the analysis. The I_A was calculated for the full and clone corrected (using only one representative of each genotype) dataset and with the multistate characters for each allele in the program MultiLocus (Agapow and Burt 2000). The statistical significance of observed data was determined by comparison with expected data for a 1000 randomly recombining datasets. Furthermore, the gametic linkage disequilibrium (non randomly associated polymorphic loci) was calculated in POPGENE with a probability of $P < 0.05$ (Hartl and Clark 1989). A percentage was thus calculated using the formula:

{Observed number of significant linkage disequilibria between two loci in a group / [x(x-1/2)] where x is the number of alleles in the group}100.

RESULTS

Phylogenetic analyses.--Among the individual gene sequence datasets that were analyzed, the EF1-α data had 8 informative sites and gave the highest level of resolution. The ITS rDNA sequence dataset had 6 informative sites (FIG. 1). The β-tubulin sequence dataset had the least variation with 5 informative sites and the clades of the tree based on these data were not well resolved (FIG. 1). The partition homogeneity test showed that DNA sequence data sets for the three regions could be combined (PHT $P = 0.74$) and the final combined dataset consisted of 1238 characters.

Based on the various analyses of the three DNA sequence datasets, *sensu lato* and *sensu stricto* groups were defined for *B. parva* (FIG. 2). The different analyses of the DNA sequence data indicated a consistent clade (66--74 % bootstrap support),

which included the ex-type and other authentic isolates of *B. parva* (FIGS. 1, 2). This clade is considered to represent *B. parva sensu stricto.* Four isolates grouped more closely to the *B. parva s.str.* than to *B. ribis*, although their positions in the various trees were not well resolved (FIGS. 1, 2). These isolates, together with those representing *B. parva s.str.*, are considered to represent a *B. parva sensu lato* clade (FIG. 2).

Isolates believed to represent *B. ribis sensu lato* consistently grouped together and were well supported in most trees (67--94 % bootstrap value) (FIGS. 1, 2). Within *B. ribis s.l.,* the sub-clade representing ex-type and other isolates of *B. ribis* from *Ribes* in New York, the origin of the type strain, was always well supported (67--99 % bootstrap support) (FIGS. 1, 2). Isolates residing in this clade are believed to represent *B. ribis sensu stricto*. The other subgroup in the *B. ribis s.l.* clade (FIGS. 1, 2) correlated with isolates from *Wollemia* in Australia and one isolate from *Eucalyptus* in Hawaii. These are clearly different to *B. ribis s.str.*, although closely related to it.

RFLP analysis.--Restriction sites were scored as present (1) or absent (0), depending on whether the enzyme digested the fragment or not. Isolates of *B. ribis* and *B. parva* were not polymorphic for the loci amplified with primers pairs BOT1 and BOT2, and BOT11 and BOT12. These loci contain polymorphisms for isolates that are closely related to *B. parva* and *B. ribis* that are not treated here. Amplicons obtained using primers BOT15 and BOT16 contained a restriction site for *Cfo*I in isolates of *B. ribis s.l.*, but not in isolates of *B. parva s.l.* (TABLE IV). The *sensu stricto* and *sensu lato* groups within each of *B. ribis* and *B. parva* could not be distinguished using RFLP data.

SSR amplification and scoring.--The eight primer pairs amplified fragments ranging from 199 bp to 437 bp, which represented 63 alleles (TABLE III). All the loci were polymorphic within and between groups of isolates representing *B. ribis* and *B. parva.* The locus amplified by primers BOT19 and BOT20 were found to be hyper-variable (TABLE IV). There were also many missing data points for this locus (TABLES III, IV). Data from this locus were, therefore, disregarded in all further analyses. Primers BOT21 and BOT22 also did not amplify a fragment for a number of isolates. The lower variability and greater size differences between alleles obtained from these primers, however, made the data from these primers acceptable for inclusion in the analyses.

Distance analyses.--UPGMA and NJ distance analyses of multistate allele characters of the SSR and RFLP markers, reflected the same relationships among isolates as those based on sequence data (FIG. 3). The relationships among the isolates were the same when only SSR marker data were used, although the groups were less well resolved (data not shown).

Isolates that had been characterized as *B. parva s.str.* based on sequence data, consistently grouped together based on SSR and RFLP marker data (FIG. 3). Isolates belonging to this group originated from all the regions studied, except New York, and from all the hosts sampled, except *Wollemia* (TABLE IV). Some of these genotypes such as genotype 5 occurred on three continents or as in the case of genotype 8, on up to five hosts (TABLE IV). There was no substructure linked to geography or host for isolates in this clade and they are considered to represent a worldwide meta-population of *B. parva s.str.* (FIG. 3).

Isolates defined as *B. parva s.l.* based on sequence data, grouped close to the *B. parva s.str.* group based on SSR and RFLP data (FIG. 3). These isolates originated from *Eucalyptus* in South Africa, Colombia and Hawaii, as well as one from *Mangifera indica* in South Africa and two isolates from *Tibouchina* in Australia (TABLE IV). One isolate (CMW2475, SSR genotype 45), however, grouped with *B. parva s.str.* based on SSR data, contrary to its placement in *B. parva s.l.*, based on sequence data.

Isolates identified as *B. ribis s.l.* and *s.str.* based on sequence data grouped together based on SSR and RFLP marker data. This is possibly because *B. ribis s.str.* was represented by only three isolates from *Ribes* in New York, which contain only two genotypes. In contrast, *B. ribis s.l.* isolates were from diverse origins such as *Eucalyptus* in Colombia and Hawaii, *Mangifera* in South Africa, as well as *Araucaria* and *Wollemia* in Australia.

Mode of reproduction.--Only *B. parva s.str.* included sufficient isolates within a defined population to justify an investigation of mode of reproduction based on I_A. The observed values for the I_A of these isolates fell within the range of the randomized dataset for the full and clone corrected datasets (FIG. 4A, B). This indicates that the alleles are distributed randomly among the isolates, suggesting random mating in the population. This is supported by a non-significant P-value for the clone corrected dataset ($P = 0.342$). However, the P-value for the observed I_A for the full dataset was statistically significant ($P = 0.003$), indicating that the null-hypothesis of non-random

mating cannot be rejected. This is most likely due to the presence of a number of isolates belonging to the same genotype in the full dataset. This would imply a high level of asexual reproduction, despite the ability of the fungus to undergo sexual reproduction. Furthermore, the value for linkage disequilibrium for alleles among isolates of *B. parva s.str.* was low (11 %), supporting the hypothesis of random mating among these isolates of *B. parva s.str.*

DISCUSSION

In this study the separation of the closely related *B. parva* and *B. ribis* was confirmed using combined gene genealogies, SSR makers and PCR RFLP fingerprints. These data and especially the RFLP profiles have also provided a rapid and effective means to distinguish between these species. Furthermore, following characterization of *B. parva* and *B. ribis,* it was shown that these fungi, and especially *B. parva*, have been moved around the world on hosts such as *Eucalyptus*. It was clear from the SSR marker data that there is recombination among a world-wide population of *B. parva*, which was the only species for which sufficient isolates were available to consider mode of reproduction. Despite *B. parva* having the capacity to undergo sexual exchange, a number of clonal lines occurring across spatial and temporal boundaries were identified for this fungus.

Using a combination of multiple gene sequence data, SSR markers and RFLPs, is was possible to define both broad and strict boundaries for *B. parva*. Based on analysis of sequence data, the clade including the ex-type and other isolates linked to the original description of *B. parva* (Pennycook and Samuels 1985) is considered to represent this species in the strict sense. These isolates are referred to as representing *B. parva sensu stricto*. They also grouped together based on SSR marker data adding to our confidence that they reflect a closely defined group.

The SSR marker data showed no substructure among isolates of *B. parva s.str.* that could be correlated to geographic origin. In contrast isolates representing distinct genotypes were randomly distributed across Australasia, South Africa and Hawaii. Isolates having identical genotypes were found to occur between geographical regions, such as genotypes 1, 5, 8, 12 and 14 that occurred in Australia, New Zealand, Hawaii and South Africa. This significant overlap in the occurrence of discrete genotypes between the regions is most likely linked to their occurrence on *Eucalyptus*. This native

Australian tree has been introduced numerous times into South Africa and Hawaii, and germplasm continues to be exchanged, mainly but not exclusively in the form of seed. *Botryosphaeria parva* is an endophyte of *Eucalyptus* (Smith et al 1996, Slippers et al 2003) and is, therefore, easily introduced into new regions on healthy plant material and probably also on seed.

No evidence of host specialization was found for isolates of *B. parva s.str.* This fungus was found on eight of the nine hosts studied. These eight hosts are from diverse plant families including both gymnosperms and angiosperms. Furthermore, single *B. parva* genotypes were found to co-infect up to five different plants. The lack of specialization found for *B. parva* in this study implies that the importation of new genotypes of this fungus on one host could negatively affect other hosts in a region.

Certain genotypes of *B. parva s.str.* were found to co-infect native and exotic hosts in Australia. For example, genotype 9 was found on *Eucalyptus*, *Mangifera* and *Tibouchina*, and genotype 8, was found on *Eucalyptus*, *Tibouchina* and *Camphor*. Native *Eucalyptus* could thus be directly affected by genotypes that are introduced with foreign plants. The introduction of foreign plant species harboring *B. parva* genotypes to which native plants might not have resistance could seriously damage these native plants. Such movement of pathogens between introduced hosts and native *Eucalyptus* trees is seen as a one of the significant threats facing these native Australian plants (Burgess and Wingfield 2001). Likewise, introduced agricultural or ornamental plants could be negatively affected by genotypes of *B. parva* that originate on native plants.

The diversity amongst isolates of *B. parva s.str.* differed between the three regions studied. This might provide some indication of the origin of this fungus. If missing data are disregarded, only three genotypes were found on *Eucalyptus* and two on *Mangifera,* amongst 21 isolates collected in South Africa. In contrast, six genotypes were found amongst only eleven Hawaiian isolates from *Eucalyptus*. Isolates from Australasia, including Australia and New Zealand were the most diverse, with 11 genotypes from 24 isolates on seven hosts. This might indicate an Australasian origin for *B. parva*. It is unlikely that the higher diversity in Australasian isolates of this fungus reflects the more diverse range of hosts sampled from this region. This is because there is no evidence for host specialization in this taxon as previously discussed.

From the SSR allele disequilibrium and I_A analyses, it is clear that the alleles are randomly associated across the global population of *B. parva s.str.* This indicates that

the fungus is undergoing sexual recombination (Brown et al 1980, Maynard Smith et al 1993, Agapow and Burt 2000). Despite this apparent ability to reproduce sexually, the recovery of identical genotypes of *B. parva s.str.* across continents and during different seasons suggests that clonal reproduction also plays and important role in structuring the population. This observation is supported by analyses when the dataset was not clone-corrected. These results regarding reproduction in *B. parva s.str.* reflect those of previous studies on other fungi, where a combination of clonal and sexual processes has been shown to significantly influence population structure (Taylor et al 1999). For example, in an extensive study of the ascomycete *Sclerotinia sclerotiorum* (Carbone et al 1999), evidence for recombination was clearly shown even though the fungus primarily reproduces clonally.

The low level of diversity in *B. parva s.str.* in countries such as South Africa is inconsistent with the notion that recombination occurs freely among isolates. A possible explanation for this result would be that the fungus is primarily homothallic. This is possible because a preliminary study on *B. eucalyptorum* (Van Geuns and Slippers, unpublished data) has shown that single ascospore cultures could give rise to pseudothecia producing viable ascospores. This does not necessarily exclude the possibility of outcrossing, because many homothallic and pseudohomothallic fungi retain a certain level of outcrossing, albeit at low levels (Taylor et al 1999).

Based on DNA sequence and SSR marker data, some isolates included in this study were more closely related to *B. parva* than to *B. ribis*, but did not group within the *B. parva s.str.* clade. These isolates are designated as *B. parva sensu lato*. The isolates residing in this *B. parva s.l.* clade also shared a common RFLP marker profile with *B. parva s.str.*, where none of the REs cut in the amplified fragments. The unresolved relationships of isolates in the *B. parva s.l.* group arise from the fact that they share some polymorphisms with both *B. ribis* and *B. parva s.str.* These shared polymorphisms might be interpreted as a sign that speciation has not occurred in these isolates. This is an unlikely explanation because each group of isolates had a number of unique alleles among loci of the sequence data, SSR marker data and restriction sites. The fact that the isolates co-occur on the same hosts and in the same areas indicates that species barriers, rather than physical or other barriers, are responsible for the differentiation among these isolates.

Another possible explanation for the shared polymorphisms between isolates of *B. parva* and *B. ribis* is that they are due to hybridization or introgression between the

isolates of the two species. This is a reasonable explanation as hybridization has recently been shown to occur amongst species of fungi and this process may not be uncommon (Brasier et al 1998, Brasier 2000, 2001, Newcombe et al 2000). Brasier (2000, 2001) points out that the probability for such hybridization is increased when geographically distinct pathogen populations are brought into contact, because such taxa often do not form complete reproductive barriers. As shown in this and other studies (Burgess et al 2003, Denman et al 2003, Chapter 5), *Botryosphaeria* spp. have been moved around the world on hosts such as *Eucalyptus*, *Pinus* and Proteaceae, thus making them ideal candidates for hybridization with native species.

