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Multiple gene sequences delimit *Botryosphaeria australis* sp. nov. as a sister species to *B. lutea*

Abstract: *Botryosphaeria lutea* (anamorph *Fusicoccum luteum*) is most easily distinguished from other *Botryosphaeria* spp. by a yellow pigment that is formed in young cultures. This fungus has been reported from a number of cultivated hosts in New Zealand and Portugal. During a survey of *Botryosphaeria* fungi that occur on native *Acacia* species in Australia, a yellow pigment was observed in some cultures. These isolates were morphologically similar to *B. lutea*, but the pigment differed slightly compared to authentic *B. lutea* isolates. Preliminary data also revealed small differences in ITS rDNA sequence data. The aim of this study was to determine whether these small differences were indicative of separate species, or merely variations within *B. lutea*. Anamorph, teleomorph and culture morphology were compared between *B. lutea* and authentic isolates from Australia. Sequence data of two other genome regions, namely the β-tubulin and EF1-α gene and intron regions, were combined with ITS rDNA sequence data to determine the phylogenetic relationship between these isolates. Isolates of *B. lutea* and those from Australian *Acacia* species were not significantly different in spore morphology. The yellow pigment was, however, much more distinct in cultures of *B. lutea* than in cultures from *Acacia*. There were only a few base pair variations in each of the analyzed gene regions, but these variations were fixed in the two groups in all regions. By combining these data it was clear that *B. lutea* and the isolates from *Acacia* were distinct species, albeit very closely related. We, therefore, propose the new epithet, *B. australis,* for the fungus from Australia. *Botryosphaeria australis* was also isolated in this study from exotic *Sequoiadendron* trees in Australia. Re-analyses of GenBank data in this study showed that *B. australis* also occurs on other native Australian hosts, namely a *Banksia* sp. and a *Eucalyptus* sp., as well as a native *Protea* sp. in South Africa and on *Pistachio* in Italy. These records from GenBank have previously been identified as *B. lutea*. The common occurrence of *B. australis* on a variety of native hosts across Australia suggests that this fungus is native to this area.

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INTRODUCTION

Various native Australian woody plants, such as *Acacia* spp., *Eucalyptus* spp., species of Proteaceae and others, make up commercial plantations world-wide (Evans 1984, Wingfield et al 2001a, b, Denman et al 2003). In order to establish and maintain these plantations, germplasm must be introduced into different countries, and this can result in the accidental introduction of exotic pathogens to new environments (Palm 1999, Wingfield et al 2001a). In this regard, a group of pathogens that are easily overlooked are *Botryosphaeria* spp., which live as endophytes in healthy plants and seeds for part of their life cycle (Smith et al 1996, Burgess and Wingfield 2002).

Once introduced into a new environment, *Botryosphaeria* spp. can threaten both native and exotic hosts (Burgess and Wingfield 2002). This is because many of these fungi have a wide host range (Wingfield et al 2001a). In order to reduce this threat, it is necessary to obtain a clear knowledge of the taxonomy and ecology of *Botryosphaeria* spp., both in their areas of natural occurrence, and in countries where these trees are commercially planted (Palm 1999, Wingfield et al 2001b).

Members from the genus *Botryosphaeria* are commonly accepted to be problematic to identify to species level. For a number of years after the circumscription of the genus, ascomatal morphology and host range were considered characteristic for different species (Cesati and De Notaris 1863, Saccardo 1877, Trotter 1928). This resulted in considerable difficulty, because the teleomorph is not frequently found in nature, and is only rarely produced in culture. It is also currently understood that some *Botryosphaeria* spp. can infect a wide variety of hosts (Stevens and Jenkins 1924, Punithalingham and Holliday 1973, Punithalingham and Waller 1973). Furthermore, teleomorph characters vary on different hosts, and are often not distinctive at species level (von Arx and Müller 1954, Slippers et al 2003).

Conidial and cultural morphology are often used to distinguish different *Botryosphaeria* spp. (Shoemaker 1964, Pennycook and Samuels 1985, Denman et al 2000, Slippers et al 2003). The anamorphs of these fungi are commonly encountered, both in nature and in culture. Distinctive features of conidia are the shape, size, length/width ratio, septation, content, color, wall thickness and ornamentation. The general growth pattern, speed and color of a colony on agar are also sometimes informative for species identification (Pennycook and Samuels 1985, Phillips et al 2002, Slippers et al 2003).

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In recent years, various DNA based techniques have been used to distinguish between *Botryosphaeria* spp. These techniques include dominant and co-dominant molecular markers such as RAPD's, ISSR's and microsatellites (Burgess et al 2001, Smith and Stanosz 2001, Zhou et al 2001), and sequence data for a number of DNA regions (Jacobs and Rehner 1998, Denman et al 2000, Zhou and Stanosz 2001a, b, Phillips et al 2002, Slippers et al 2003). These data have, however, not always been sufficient to distinguish boundaries between closely related or cryptic species. In such cases, multiple gene genealogies have been used (De Wet et al 2003, Slippers et al 2003). These molecular data, combined with morphological and ecological data, allow for robust identification of *Botryosphaeria* spp.

Pennycook and Samuels (1985) described an anamorph of a *Botryosphaeria* species, *Fusicoccum luteum* Pennycook & Samuels, from New Zealand. This species could most easily be distinguished from other Botryosphaeriaceous fungi from *Malus* sp., *Populus* sp. and *Actinidia deliciosa* by a yellow pigment produced in young cultures. The teleomorph was unknown. A few years later, Phillips et al (2002) also noticed a yellow pigment in cultures derived from *Botryosphaeria* ascomata on *Vitis vinifera* in Portugal. Using ribosomal DNA (rDNA) sequence and SSCP, RAPD and morphological data, the Portugese