Shared polymorphisms between isolates of *B. parva* and *B. ribis* could also be present because their lineages are not completely resolved. Taylor et al (2000) have shown that that fungi that have recently undergone speciation, will share many polymorphisms. This is due to the fact that the amount of lineage sorting is directly proportional to time after speciation has occurred. The few fixed polymorphisms between *B. parva* and *B. ribis* compared to other closely related *Botryosphaeria* spp. indicate that they have recently undergone speciation. They might, therefore, be expected to still share a number of polymorphisms.

Isolates representing the broader boundary of *B. ribis*, which is referred to as *B. ribis sensu lato*, grouped together based on DNA sequence, SSR and RFLP marker data. The fragment amplified using the primers BOT15 and BOT16 had a unique restriction site for RE *Cfo*I, which presents a simple method to identify isolates residing in this group. Multiple gene sequence phylogeny identified more than one well supported clade, amongst the isolates representing *B. ribis s.l.* For example, the ex-type culture of *F. ribis* Slippers, Crous & M.J. Wingf. and other isolates from the *Ribes* in New York, USA, were distinct within this clade, in all analyses. These isolates are regarded as representing *B. ribis sensu stricto*. Another sub-clade contained strains isolated from *Wollemia* in Australia and *Eucalyptus* in Hawaii. Representative isolates from populations will be required to determine whether these subgroups indicate species barriers or distinct populations of the same species.

The consistencies between the SSR and RFLP polymorphic marker analysis and multiple gene sequence data shows that co-dominant marker data are phylogenetically informative. The phylogenetic value of SSR marker data has been questioned because of constraints on the range of allele sizes and their high rate of mutation, resulting in high incidences of homoplasy (Nauta and Weissing 1996, Orti et al 1997, Fisher et al

2000). Other studies on fungi such as *D. pinea* and *Coccidioides immitis* have, however, also shown that that SSR data can be phylogenetically informative (Fisher et al 1997, Burgess et al 2001, De Wet et al 2003). These markers will thus be very useful to confirm speciation event between closely related groups that are less clearly resolved using other techniques.

The subgroups within *B. ribis s.l.* and *B. parva s.l.* are easily identifiable by the SSR marker data used in this study. The ability to identify isolates that represent these groups will facilitate future selection of isolates needed to determine whether these groups indicate species barriers or distinct populations of the same species. Previous authors (Davis and Nixon 1992, Taylor et al 1999) have noted the inherent difficulties in defining and identifying populations of closely related species or subspecies from which to collect samples. Thus, if populations selected to represent two closely related groups contains a mixture of isolates from both of them, then reproductive isolation might not be detected. This is especially true for fungi such as the *Botryosphaeria* spp. studied here. Because of their cosmopolitan distribution and sympatric occurrence on certain hosts, the delimitation of populations is highly problematic. Fisher et al (2000) also found that SSR data can serve the dual purpose of identifying inter- and intra specific variation among a group of isolates to enable further testing of population and species hypotheses. Results of this study provide added evidence supporting this view.

Polymorphic SSR marker data combined with multiple gene sequence data in this study have provided a substantially increased the available information about the diversity present amongst isolates in the *B. ribis* and *B. parva* complex. The combination of these datasets can now be used to determine species boundaries between subgroups within *B. parva s.l.* and *B. ribis s.l.* This study has highlighted the fact that identification of cryptic species in *Botryosphaeria* cannot rest on phenotypic or single gene phylogenies. Furthermore, the importance of representative numbers of samples is emphasized in this study. Species exist as populations that are not necessarily represented by a small number of isolates. The ability to characterize the inter-specific boundaries between *B. parva* and *B. ribis*, as illustrated in this study, can now be used to further explore the reproductive and evolutionary forces that shape populations of these species. Understanding the variation, interaction and mode of reproduction within species of *Botryosphaeria* is an important prerequisite to understanding the global distribution and pace of evolution of these important plant pathogens.

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TABLE I. Geographical origin and hosts from which isolates were obtained in this study. Isolates from native hosts are highlighted in grey.

Origin	Host	Culture number[1]
Australia	*Araucaria*	3388
	Cinnamomum	6814
	Eucalyptus	6798, 6799, 6802
	Mangifera	7799, 7025, 7026
	Ribes	9071
	Tibouchina	6235, 6236, 6536, 6795, 6797
	Wollemia	3389, 9070
Colombia	*Eucalyptus*	8930, 8931, 8934--8940, 8942--8948, 8952, 8953, 8955, 8957, 8958, 8964--8966
Hawaii	*Eucalyptus*	7882--7888, 7891, 7892, 7893a, b, 7894a, b, 7895, 7896--7899
New Zealand	*Actinidia*	9077--9079
	Araucaria	10120, 10121
	Populus	9080, 9081
	Tibouchina	10117--10119, 10127--10129
New York, USA	*Ribes*	7772, 7773, 7054
South Africa	*Eucalyptus*	937, 944, 947, 1217, 1238, 1242, 1304, 1322, 1396, 1492, 1625, 1782, BOT7, BOT19
	Mangifera	BOT2283, BOT2292, BOT2294, BOT2298, BOT2299, BOT2324, BOT2325, BOT2330, BOT2341, BOT2347, BOT2370, BOT2390, BOT2404

[1] Numbers are those of CMW = Culture collection of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa. BOT numbers refer to a *Botryosphaeria* sub-collection of CMW.

TABLE II. Isolates of *Botryosphaeria parva* and *B. ribis* considered in the phylogenetic study.

Culture no.[1,2]	**SSR genotype**[1]	**Identity**[3]	**Host**	**Location**	**Collector**	**Reference**[4]
CMW9071	11	*Botryosphaeria parva s.str.*	*Ribes* sp.	Australia	M.J. Wingfield	Slippers et al 2003
CMW9077	1	*B. parva s.str.*	*Actinidia deliciosa*	New Zealand	S.R. Pennycook	"
CMW9078	12	*B. parva s.str.*	*A. deliciosa*	New Zealand	S.R. Pennycook	"
CMW9079	13	*B. parva s.str.*	*A. deliciosa*	New Zealand	S.R. Pennycook	"
CMW9080	8	*B. parva s.str.*	*Populus nigra*	New Zealand	G.J. Samuels	"
CMW9081	14	*B. parva s.str.*	*P. nigra*	New Zealand	G.J. Samuels	"
CMW10120	8	*B. parva s.str.*	*Araucaria heterophylla*	New Zealand	M.J. Wingfield	"
CMW10121	5	*B. parva s.str.*	*A. heterophylla*	New Zealand	M.J. Wingfield	"
CMW10123	5	*B. parva s.str.*	*E. smithii*	South Africa	H. Smith	"
CMW6235	28	*B. parva s.l.*	*Tibouchina lepidota*	Australia	M.J. Wingfield	Chapter 5
CMW6237	28	*B. parva s.l.*	*T. urvilleana*	Australia	M.J. Wingfield	"
BOT2475	45	*B. parva s.l.*	*Eucalyptus* sp.	Colombia	C. Rodas	Rodas 2003
BOT2404	35	*B. parva s.l.*	*Mangifera indica*	South Africa	R. Jacobs	Jacobs 2002

TABLE II. Continued.

Culture no.[1,2]	**SSR genotype**[1]	**Identity**[3]	**Host**	**Location**	**Collector**	**Reference**[4]
CMW7772	31	*B. ribis s.str.*	*Ribes* sp.	New York, USA	B. Slippers/G. Hudler	Slippers et al 2003
CMW7773	31	*B. ribis s.str.*	*Ribes* sp.	New York, USA	B. Slippers/G. Hudler	"
CMW7054	32	*B. ribis s.str.*	*R. rubrum*	New York, USA	N.E. Stevens	"
CMW3388	33	*B. ribis s.l.*	*Araucaria cunninghamii*	Australia	M. Ivory	Chapter 6
CMW3389	34	*B. ribis s.l.*	*Wollemia nobilis*	Australia	M. Ivory	"
CMW9070	34	*B. ribis s.l.*	*W. nobilis*	Australia	B. Summerell	"
CMW7885	53	*B. ribis s.l.*	*Eucalyptus* sp.	Hawaii	M.J. Wingfield	Slippers et al 2003

[1] Isolates in bold are ex-type.
[2] Designation of genotypes and groups as used later in the study (TABLE IV).
[3] Identities as determined in this study for *sensu lato* (*s.l.*) and *sensu stricto* (*s.str.*) groups of *B. parva* and *B. ribis*.
[4] Study where DNA sequence of the isolate was determined.

TABLE III. SSR primers, labels and size ranges for amplicons obtained in this study.

Primer number	Label	Size ranges (bp)	No. of alleles	No. not amplified
BOT 11&12	NED	420-437	5	1
BOT 15&16	PET	365-395	3	0
BOT 17&18	NED	228-258	9	5
BOT 19&20	6-FAM	271-311	16	13
BOT 21&22	VIC	199-231	5	18
BOT 23&24	6-FAM	415-427	4	0
BOT 35&36	NED	221-261	9	2
BOT 37&38	VIC	298-334	5	2

TABLE IV. Genotypes and RFLP profiles of isolates used in this study. Isolates are grouped according to the species groups identified using sequence data.

Genotype description[1]	SRR loci[2]							SSR genotype	RFLP loci			RFLP genotype	Not used
	11&12	15&16	17&18	21&22	23&24	35&36	37&38		1&2r	11&12r	15&16r		19&20
B. parva sensu stricto													
SA Euc 1217	B	B	J	D	B	B	B	sr 1	0	0	0	rf 1	K
SA Euc 1238	B	B	J	D	B	B	B	sr 1	0	0	0	rf 1	K
SA Euc 1242	B	B	J	D	B	B	B	sr 1	0	0	0	rf 1	K
SA Euc 1396	B	B	J	D	B	B	B	sr 1	0	0	0	rf 1	J
SA Euc 1625	B	B	J	D	B	B	B	sr 1	0	0	0	rf 1	K
SA Euc 937	B	B	J	D	B	B	B	sr 1	0	0	0	rf 1	L
SA Euc 1782	B	B	J	D	B	B	B	sr 1	0	0	0	rf 1	K
SA Euc 947	B	B	J	D	B	B	B	sr 1	0	0	0	rf 1	K
NZ Act 9077	B	B	J	D	B	B	B	sr 1	0	0	0	rf 1	Q
SA Euc 1242	B	B	J	?	B	B	B	sr 2	0	0	0	rf 1	K
SA Euc BOT7	B	B	J	?	B	B	B	sr 2	0	0	0	rf 1	K
SA Euc 1492	B	B	J	D	B	C	B	sr 3	0	0	0	rf 1	K
SA Euc 944	B	B	J	D	B	C	B	sr 3	0	0	0	rf 1	M
SA Euc 1304	B	B	J	D	B	B	?	sr 4	0	0	0	rf 1	K
SA Euc BOT19	B	B	F	C	B	C	B	sr 5	0	0	0	rf 1	E
Aus Tib 6235	B	B	F	C	B	C	B	sr 5	0	0	0	rf 1	E
Aus Man 7026	B	B	F	C	B	C	B	sr 5	0	0	0	rf 1	E
NZ Tib 10127	B	B	F	C	B	C	B	sr 5	0	0	0	rf 1	E
SA Man BOT2298	B	B	I	C	B	A	B	sr 6	0	0	0	rf 1	O
SA Man BOT2347	B	B	I	C	B	A	B	sr 6	0	0	0	rf 1	I
SA Man BOT2292	B	B	I	C	B	A	B	sr 6	0	0	0	rf 1	J
SA Man BOT2299	B	B	I	D	B	A	B	sr 7	0	0	0	rf 1	H
SA Man BOT2370	B	B	I	D	B	A	B	sr 7	0	0	0	rf 1	H
SA Man BOT2283	B	B	I	D	B	A	B	sr 7	0	0	0	rf 1	I
SA Man BOT2324	B	B	I	D	B	A	B	sr 7	0	0	0	rf 1	I

TABLE IV. Continued.

Genotype description[1]	SRR loci[2]							SSR genotype	RFLP loci			RFLP genotype	Not used
	11&12	15&16	17&18	21&22	23&24	35&36	37&38		1&2r	11&12r	15&16r		19&20
B. parva sensu stricto (continued)													
Aus Euc 6798	B	B	F	C	B	A	B	sr 8	0	0	0	rf 1	G
Aus Tib 6797	B	B	F	C	B	A	B	sr 8	0	0	0	rf 1	?
Aus Cam 6814	B	B	F	C	B	A	B	sr 8	0	0	0	rf 1	G
NZ Pop 9080	B	B	F	C	B	A	B	sr 8	0	0	0	rf 1	E
NZ Tib 10117	B	B	F	C	B	A	B	sr 8	0	0	0	rf 1	E
NZ Ara 10120	B	B	F	C	B	A	B	sr 8	0	0	0	rf 1	?
Aus Euc 6799	B	B	F	?	B	A	B	sr 9	0	0	0	rf 1	D
Aus Euc 6802	B	B	F	?	B	A	B	sr 9	0	0	0	rf 1	E
Aus Tib 6795	B	B	F	?	B	A	B	sr 9	0	0	0	rf 1	E
Aus Man 7799	B	B	F	?	B	A	B	sr 9	0	0	0	rf 1	D
Aus Man 7025	B	B	?	D	B	A	B	sr 10	0	0	0	rf 1	E
Aus Rib 9071	B	B	K	C	B	B	B	sr 11	0	0	0	rf 1	N
NZ Act 9078	B	B	F	D	B	A	B	sr 12	0	0	0	rf 1	G
Haw Euc 7887	B	B	F	D	B	A	B	sr 12	0	0	0	rf 1	J
NZ Act 9079	B	B	F	D	B	C	B	sr 13	0	0	0	rf 1	E
NZ Tib 10128	B	B	F	D	B	C	B	sr 13	0	0	0	rf 1	E
NZ Pop 9081	B	B	F	A	B	C	B	sr 14	0	0	0	rf 1	E
Haw Euc 7897	B	B	F	A	B	C	B	sr 14	0	0	0	rf 1	E
NZ Tib 10129	B	B	I	?	B	A	B	sr 15	0	0	0	rf 1	E
NZ Tib 10118	B	B	J	C	A	A	B	sr 16	0	0	0	rf 1	C
NZ Tib 10119	B	B	G	C	B	C	B	sr 17	0	0	0	rf 1	E
Haw Euc 7882	B	B	J	?	B	A	B	sr 18	0	0	0	rf 1	?
Haw Euc 7894b	B	B	J	?	B	A	B	sr 18	0	0	0	rf 1	J
Haw Euc 7888	B	B	G	?	B	A	B	sr 19	0	0	0	rf 1	K
Haw Euc 7893a	B	B	K	C	B	A	B	sr 20	0	0	0	rf 1	I
Haw Euc 7893b	B	B	K	C	B	A	B	sr 20	0	0	0	rf 1	J
Haw Euc 7894a	B	B	J	C	B	A	B	sr 21	0	0	0	rf 1	I
Haw Euc 7896	B	B	F	?	B	C	B	sr 22	0	0	0	rf 1	G
Haw Euc 7898	C	B	F	?	B	B	B	sr 23	0	0	0	rf 1	I
Haw Euc 7899	C	B	F	A	B	B	B	sr 24	0	0	0	rf 1	K

TABLE IV. Continued.

Genotype description[1]	SRR loci[2]							SSR genotype	RFLP loci			RFLP genotype	Not used
	11&12	15&16	17&18	21&22	23&24	35&36	37&38		1&2r	11&12r	15&16r		19&20
B. parva sensu lato													
Aus Tib 6236	B	C	C	C	B	A	B	sr 28	0	0	0	rf 1	L
Aus Tib 6536	B	C	C	C	B	A	B	sr 28	0	0	0	rf 1	L
Haw Euc 7883	A	B	F	E	D	B	B	sr 29	0	0	0	rf 1	K
Haw Euc 7884	A	B	F	E	D	B	B	sr 29	0	0	0	rf 1	K
Haw Euc 7895	B	B	F	E	B	D	B	sr 30	0	0	0	rf 1	P
SA Man BOT2404	D	B	D	C	D	?	B	sr 35	0	0	0	rf 1	B
SA Man BOT2294	B	B	D	C	D	B	B	sr 37	0	0	0	rf 1	C
SA Man BOT2390	?	B	D	C	D	L	B	sr 38	0	0	0	rf 1	N
SA Man BOT2330	D	B	D	C	D	L	B	sr 39	0	0	0	rf 1	O
SA Man BOT2341	D	B	D	C	D	A	B	sr 40	0	0	0	rf 1	O
Col Euc 8955	B	B	?	C	D	G	B	sr 44	0	0	0	rf 1	K
Col Euc 8966	B	B	?	C	B	F	B	sr 45	0	0	0	rf 1	K
Col Euc 8958	C	B	D	C	B	F	B	sr 46	0	0	0	rf 1	K
Col Euc 8948	B	B	D	C	D	F	B	sr 47	0	0	0	rf 1	H
Col Euc 8930	B	B	D	C	B	F	B	sr 48	0	0	0	rf 1	K
Col Euc 8931	B	B	D	C	B	F	B	sr 48	0	0	0	rf 1	K
Col Euc 8935	B	B	D	C	D	F	B	sr 47	0	0	0	rf 1	K
Col Euc 8936	B	B	D	C	D	F	B	sr 47	0	0	0	rf 1	K
Col Euc 8937	B	B	D	C	D	E	B	sr 49	0	0	0	rf 1	J
Col Euc 8938	C	B	D	C	D	F	B	sr 50	0	0	0	rf 1	K
Col Euc 8940	C	B	D	C	B	F	B	sr 46	0	0	0	rf 1	?
Col Euc 8942	B	B	D	C	D	?	B	sr 51	0	0	0	rf 1	K
Col Euc 8944	B	B	D	C	D	F	B	sr 47	0	0	0	rf 1	H
Col Euc 8945	B	B	D	C	D	F	B	sr 47	0	0	0	rf 1	I
Col Euc 8946	B	B	D	C	B	F	B	sr 48	0	0	0	rf 1	M
Col Euc 8947	C	B	D	C	C	G	B	sr 52	0	0	0	rf 1	G

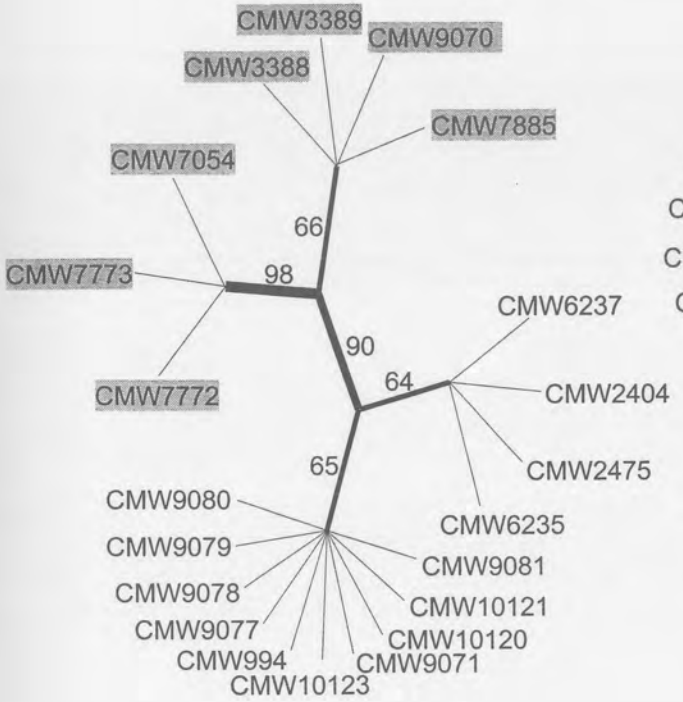
TABLE IV. Continued.

Genotype description[1]	SRR loci[2]							SSR genotype	RFLP loci			RFLP genotype	Not used
	11&12	15&16	17&18	21&22	23&24	35&36	37&38		1&2r	11&12r	15&16r		19&20
B. ribis sensu stricto													
NY Rib 7773	D	B	B	?	D	F	E	sr 31	0	0	1	rf 2	F
NY Rib 7772	D	B	B	?	D	F	E	sr 31	0	0	1	rf 2	G
NY Rib 7054	D	B	B	C	D	F	E	sr 32	0	0	1	rf 2	G
B. ribis sensu lato													
Col Euc 8965	C	D	B	C	B	B	C	sr 25	0	0	1	rf 2	?
Col Euc 8934	C	D	B	C	B	B	C	sr 25	0	0	1	rf 2	?
Col Euc 8953	C	D	B	?	B	B	C	sr 26	0	0	1	rf 2	?
Col Euc 8939	C	D	?	C	B	B	C	sr 27	0	0	1	rf 2	?
Aus Ara 3388	E	B	B	C	D	E	C	sr 33	0	0	1	rf 2	B
Aus Wol 3389	E	B	B	?	D	E	C	sr 34	0	0	1	rf 2	B
Aus Wol 9070	E	B	B	?	D	E	C	sr 34	0	0	1	rf 2	B
SA Man BOT2325	D	B	B	B	B	G	B	sr 36	0	0	1	rf 2	I
Col Euc 8964	B	B	?	C	D	H	?	sr 41	0	0	1	rf 2	F
Col Euc 8957	D	B	B	C	D	F	D	sr 42	0	0	1	rf 2	J
Col Euc 8952	D	B	B	C	D	H	C	sr 43	0	0	1	rf 2	C
Col Euc 8943	D	B	B	C	D	H	C	sr 43	0	0	1	rf 2	D
Haw Euc 7885	E	B	E	A	B	E	B	sr 53	0	0	1	rf 2	?
Haw Euc 7886	E	B	E	A	B	E	B	sr 53	0	0	1	rf 2	?
Haw Euc 7891	E	B	E	A	B	E	B	sr 53	0	0	1	rf 2	B
Haw Euc 7892	E	B	E	A	B	E	B	sr 53	0	0	1	rf 2	?

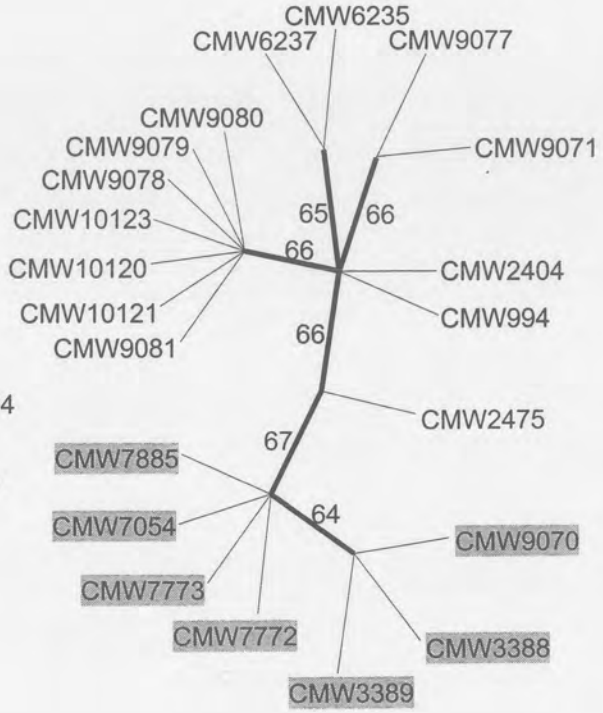
[1] First letters indicate the origin (Aus = Australia; Haw = Hawaii; Col = Colombia; NZ = New Zealand; NY = New York, USA; SA = South Africa). Second letters indicate host (Act = *Actinidia*; Ara = *Araucaria*; Cam = *Camphor*; Euc = *Eucalyptus*; Man = *Mangifera*; Pop = *Populus*; Rib = *Ribes*; Tib = *Tibouchina*; Wol = *Wollemia*). Numbers are CMW numbers (Culture collection of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria), except numbers indicated as BOT, which is refers to the *Botryosphaeria* sub-collection of CMW.

[2] Missing data are indicated with a '?'. Numbers under SSR or RFLP loci (e.g. 1&2) indicate the BOT primer pair used to amplify the locus. Alleles amplified using the primer pair BOT 19&20 is also included, although it was not used in the final analyses.

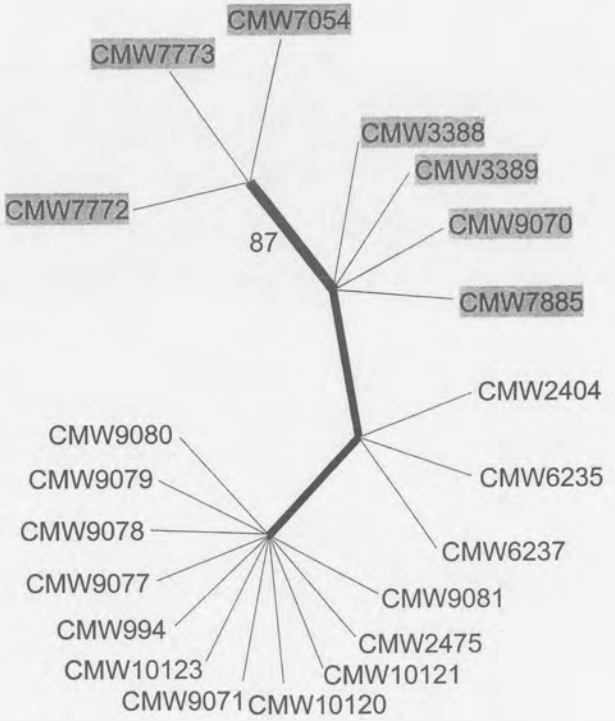
FIG. 1. Unrooted cladograms of the most parsimonious (MP) trees obtained after heuristic searches in PAUP. The thickness of the branches indicates the number of steps, and bootstrap supports (1000 replicates) for the clades are indicated next to the branches. Isolates representing *Botryosphaeria ribis* are shaded, while those representing *B. parva* are not. Trees were obtained from ITS rDNA, β–tubulin and EF1-α sequence datasets. Number of trees and tree statistics are indicated below the trees, including consistency index (CI), retention index (RI) and phylogenetic signal (g1).



EF1-*α*
1 MP tree
8 steps
CI = 1.0
RI = 1.0
g1 = - 0.85



ITS
1 MP tree
6 steps
CI = 1.0
RI = 1.0
g1 = - 0.57



β-tubulin
1 of 6 MP trees
7 Steps
CI = 0.71
RI = 0.92
g1 = - 0.67

FIG. 2. An unrooted cladogram indicating the relationship between *sensu stricto* and *sensu lato* groups of *Botryosphaeria parva* and *B. ribis*, indicated by differentially shaded groups. The tree was obtained from heuristic searches of combined dataset of the ITS rDNA, β–tubulin and EF1-α sequences. Ex-type isolates are underlined.

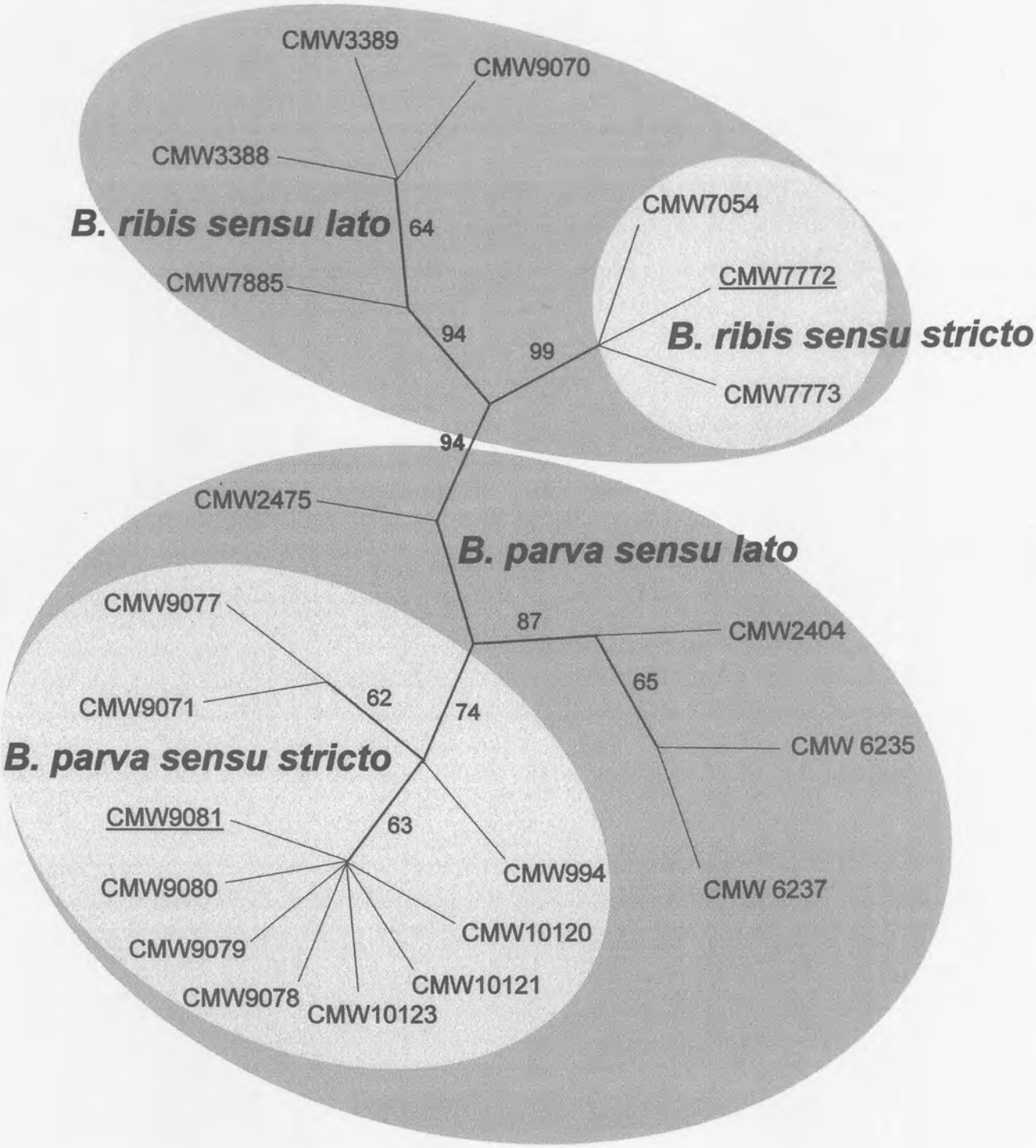
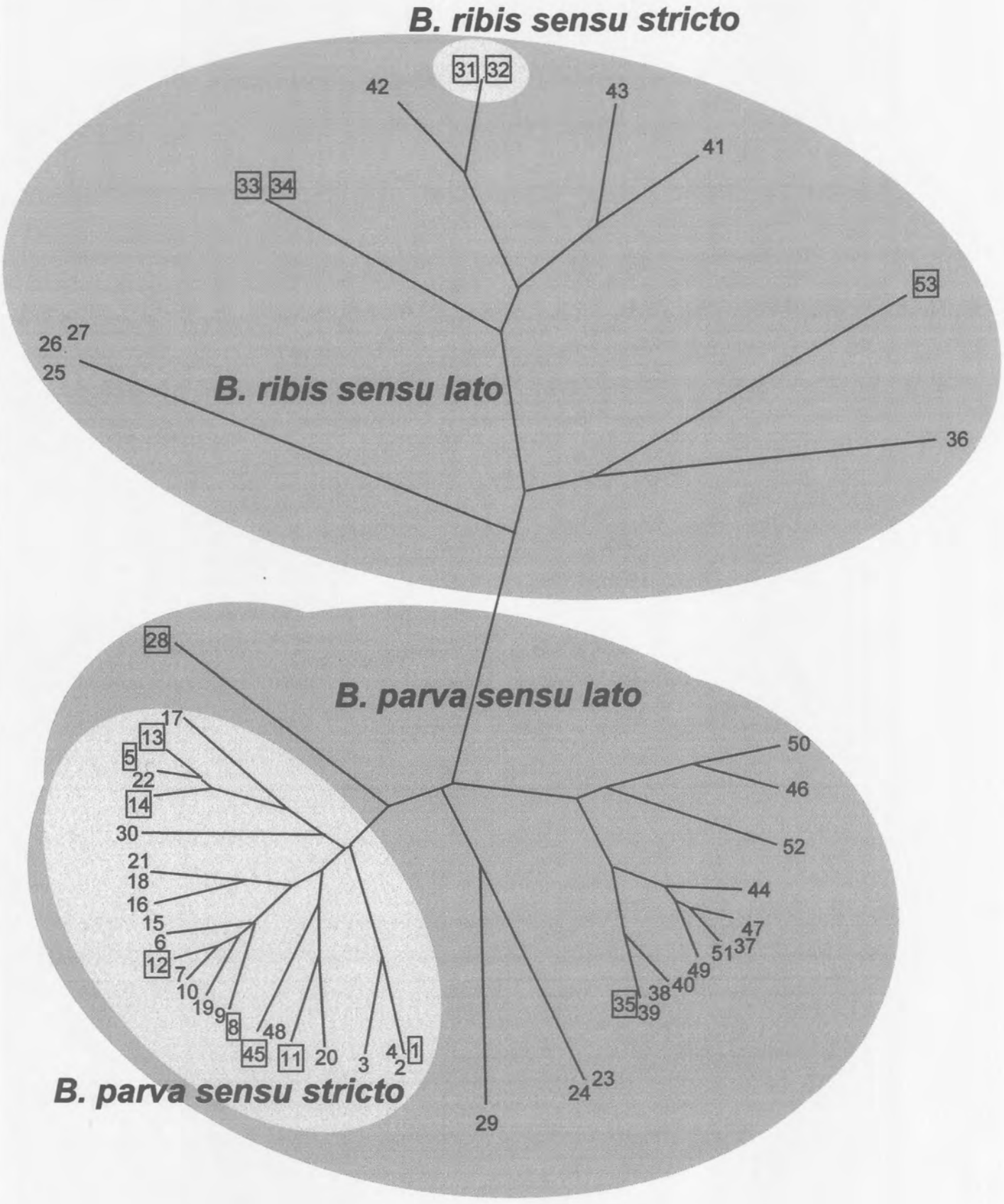
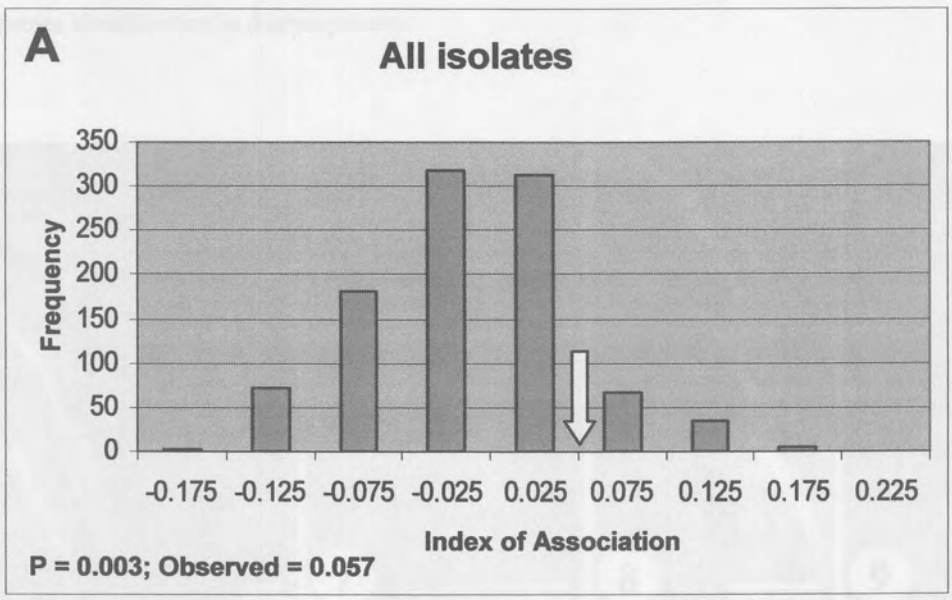


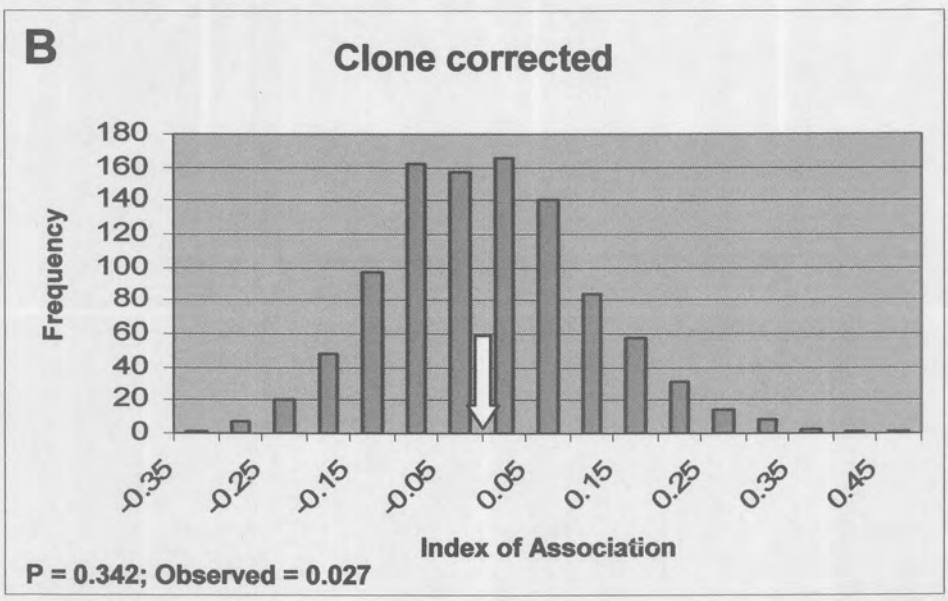
FIG. 3. An unrooted UPGMA dendrogram depicting the distance relationships between genotypes identified using polymorphic SSR markers. Genotypes that were sequenced and formed part of the phylogenetic study are in blocks. The tree was calculated from a clone corrected dataset of SSR and RFLP marker data. *Sensu lato* and *sensu stricto* groups of *Botryosphaeria parva* and *B. ribis*, as initially determined by multiple sequence data, are indicated by differentially shaded areas.



0.05 changes

FIG. 4. The position of the observed (arrow) Index of Association (I_A) of alleles for *Botryosphaeria parva sensu stricto* plotted against a randomized dataset, which represent the distribution when the population is randomly mating. The I_A was calculated for (A) a dataset containing all isolates of *B. parva sensu stricto* and (B) from a clone corrected dataset where only one representative of a genotype was used. The significance (P value) of the difference between the observed value and that of the randomized dataset is indicated at the bottom of the graph.





10

Evolving species concepts in *Botryosphaeria* over 140 years

Abstract: For many groups of living and extant organisms, the identification of species is a controversial issue. These concepts are often influenced by the current knowledge or tools available to delimit the boundaries between taxa. Since the description of the genus *Botryosphaeria* 140 years ago, numerous species have been added. The concepts and techniques that have been used to identify these species have, however, changed over this time. This has led to conflicting views and considerable confusion in *Botryosphaeria* taxonomy. Recent studies employing various molecular tools have, however, helped to delimit natural boundaries between species of *Botryosphaeria.* In this review, past and present species concepts used to identify species of *Botryosphaeria* are compared. An attempt is also made to circumscribe methods to identify and describe species of *Botryosphaeria* that will prevent a recurrence of many years of confusion that has plagued the taxonomy of this genus.

INTRODUCTION

The ascomycete genus *Botryosphaeria* Ces. & De Not. is a cosmopolitan group of fungi that has an exceptionally large host range, including gymnosperms and angiosperms (von Arx and Müller 1954, Barr 1972). These fungi can be saprophytes on dead or dying stems, branches, stalks, culms or leaves of plants. They are, however, best known as pathogens of woody plants, typically causing cankers, die-back and eventually the death of affected trees (von Arx 1987, Sinclair et al 1987). These diseases mostly follow the onset of stress due to factors other than *Botryosphaeria* infection (Christ and Schoeneweiss 1975, Wene 1979, Wene and Schoeneweiss 1980).

Infection by *Botryosphaeria* spp. has traditionally been thought to occur via wounds and this group has generally been referred to as wound pathogens (von Arx and Müller 1954) Recent discoveries have, however, shown that they can infect directly through lenticels, stomata or other openings on healthy plants without necessarily causing symptoms (Brown and Hendrix 1981, Michailides 1991, Smith et al 1996). After such infection these fungi can live endophytically in asymptomatic tissue until the host is stressed (Johnson et al 1992, Fisher et al 1993, Smith et al 1996). Given the wide host range and mode of infection, most woody plants appear have some level of *Botryosphaeria* infection.

The genus *Botryosphaeria* was erected in 1863 by Cesati and De Notaris for twelve ascomycete taxa. These include *B. dothidea* (Moug.:Fr.) Ces. & De Not., a new binomial for *Sphaeria dothidea* Moug.:Fr. described by Mougeot in Fries (1823). This species is regarded as the type of the genus. Subsequently, more than 140 species were described (Denman et al 2000).

There are few morphological distinctions between many of the *Botryosphaeria* spp. In their revision of the genus, von Arx and Müller (1954) synonymized a large number of species, particularly using the names *B. quercuum* (Schwein.) Sacc. and *B. dothidea*. *Botryosphaeria quercuum* was soon afterwards split into a number of distinct taxa (Shoemaker 1964). Although not all researchers accepted all the species synonymized under *B. dothidea*, this taxon was not revised again. *Botryosphaeria dothidea* and some of its synonyms (e.g. *B. ribis* Grossenb. & Duggar and *B. berengeriana* De Not.) have, thus been used inconsistently in the literature for many years. In terms of pathology, this has resulted in considerable confusion. Slippers et al

(2003) epitypified *B. dothidea* and characterized the taxa formerly treated under this name, including *B. ribis* and *B. parva* Pennycook & Samuels. However, the confusion of names used under these and other *Botryosphaeria* binomials in past studies remains problematic.

The most commonly used operational species concepts (OSC) for fungi include the morphological (MSC), biological (BSC), ecological (ESC) and, more recently, phylogenetic (PSC) species concepts (Brasier 1997, Harrington and Rizzo 1999, Taylor et al 2000). In *Botryosphaeria*, like in most other fungi, the MSC has been most dominantly used to identify and describe new species. In general this species concept underestimates the true diversity, by a factor of two to three as estimated based on a growing number of recent examples (Taylor et al 2000). This order of magnitude is much greater for the morphological concepts of *B. quercuum* and *B. dothidea* sensu von Arx and Müller (1954).

The OSC in fungi has been greatly aided by the application of the BSC since the 1950's (Brasier 1997, Harrington and Rizzo 1999, Taylor et al 2000). The BSC concept has, however, never been used for *Botryosphaeria* spp., because they rarely produce ascomata in culture. An ESC, focussed on host specialization, has been widely used to identify new taxa in *Botryosphaeria* (Cesati and De Notaris 1863, De Notaris 1863, Saccardo 1877, 1882, Grossenbacher and Duggar 1911, Putterill 1919, Trotter 1928, Grove 1937). Host specialization is, however, not always practical, because some *Botryosphaeria* species have very wide host ranges, e.g. *B. obtusa* (Schwein.) Shoemaker, *B. parva*, etc. (Stevens and Jenkins 1924, Punithalingam and Waller 1973, Pennycook and Samuels 1985).

In recent years, molecular data have been used increasingly to identify groups and species amongst the fungi, including *Botryosphaeria* (Palmer et al 1987, Stanosz et al 1996, Jacobs and Rehner 1998, Smith and Stanosz 2001, Zhou and Stanosz 2001a, b). Of these DNA sequence data have been most commonly applied in recent years and they have had a profound impact on the taxonomy of *Botryosphaeria* spp. Differences in DNA sequences have also been successfully combined with morphological characteristics to identify and describe *Botryosphaeria* species (Smith et al 2001, Phillips et al 2002, Denman et al 2003).

Most taxonomic studies on *Botryosphaeria* using DNA sequence differences have used ITS rDNA phylogenies. Use of this single gene can underestimate the true species diversity especially among closely related or cryptic species (Taylor et al

2000). In this regard, multiple gene sequence concordance phylogenies have been successfully applied to identify cryptic *Botryosphaeria* species, previously overlooked or of uncertain identity (De Wet et al 2003, Slippers et al 2003, Chapters 4, 6, 7).

A number of recent reviews have provided thorough discourse on species and species concepts in fungi, including contemporary techniques (Brasier 1997, Harrington and Rizzo 1999, Taylor et al 1999, 2000). The taxonomy of *Botryosphaeria* and its anamorphs has also been adequately reviewed, including the provision of keys to important species (Denman et al, 2000, Phillips 2002 www.crem.fct.unl.pt/botryosphaeria_site). In this review, we refer to these issues only in summary. Our aim is rather to assess the influence that changing theoretical and practical ideas, and techniques, have had on the taxonomy and the understanding of the biology of *Botryosphaeria.*

SPECIES CONCEPTS

At the outset, it is important that we define our view of *Botryosphaeria* species, as this clearly influences the views that we will present throughout this paper. We favor the definitions of Brasier (1997) and Taylor et al (2000) that consider a fungal species as an identifiable unit of biodiversity, or an identifiable unit from which other species will evolve, whether an organism has a sexual or asexual life-cycle. Furthermore, our preference is for a phylogenetic species concept implying that there is no internal phylogenetic structure within a species. The species concept defined by Nixon and Wheeler (1990), and applied to fungi (Harrington and Rizzo 1999, Taylor et al 1999, 2000), suggesting that a species is the smallest aggregate of populations identifiable by a unique character, is followed in this review.

Harrington and Rizzo (1999) and Brasier (1997) suggest that species delimiting characters should be phenotypic. Thus, multiple gene genealogies or other molecular techniques can indicate species boundaries, but in the absence of distinctive phenotypic characters, should not be used to describe taxa. If two species represent a continuum of populations, then identifying a recent barrier to recombination without further relevant phenotypic information makes the description of species obsolete. The objection that the systematic system then lacks a piece of recent evolutionary information is discarded, because this unit contains no or very little unique characters (Harrington and Rizzo 1999, Brasier 1997). This is a weakness as differentiating

phenotypic characters might not yet have been discovered or their phylogenetic value could have been overlooked. This is especially true for cryptic species. An effort should thus be made to re-evaluate known phenotypic characters and to look for new distinguishing characters following molecular identification of recent species barriers (Taylor et al 2000).

TRADITIONAL PHENOTYPIC AND ECOLOGICAL IDENTIFICATION AND CLASSIFICATION

Morphology has been the most widely used phenotypic character to distinguish between and describe species of *Botryosphaeria*. This is also true for most other fungi. There are, however, a limited number of morphological characters that are consistently useful to distinguish between these species. Furthermore, the morphological features that are most often used such as spore morphology are highly variable and sexual fruiting structures are not always present. As a consequence, these continuous characters overlap between species, making identifications very difficult. This problem is enhanced in closely related, sibling species. Other phenotypic characters such as physiology, chemical products and life-cycle have not been widely used to identify *Botryosphaeria* species. Where they have been used, they have been found to be inordinately variable within species to be of taxonomic value (Kay et al 2002).

Teleomorph.--For the description of the genus *Botryosphaeria* and until the 1950's, the general morphology of the ascospores (hyaline, aseptate, shape, etc,) and stromatal and ascomatal morphology was considered taxonomically and phylogenetically informative. These characters were later shown to be inordinately variable within species and of little value for distinction between species.

Cesati and De Notaris (1863) described the genus *Botryosphaeria* for a group previously named *Sphaeria*, *Gibbera* and *Dothidea* spp. Sacccardo (1877) amended the initial description to exclude species with hypocreaceous stromata or ascomata. During this time, the ascomata carried in botryose clusters in well-developed stromata and ascospores (8-spored, hyaline, ovoid to ellipsoidal, unicellular or rarely septate) were considered important characters. Between 1863 and the 1920's these characters

were used to describe a large number of species (De Notaris 1863, Winter 1886, Saccardo 1877, 1882, Trotter 1928 and others).

Von Arx and Müller (1954) recognized the variation of ascomata within species of *Botryosphaeria*. In their revision of the genus they placed substantial emphasis on ascospore size to distinguish taxa. These authors studied herbarium specimens of *Botryosphaeria* spp. and other taxa and synonymized 108 species under *B. quercuum* (ascospores 24--42 x 10--18 µm) and 24 species under *B. dothidea* (ascospores 15--26 x 6--9µm). This study represented an important revision and consolidation of a diverse group of fungi. The very broad species concept used by von Arx and Müller was, however, not widely accepted. *Botryosphaeria quercuum* and *B. dothidea* were subsequently shown to include numerous distinct species (Shoemaker 1964, Slippers et al 2003).

There are a number of objections to the use of ascospore size or morphology as a primary character to delimit species of *Botryosphaeria*. While some species are easily distinguishable based on the ascospore size, in others these spores have exactly the same size and shape (Shoemaker 1964). The morphological variation that exists within some species makes it difficult to determine the relevance of a specimen such as those represented in many herbarium collections. Furthermore, in some species, the teleomorph structures are infrequently found in nature Teleomorph structures and spores are also very rarely induced in culture, which makes this character useless to identify pathogens that are isolated from disease symptoms.

Botryosphaeria asci and ascospores develop and mature slowly. Consequently, they are often collected in an immature state (von Arx and Müller 1954, Slippers et al 2003). The immature spores generally do not have the same dimensions as those of mature spores. They mostly lack important characters such as septa that develop with age. As previously stated, spore size was not of primary importance during the initial years when most *Botryosphaeria* species were described. Many *Botryosphaeria* species are, therefore, represented by herbarium specimens bearing immature spores. These include important species such as the type specimen of *B. dothidea*.

A possible solution to some of the problems experienced when working with early species descriptions or indistinct herbarium specimens of *Botryosphaeria* species, is to select epitype samples that better represent the specific taxon. An added advantage of these collections is that ex-epitype culture can be obtained from the

epitype specimen. Such isolates are essential when assessing species concepts using contemporary DNA based tools. When selecting an epitype, effort should also be made to collect additional specimens and isolates representative of the diversity of the population from which the ex-type isolate originated.

Botryosphaeria dothidea, the type species of the genus, is the only species that has been epitypified in this genus (Slippers et al 2003). An ex-type isolate from this sample has been deposited in major culture collections and its DNA sequences deposited in GenBank. These data and collections now allow for easy identification of isolates thought to belong to this taxon.

Currently, *Botryosphaeria* includes species that lack or have only inconspicuous stromata that are imbedded or immersed in the host tissue and that range from uniloculate to multiloculate, with or without a papilla, (FIGS. 1-5). Consistent characters are the 8-spored, bi-tunicate, clavate asci, and the filliform pseudoparaphyses between the asci (FIGS. 6-8). The ascospores of *Botryosphaeria* are hyaline, ovoid to ellipsoid, and mostly aseptate, although some species (e.g. *B. quercuum*, *B. obtusa*) may become septate with age (FIGS. 6-8). They range from 18--50 µm x 6--18 µm in size. The ascospores of *Botryosphaeria* species vary and overlap in size, but other features are useful for species identification. Thus, the brown discoloration and up to three septa in aging ascospores of *B. quercuum* is characteristic (Shoemaker 1964). The value of teleomorph characters should thus not be completely disregarded.

The higher classification of *Botryosphaeria* is based on the morphology of the ascomata and asci. Characters such as the ontogeny of the ascomata and the interthecial tissues have also been used at the higher taxonomic placement of *Botryosphaeria*. This genus does not have true perithecial walls (pseudothecia) and has pseudoparaphyses that form in the ascomal cavities before the asci (von Arx and Müller 1954, Luttrell 1965, Eriksson 1981). Based on these characters and DNA sequence data, this genus is currently classified in the family Botryosphaeriaceae in the order Dothideales, class Loculoascomycetes (Sivanesan 1984, Kirk et al 2001, Denman et al 2000).

Anamorph.--Since the 1960's the value of using morphological characters of the anamorphs to delimit and identify *Botryosphaeria* spp. has been realized and widely

applied (Shoemaker 1964, Pennycook and Samuels 1985, Morgan-Jones and White 1987, Denman et al 2000, Phillips et al 2002, Slippers et al 2003). There are many advantages to using anamorphs to identify the associated *Botryosphaeria* species. These forms are more frequently found in nature than teleomorphs and are easily induced in culture. Conidia are also much more diverse in shape and size than the associated ascospores. Conidial characters that can be used include wall thickness, ornamentation, maturation, color, septation, shape and size (length, width, l/w and lxw) (FIGS. 6-21).

Early taxonomy of *Botryosphaeria* anamorphs has been very confusing. Eighteen anamorphs have been linked to this teleomorph genus, the most common of which include *Botryodiplodia* (Sacc.) Sacc., *Diplodia* Fr., *Dothiorella* Sacc., *Fusicoccum* Corda, *Lasiodiplodia* Ellis & Everh., *Macrophoma* (Sacc.) Berl. & Voglino and *Sphaeropsis* Sacc. (Sivanesan 1984, Denman et al 2000). Recent studies (Denman et al 2000) have, however, suggested that many of these taxa need to be synonymized. For example, the type species of *Dothiorella* has been reduced to synonymy with *Diplodia*, while *Botryodiplodia* was shown to be a *nomen dubium* (Crous and Palm 1999). *Macrophoma* was reduced to synonymy with *Sphaeropsis* (Sutton 1980).

There are questions regarding the validity of separating *Sphaeropsis*, *Diplodia* and *Lasiodiplodia* (Denman et al 2000). *Sphaeropsis* and *Diplodia* differ only in percurrent proliferation in the conidiogenous cells in *Sphaeropsis* and the time of septation of ageing spores. Neither of these characters is unique to either group, or are synapomorphies, because both features are also seen in *Fusicoccum*. *Lasiodiplodia* differs from other *Diplodia* species only by prominent melanin deposits or striations in the conidial walls (Denman et al 2000). The taxonomic value of these deposits is doubtful because they also occur in other *Diplodia* spp.

Denman et al (2000) reviewed anamorph taxa of *Botryosphaeria* and concluded that *Fusicoccum* and *Diplodia* were the only two genera that can be validated. These taxa were delineated as having hyaline conidia (*Fusicoccum*) or conidia that are often pigmented when they age (*Diplodia*) (FIGS. 6-21). Phillips (2002) supports the retention of only these two anamorph genera, but has suggested that they are better defined by cell wall thickness and conidial width. *Diplodia* species have wider conidia (normally more than 10 µm) with thicker walls (0.5--2 µm), while

Fusicoccum has narrower conidia (normally <10 μm) with thinner walls (<0.5 μm) (Phillips 2002) (FIGS. 6-21). Von Arx and Müller (1954) note that a conglomeration of species under the *B. quercuum* has *Diplodia* (reported as *Botryodiplodia*) anamorph states. They also note that species synonymized with *B. dothidea* have *Fusicoccum* anamorph states (reported as *Dothiorella*).

Amongst *Botryosphaeria* anamorph characters, only the conidia are consistently used to distinguish taxa. The pycnidia are often indistinguishable from ascomata and of equally little value when distinguishing species boundaries (FIGS. 1-5). Conidiophores and conidial development have also not been valuable in delimiting species (FIGS. 16-17). Microconidia or spermatia have occasionally been reported (Pennycook and Samuels 1985, Denman et al 1999, 2003, Phillips et al 2002, Slippers et al 2003) (FIG. 9). They, however, appear to be less common and easily overlooked. It is not clear whether microconidia have simply been overlooked in studies that do not record their presence or whether they have any phylogenetic or taxonomic value.

Despite the value of conidial morphology, for some species considerable overlap exists when using this character. This overlap is most problematic for recently diverged species. For example, the conidia of *F. eucalyptorum* Crous, Smith & M.J. Wingf. and *F. irregulare* Slippers, Crous & M.J. Wingf. are virtually the same in shape, length and width, maturation, etc. (Chapter 5), although these species differ slightly in overall size or dimensions of the conidia. Another example is that of *B. parva* and *B. ribis*, where young conidia are indistinguishable from each other (Slippers et al 2003). *Botryosphaeria parva*, however, commonly produces two-septate conidia with a darker middle cell, unlike the single septum and consistently colored conidia in *B. ribis*. *Botryosphaeria lutea* A.J.L. Phillips and *B. australis* Slippers, Crous & M.J. Wingf. can also not be easily distinguished from each other based on conidial morphology (Chapter 3), but can be distinguished based on differences in cultural morphology. The above-mentioned differences are so slight that, in the absence of DNA based comparisons, they could easily have been considered as encompassing variation within a species.

Cultural characteristics.--Since the mid 1980's cultural characteristics have been commonly used to augment other characters. In general, cultures of *Botryosphaeria* spp. are easily distinguished from most other fungi by their grey to black, aerial mycelium and the grey to indigo grey or black pigment that is visible from the reverse

side of Petri dishes. The appearance and color of aerial mycelium and pigments have also aided in the delimitation and rapid identification of *Botryosphaeria* taxa that are otherwise morphologically similar.

Pigments were not always recognized, e.g. Witcher and Clayton (1963) disregarded the yellow pigment in cultures of '*B. dothidea*' as variation in the species. More recently, pigments produced in culture have, however, been recognized as valuable in distinguishing certain *Botryosphaeria* species. Pennycook and Samuels (1985) found that conidial morphology overlapped between *F. luteum* Pennycook & Samuels and anamorphs of both *B. dothidea* and *B. parva*. In young cultures, *F. luteum* produces a unique bright yellow pigment. Phillips et al (2002) also noted this yellow pigment in cultures from *Vitis* and subsequently described the teleomorph, *B. lutea*. Another example is that of Jacobs (2002) who found that the only phenotypic characteristic that separates the *F. indigoticum* Jacobs, Slippers & M.J. Wingf. prov nom. from closely related taxa, is the distinctly darker (dark indigo-black) pigment in cultures of this species.

Apart from pigments, the extent and color of aerial mycelium and rate of growth of cultures has been used to characterize taxa or subspecies groups in *Botryosphaeria*. The morphotypes of the anamorph *Diplodia pinea* (Desm.) J. Kickx (= *Sphaeropsis sapinea* (Fr.) Dyko & B. Sutton) could thus be distinguished based on the mycelial texture and aerial mycelium in cultures (Palmer et al 1987). Study of greater numbers of isolates eventually showed that these characteristics are insufficiently variable to consistently distinguish all of the groups (De Wet et al 2002, Kay et al 2002). *Botryosphaeria dothidea* and *B. lutea* also have thinner mycelial mats and less aerial mycelium than *B. parva* and *B. ribis* (Pennycook and Samuels 1985, Slippers et al 2003). Pennycook and Samuels (1985) showed distinct growth rates between *B. dothidea*, *B. parva* and *F. luteum* at different temperatures. However, intra species variation, effect of different media and age of cultures, reduce the value of this character.

Host relations.--Ever since the first description of *Botryosphaeria*, ecology and biology have influenced the view regarding species delimitation. Two main views are prevalent. Many taxonomists have considered as important, the ability to infect a specific suite of hosts. Many *Botryosphaeria* species and their anamorphs have thus been described based on host. Examples include *B. quercuum* (from *Quercus*), *B. ribis*

Vitis), and many other names (Grossenbacher and Duggar 1911, Putterill 1919, Trotter 1928). This view of strict host pathogen evolution arose from a plant pathology and a co-evolutionary perspective. Grove's (1937) statement best illustrates the logical argument that led to this approach:

> 'The majority of the species of *Diplodia* and *Botryodiplodia* are extremely similar to one another, especially in regard to the spores. They can be discriminated only by host-plant…a shrub can be seen to be badly attacked by a *Diplodia*, and yet surrounding shrubs may show no sign of infection although they belong to a species which, in other localities, can be found abundantly infested with a *Diplodia* having spores apparently exactly like those of the first mentioned."

A second and strongly held view for many years has been that *Botryosphaeria* species are mainly wound infecting or saprophytic fungi (Von Arx and Müller 1954). If this were correct, they would need no host specialization. This view contributed to a very broad, 'super-species' concept for *Botryosphaeria*. Stevens and Jenkins (1924), Punithalingam and Waller (1973), Punithalingam and Holliday (1973) and others also report extremely wide host ranges for *B. ribis* and *B. obtusa* that include dicotyledenous and monocotyledenous plants.

Both the views that *Botryosphaeria* species are host specialized pathogens or that they are secondary, often saprophytic fungi are considered correct. Certain well-studied species infect a wide range of hosts, e.g. *B. obtusa*, *B. parva*, *B. dothidea, B. rhodina* (Berk. & Curtis) Arx (Punithalingam and Waller 1973, Pennycook and Samuels 1985, Phillips et al 2002, Slippers et al 2003). On the other hand, some well characterized species are clearly specialized on certain hosts e.g. *D. pinea* on *Pinus* and other conifers, *B. eucalyptorum* Smith, Crous & M.J. Wingf. and *B. irregularis* Slippers, Crous & M.J. Wingf. on *Eucalyptus*, *B. protea* (Wakef.) Denman & Crous and *B. protearum* Denman & Crous on Proteaceae species (Smith et al 1996, Smith et al 2001, Burgess et al 2003, Phillips et al 2002, Denman et al 2003, Slippers et al 2003). In these examples, closer examination of *Botryosphaeria* isolates from indigenous hosts has often revealed these distinct species. They have commonly been overlooked in the past, because of morphological similarities or uncertainty regarding the phylogenetic value of small morphological differences. Many of these species also occur sympatrically.

INFLUENCE OF MOLECULAR TECHNIQUES

A number of molecular techniques have been used to study variation and relationships in the fungi. The value of all these techniques to identify species, however, varies, particularly where cryptic species are concerned. Many DNA-based and other molecular tools have been developed for studies at the population level. These tools are increasingly used to understand questions at the interface between populations and species. For this reason, we also include a discussion on some population level approaches and reflect on how they have increased our understanding of the evolution and speciation in *Botryosphaeria*.

Isozymes.--Isozyme patterns were used to distinguish the A and B morphotypes of *D. pinea* (Stanosz et al 1999). These morphotypes have more recently been shown to represent two distinct taxa, *D. pinea* and *D. scrobiculata* De Wet, Slippers & M.J. Wingf., following the production of extensive multigene phylogenies (De Wet et al 2003). Interpretation of the phylogenetic value of isozymes for *Botryosphaeria* systematics is difficult given that it provides little information. From the example discussed here, it seems that variation in these markers could indicate speciation, thus validate further investigation.

RAPD's.--Randomly Amplified Polymorphic DNA markers have been used to distinguish between groups within or between established morpho-species (Smith and Stanosz 1995, Stanosz et al 1999, Smith and Stanosz 2001). The advantage of using RAPD's is that they give a genome-wide perspective. A distinct disadvantage is that they are not always reproducible. This concern is diminishing with increasingly refined technology and standardized methods and reagents. They are, however, dominant markers and the level of homoplasy cannot be determined from their data (except where further analysis is done through hybridization). For this reason, RAPD data have not impacted on the identification or formal taxonomy of *Botryosphaeria*. They have, however, in some cases provided useful preliminary data used to justify further phylogenetic and taxonomic studies.

Data from RAPD's often provide support for *Botryosphaeria* groups that have been defined based on other data, such as morphology or sequence data (Smith and Stanosz 1995, 2001). This is, however, not true for comparisons between closely

related taxa. In these cases RAPD's tend to under-estimate species diversity. For example, RAPD's did not distinguish between *B. ribis* and *B. parva,* nor between *B. lutea* and *B. australis* (Smith and Stanosz 2001). RAPD's can also over-estimate species diversity. Thus RAPD's identified four distinct groups in *D. pinea* (Stanosz et al 1996, De Wet et al 2000, Hausner et al 1999). These groups were later shown to represent three species, namely *D. pinea*, *D. scrobiculata* and *B. obtusa*.

Given the difficulty in interpreting the phylogenetic value of RAPD's, this technique is probably best restricted to comparisons within species and not those between taxa. In other words, it would be more useful in population studies following appropriate identification of species (Ma et al 2001).

ISSR's.--Inter simple sequence repeat (ISSR) PCR, which is also known as microsatellite primed PCR, has been used only once to test phylogenetic species concepts in *Botryosphaeria* (Zhou et al 2001). Unlike RAPD's the results emerging for this technique were comparable with those of other phylogenetic studies (Zhou et al 2001). ISSR markers provide a genome-wide perspective, which is similar to RAPD's. However, they are generally more reproducible, because longer primers and higher annealing temperatures are used (McDonald 1997). The data from the latter study was used to successfully distinguish between well defined species (e.g. *B. dothidea*, *B. lutea* and *B. ribis*), as well as recently diverged species (e.g. *B. ribis* and *B. parva*). In the distinction of *B. parva* and *B. ribis* ISSR data were more useful than ITS rDNA sequences, RAPD's or morphology. This technique, therefore, appears to hold promise for indicating species boundaries amongst large numbers of isolates of *Botryosphaeria*. The data obtained from ISSR markers are, however, dominant and need to be complemented by other phylogenetically informative data before taxonomic proposals can be considered. These markers have also been useful to study intra-specific variation in *B. dothidea* (Ma et al 2001).

SSR markers.--Simple Sequence Repeat (SSR) markers are co-dominant that are useful to study population level questions in fungi. SSR markers have recently been developed to study populations of *D. pinea* and *D. scrobiculata, B. rhodina*, and *B. parva* as well as other *Botryosphaeria* species with *Fusicoccum* anamorphs (Burgess et al 2001a, b, 2003, Chapter 8). These markers have significantly increased our

understanding of the population structure, mode of reproduction and spread of these important pathogens (Burgess et al 2003, Chapter 9).

The phylogenetic value of data from SSR makers has been questioned, because of the high mutation rate and constraints on maximum size, which increase the level of homoplasy. Studies by Fisher et al 2000 (*Coccidioides immitis* G.W. Stiles) and Burgess et al 2001a (*D. pinea*) have, however, shown that these markers can reflect species boundaries, even between cryptic species. In both these cases the SSR marker data were, however, interpreted by comparing results from multiple gene genealogies, which confirmed their phylogenetic value (Kofoupanou et al 1997, Fisher et al 2000, De Wet et al 2003).

The superiority of SSR markers over other methods used to indicate species boundaries for cryptic species is well illustrated in studies of the *Botryosphaeria* anamorphs, *Diplodia pinea*. This fungus is a well known pine pathogen that has been studied extensively for many years (Punithalingam and Waterston 1970). Identification has primarily been based on the host, as well as conidial ontogeny and morphology and a large number of synonyms have emerged for it (Sutton 1980). Recent studies using RAPD markers, combined with morphology and epidemiology, have shown that there are as many as four 'morphotypes' for this taxon and these have become known as the A, B, C and I morphotypes (Palmer et al 1987, Smith and Stanosz 1995, Hausner et al 1999, de Wet et al 2000). Some studies have, however, shown considerable variation in the characters used to distinguish these morphotypes (Swart et al 1991, 1993, Kay et al 2002). Single gene phylogenies also do not separate all these morphotypes, or even clearly distinguish them from the closely related *B. obtusa*. Microsatellite markers have revealed that there is no genetic exchange between the B morphotype and the A and C morphotypes (Burgess et al 2001a). Subsequently, comparison of sequence data for some of these markers, as well as for some gene coding regions, has confirmed that the B morphotype is a distinct species, namely *D. scrobiculata* (De Wet et al 2003). These markers also showed that the 'I' morphotype represents *B. obtusa* (Burgess et al 2001a).

In one study in this thesis (Chapter 9) the separation of the closely related species *B. parva* and *B. ribis* is confirmed using combined gene genealogies, SSR makers and PCR RFLP fingerprints. These data, especially the RFLP profiles, also provided a quick and effective way to distinguish these species. Furthermore, following characterization of *B. parva* and *B. ribis* their distribution was also studied,

and it was shown that these fungi, especially *B. parva*, have been moved around the world on hosts such as *Eucalyptus*. It was also clear from the SSR marker data that there is free recombination among a world-wide population of *B. parva*, which was the only species for which sufficient isolates were available to make comparisons at a population level. Despite the ability of *B. parva* to reproduce sexually a number of clonal lines were, however, identified that occurred across spatial and temporal boundaries.

SSR markers can differentiate between inter- and intra specific variation where species boundaries are unclear and this is an advantage over other techniques used to distinguish species or study populations. For example, SSR markers have not only revealed population structures in *B. parva* and *B. ribis*, but they were also used in combination with multiple gene genealogies to identify *sensu lato* and *sensu stricto* groups in these species. As a result of studies of this nature, the identified sets of isolates can be used to further characterize phenotypic distinctions between, and variation within the species. Selecting representative isolates to characterize a species, especially for cryptic species, is often difficult using other techniques (Davis and Nixon 1992, Taylor et al 1999). Fisher et al (2000) also found that SSR data can serve the dual purpose of identifying inter- and intra specific variation among a group of isolates to enable further testing of population and species hypotheses.

Single locus sequence data.--The Internal Transcribed Spacers and 5.8S gene of the ribosomal DNA complex (ITS rDNA) has been the most widely used in *Botryosphaeria* phylogenetics and systematics. Despite significant contributions, data from this region alone is insufficient to distinguish closely related sibling species.

The first study to test phylogenetic hypotheses for species of *Botryosphaeria* using rDNA sequence data was by Jacobs and Rehner (1998). These authors combined sequence data with morphological characters and showed that *B. dothidea* was paraphyletic. Subsequently, this paraphyletic group has been shown to represent *B. ribis* and *B. dothidea* (Smith and Stanosz 2001, Zhou and Stanosz 2001a, Zhou et al 2001, Slippers et al 2003).

A number of studies followed that of Jacobs and Rehner (1998) in using the ITS rDNA region to determine phylogenetic relationships among *Botryosphaeria* species. Data from this region was used in combination with RAPD or ISSR data (Smith and Stanosz 2001, Zhou et al 2001), morphology (Smith et al 2001, Zhou and

Stanosz 2001a, Denman et al 2003) or both (Phillips et al 2002). In most cases where these data have been combined with morphology, taxonomic proposals could be made. In some cases, however, the ITS data combined with morphology and RAPD's could not distinguish closely related species or resolve the taxonomic issues, for example in the distinction of *B. parva* and *B. ribis* (Smith and Stanosz 2001).

All the ITS-rDNA studies have shown that there is a separation between *Botryosphaeria* spp. with *Fusicoccum*-like and *Diplodia*-like anamorphs. Jacobs and Rehner (1998) first noticed this subdivision. Denman et al (2000) refers to these groups as representative of the *Fusicoccum* and *Diplodia* groups, which are the only anamorphs that they consider relevant to the genus. Zhou and Stanosz (2001a) supported these findings and refer to these groups as section Hyala and section Brunnea. However, not all *Botryosphaeria* species group in one of these two sections, e.g. *B. zeae* (G.L. Stout) Arx & E. Müll. and *B. proteae* (Denman et al 2000, FIG. 22). When a larger number of *Botryosphaeria* species representative of the true diversity of the genus are thus considered, the separation of the genus into only two groups appears to be of lower phylogenetic value.

Mitochondrial small subunit sequence data have been applied only once to phylogenetic questions in *Botryosphaeria* (Zhou and Stanosz 2001b). The phylogenies from these data were not congruent with those developed using ITS rDNA data or those data from other gene regions as discussed below. For example, the split between *Fusicoccum* and *Diplodia* was not supported by the mitochondrial small subunit DNA sequence data. *Botryosphaeria dothidea*, *B. corticis* (Demaree & Wilcox) Arx & E. Müll. and *B. mamane* D.E. Gardner, which all have *Fusicoccum* anamorphs, grouped within the *Diplodia* clade. mtSSU data, however, clearly revealed the distinction between the genus *Botryosphaeria* and *Guignardia* species that were previously described within it. The incongruence between the mitochondrial and nuclear gene sequence datasets is not unexpected given the fact that these genomic regions have independent evolutionary histories.

Multiple gene genealogies.--There are dangers in using single gene phylogenies to infer species phylogenies, because diversity can be either over or under estimated, depending on the evolutionary history of the genes and organisms studied (Taylor et al 2000). For this reason, a phylogenetic species concept based on concordance between unlinked gene genealogies has increasingly been promoted and applied to

identify species units in fungi (Koufoponou et al 1997, Geiser et al 1998, O'Donnell et al 1998, Taylor et al 1999, 2000, Steenkamp et al 2002). Following this concept, independent gene genealogies will only be consistent in identifying a distinction between taxa after genetic isolation. Any continued genetic exchange, or even remnants of recent genetic exchange, will randomize the polymorphisms in each genealogy and thus cause discrepancies between the individual phylogenies that they indicated. Concordance and combination of multiple gene genealogies have also played an important role in defining species boundaries between closely related and cryptic *Botryosphaeria* species (De Wet et al 2003, Slippers et al 2003, Chapter 3, 5).

The inability of single gene phylogenies, co-dominant molecular data or any specific phenotypic character to confirm the distinction between the cryptic species *Diplodia pinea* and *D. scrobiculata* have been discussed above. *Botryosphaeria parva* and *B. ribis* were equally difficult to distinguish from each other. In both these species complexes, SSR markers confirmed genetic isolation between groups of isolates representing these species (Burgess et al 2001a, Chapter 9). It was, however, only through the use of combined multiple gene genealogies that phylogenetic distinction between these species could be confirmed. Following the identification of the phylogenetic species, the small phenotypic differences between these taxa could be interpreted as useful for identification.

Single gene phylogenies have indicated the distinction between *B. australis* and *B. lutea*, as well as between *B. eucalyptorum* and *B. irregularis* (Chapters 3, 5). However, these species are so closely related that the lengths of the branches defining each species are insignificant in comparison with those between more robustly defined *Botryosphaeria* species. These small differences, combined with the fact that these species are morphologically almost identical, make it difficult to justify their separation. However, when data from three independent gene regions are compared, it is clear that the polymorphisms in each genealogy group the same isolates together (Chapters 3, 5). These fixed polymorphisms in each group for the different genealogies are indicative of reproductive isolation, despite their sympatric occurrence. When these DNA sequence datasets were combined they then provided strong support for the separation of these species.

The number of genes that need to be sequenced to accurately reflect species boundaries will depend on how closely related the species are. Our experience has shown that for *Botryosphaeria*, if only two regions had to be chosen, the ITS rDNA

and EF1-α regions would be preferable (Chapter 9). Data for the ITS rDNA region is the most widely available for *Botryosphaeria* species and is useful to place species in tentative groups. The EF1-α has been the most variable and thus useful to indicate distinctions between closely related species. β-tubulin sequences lacked one of the larger introns normally found in this region (B. Slippers unpublished data) and generally had little variation in most of the studies where it was used. The other gene regions (Calmodulin, Actin, rDNA Intergenic spacer) have only been used for *D. pinea* and *D. scrobiculata* and it is difficult to assess their value for other species (De Wet et al 2003).

Species-specific primers and PCR RFLP identification.--After the identification of a species using the methods described above, there is usually a need for a rapid and effective tool to identify the species. This is especially true where a specific host (especially cultivated and introduced hosts) is infected by a complex of *Botryosphaeria* species (Jacobs 2002, Chapters 5, 6). As discussed above, phenotypic characters are often inadequate to distinguish closely related species and sequencing large numbers of species for routine identification is impractical. In this regard species specific primers and PCR RFLP profiles have proven useful identification tools for various *Botryosphaeria* species.

Species-specific primers have been applied to identify subspecies or species groups in *Botryosphaeria* (Ma and Michailides 2002a). These primers were designed in the ITS1 region. Unfortunately, the groups for which the primers were designed, has not been identified to species level. This tool can be robust for the identification of *Botryosphaeria* species if correctly applied, and should be further explored.

There are some factors that hamper the development of species-specific primers for some *Botryosphaeria* species. The ITS rDNA region has been shown to be insufficient to distinguish closely related species (De Wet et al 2003, Slippers et al 2003). Even where ITS sequences are sufficient to distinguish closely related species, such as *B. eucalyptorum* and *B. irregularis,* and *B. lutea* and *B. eucalyptorum*, the polymorphisms are spread across the fragment (Chapter 5). Primers will, therefore, differ only by one base pair, which might not be sufficient or robust under all conditions. Species-specific primers between these closely related species will need to be developed for other regions of the genome. For example, the EF1-α region is consistently more variable than the ITS rDNA region (Chapter 3, 5, 9). Unfortunately,

polymorphic sequences between the most closely related species, e.g. *B. ribis* and *B. parva*, and *D. pinea* and *D. scrobiculata*, are not close enough in any part of the regions sequenced thus far, to allow the development of robust primers.

Like species-specific primers, PCR RFLP fingerprints can provide effective tools to rapidly and reliably identify larger numbers of isolates that would be impractical to identify otherwise. RFLP profiles of the ITS rDNA region has been used to distinguish among distant and closely related *Botryosphaeria* species (Jacobs 2002, Chapters 4, 5). This technique overcomes the need for a continuous group of polymorphic bases, because restriction enzymes (RE) recognize single base pair changes. Unfortunately, not all polymorphisms will present restriction sites. Closely related species like *B. parva* and *B. ribis* could thus not be distinguished using this technique when ITS rDNA, β-tubulin and EF1-α sequence data were studied. Subsequent studies have, however, identified non-coding regions that are useful for RFLP distinction of these species (Chapter 9).

DISCUSSION, CONCLUSIONS AND FUTURE WORK

In most DNA-based and other molecular studies, *Botryosphaeria* spp. group according to their associated anamorph species. One group (section Hyala) has anamorphs in *Fusicoccum*, while the other (section Brunea) have anamorphs in *Diplodia*. The question arises as to whether the genus should be sub-divided to reflect these groups. Our view is that it is currently not necessary or defendable to subdivide the genus. *Botryosphaeria* species form a monophyletic clade with respect to all other ascomycete genera, including the most closely related genus, *Guignardia*. The genus *Botryosphaeria*, as it has been used for the past 140 years, is well recognizable, because of the similar morphology and ecological niche and role of its species. It should be noted that the apparent split is not based on a representative sample of species in the genus. Furthermore, DNA data from species such as *B. zeae* and *B. proteae*, as well as mtSSU DNA sequence data, do not support the clear split in the genus (Denman et al 2000, Zhou and Stanosz 2001b, FIG. 22). The apparent phylogenetic substructure in the genus *Botryosphaeria* is thus not well enough characterized or understood at present to validate a division of the genus. In our view, this should await a more comprehensive study of a representative number of species.

If the teleomorph name for *Botryosphaeria* is not separated into two groups, it could be argued to synonymize the anamorph species under the older name *Fusicoccum*. This too seems unnecessary for a number of reasons. Where a holomorph concept is known, the teleomorph name will take preference. DNA based comparisons are making it easier to link the anamorph names to *Botryosphaeria* taxa. It is thus expected that the use of anamorph names will gradually diminish. Furthermore, *Diplodia* is a large genus. Changing a great number of names in this genus will take considerable effort and will also make the interpretation of past literature more complicated. A more valuable approach would be to epitypify these species and to link them to *Botryosphaeria* taxa.

The value of different characters or datasets to define species phylogenies is debatable, and might be unique for each species. Apart from multiple gene sequence and SSR markers, which will almost certainly indicate species boundaries when representative samples of populations are studied, a combination of other sets of information might indicate species boundaries. No single phenotypic character, whether from the teleomorph, anamorph or ecological data, will, however, conclusively distinguish closely related species in *Botryosphaeria*. Therefore, we would base conspecificity on the congruence between at least three of the following data sets, taking the possible variation within species into consideration as explained before: conidial spore size/shape (l/w and lxw), color, septation and discoloration pattern; cultural morphology; ITS rDNA sequence; predetermined RFLP patterns or species-specific primers; ISSR's; host and geographic origin

Apart from the value of specific characters and datasets in identifying species, there are a number of important considerations that will minimize mistakes when describing a new *Botryosphaeria* species. These include:

A. Identification of potential new species should be based on a representative number of samples. At the minimum three collections from different individual host plants, but if possible more. Unless differences between species are large, smaller sample sizes will underestimate species diversity based on phenotypic characters and overestimate them based on molecular data.

B. The most closely related species to a specific isolate can easily be determined by comparing its ITS rDNA sequence data to that in public databases. Care should, however, be taken concerning the identity of

isolates in such databases. Identifications should preferably be based on ex-type or otherwise authenticated isolates where possible. The use of ad hoc identified samples is of little taxonomic value. For example, the names *B. dothidea*, *B. ribis* and *B. parva* are not correctly applied to all isolates in GenBank.

C. Physical comparisons with type and isolates should also be accompanied by a thorough investigation of possible synonyms for any new name, based on published morphological or ecological data (same host and geographical area might be a good starting point).

D. If the data is not congruent with that of known species or some consistent variation exist, and there is doubt about the phylogenetic value of such variation, multiple gene genealogies should be used to determine species boundaries.

E. Identification of new species should be followed by a description based on relevant phenotypic characters, ecological data, etc. Molecular data in isolation might identify a species, but does not circumscribe it. Morphological data alone also has little biological relevance in *Botryosphaeria*. It is the combination of molecular data with morphological characters, geographical distribution, host associations, life-cycle, etc. that gives biological identity and meaning to a *Botryosphaeria* species. Furthermore, the identification of separate groups in molecular studies that are not followed by taxonomic proposals, will lead to confusion.

F. Type material should be mature and preferably be associated with deposited anamorph material. If the anamorph is not known from nature, it can be easily induced in the laboratory. Apart from type material, it is imperative to deposit representative isolates (including ex-type) in recognized culture collections to allow future comparisons based on molecular and cultural characters. When type material or published data is insufficient to circumscribe a species, it is preferable to epitypify the taxon rather than merely rejecting the name. This will minimize an unnecessary proliferation of names, confusion and later work to amend the description.

G. An effort should be made to provide an easy identification tool (RFLP, Primers, etc.) and/or key for relevant taxa. Especially species-specific

primers could be applied more widely. The intron of the EF1-α gene region seems particularly suited for this purpose, because it is more variable than any other studied thus far.

Mishler and Donoghue (1982) concluded that researchers identify species based on their own field of experience. Such an approach logically leads to conflicting views. It is hoped that the broader approach advocated here, will diminish taxonomic problems in *Botryosphaeria* and result in a more rigorous, useful and stable systematic system for this group of fungi. One prerequisite, if such a goal is to be attained, is easily accessible, updated and complete information about this group of important fungi. A first attempt at such a system has been put in place by Phillips (2002) (www.crem.fct.unl.pt/botryosphaeria_site).

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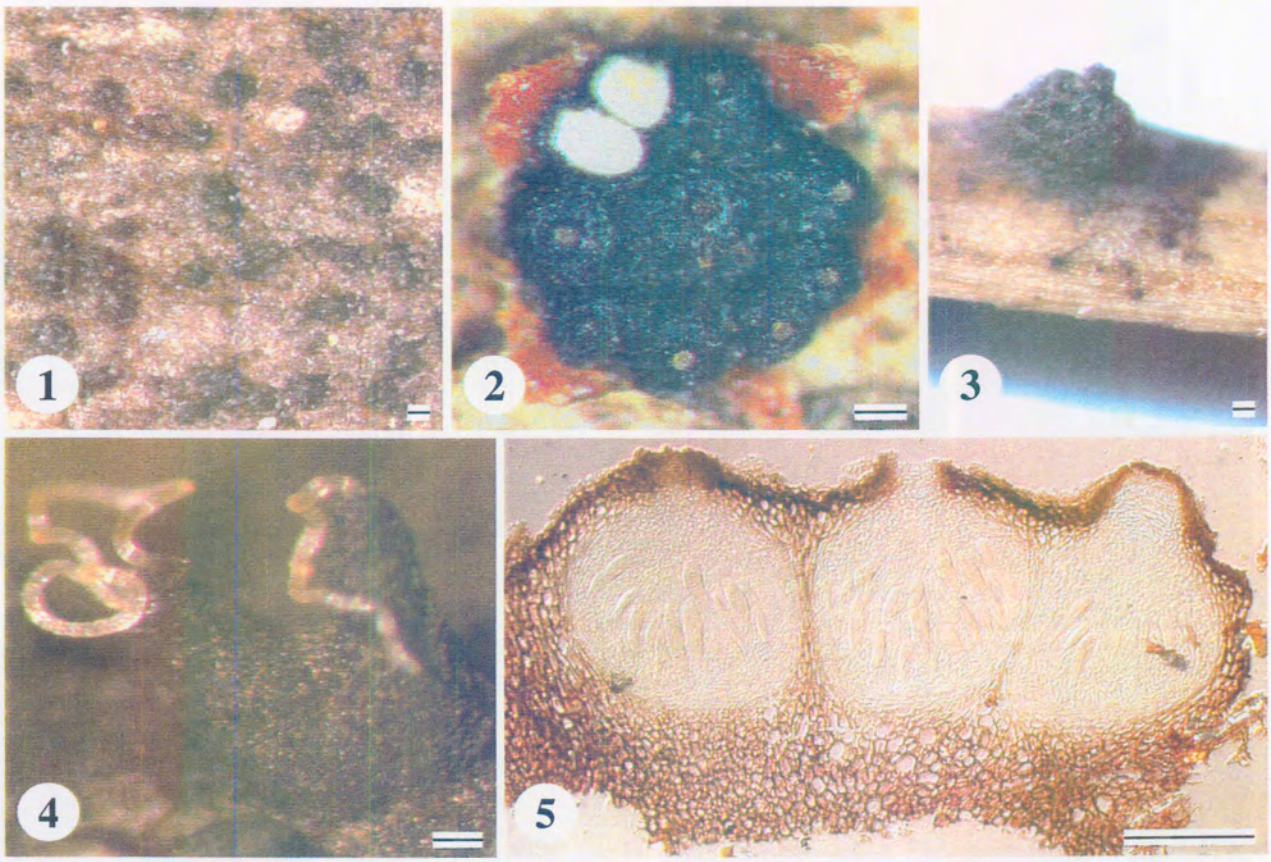
FIGS. 1-5. Dissecting microscope and phase contrast compound-microscope micrographs of ascostroma and pycnidia of *Botryosphaeria* species. 1. *B. irregularis*. Singular, semi-erumpent ascostroma. 2. *B. dothidea*. Botryose, erumpent ascomata with a central ostiole. The apices of two ascomata have been removed to show the typical white centrum contents. 3. *B. parva*. Singular pycnidium covered with grey mycelium, which was formed in culture on a pine needle. 4. *Botryosphaeria* sp. Mature pycnidia oozing conidia from an ostiole at the end of an extensive conical neck. 5. Median, longitudinal section through a mature ascoma of *B. dothidea*. Bars = 100 µm.



FIGS. 6-8. Light micrographs of sexual structures of *Botryosphaeria* species. 6. *B. australis.* Immature (right) and mature (left) asci. 7. *B. australis* and 8. *B. irregularis.* Mature ascospores can be ellipsoidal to spindle shaped, smooth or with granular contents. Bars = 10 µm.

FIGS. 9-16. Light micrographs some morphological characteristics of *Fusicoccum* anamorphs of *Botryosphaeria* species. Conidia of these species are normally hyaline, thin walled and narrower than those of *Diplodia* species. 9. *B. dothidea.* Spermatiophores and spermatia. 10. *B. parva.* Fusiform conidia of medium length (~17--20 µm x 5--6 µm). 11. *B. dothidea.* Narrow fusiform and long conidia (22--25 µm x 5--6 µm). 12. *F. mangiferum.* Short, fusiform conidia (>15 µm x 5 µm). 13. *Fusicoccum* sp. (previously *Dothiorella* 'long'). Long and broad bacilliform or cylindrical conidia (24--30 x 6 µm). 14. *B. eucalyptorum.* Hyaline, septate, germinating conidia. 15. *B. parva.* Septate, mature conidia with a pigmented middle cell. 16. *B. dothidea.* Immature conidia and conidiogenous cells. Bars = 10 µm.

FIGS. 17-21. Light micrographs some morphological characteristics of *Diplodia* anamorphs of *Botryosphaeria* species. Conidia of these species are normally thick walled, broader than those of *Fusicoccum* species, regularly septate and pigmented with age. 17. *B. stevensii.* Immature conidia and conidiogenous cells. 18. *B. quercuum.* Young hyaline conidia with thick glassy walls. 19. *B. obtusa.* Aseptate, pigmented conidia with rough walls. 20. Immature hyaline and mature conidia that are pigmented and septate. 21. *B. quercuum.* Three septate and pigmented conidium. Bars = 10 µm.

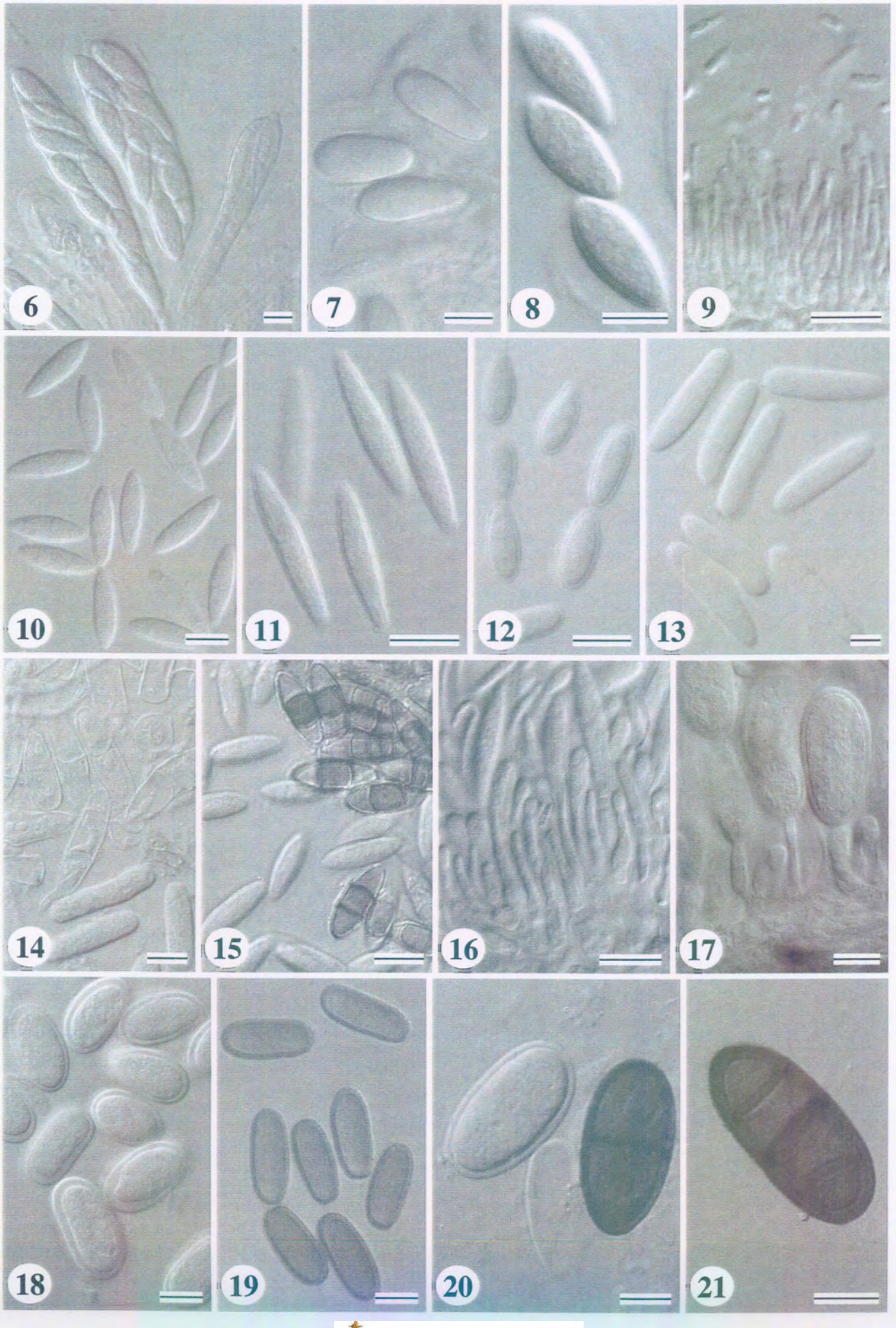


FIG. 22. One of two most parsimonious trees of 401 steps (g1 = -0.43, CI = 0.71, RI = 0.83) obtained from heuristic searches of DNA sequence data of the ITS rDNA region indicating the phylogenetic relationships between various *Botryosphaeria* species and *Diplodia pinea*. Fifty characters from the hypervariable ITS1 region were excluded, because of ambiguous alignments. Bootstrap values (1000 replicates) are indicated above or below the branches. The tree is rooted to the outgroup taxa, *Mycosphaerella africana* and *Guignardia bidwellii*. Isolate numbers refer to: ATCC = American Type Culture Collection, Manassas, VA; CAP = Culture collection of AJL Phillips, Lisbon, Portugal; CBS = Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; CMW = Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; ICMP = International Collection of Microorganisms from Plants, Auckland, New Zealand; KJ = Jacobs and Rehner (1998); STE = Department of Plant Pathology, University of Stellenbosch, South Africa; ZS = Zhou and Stanosz (2001a).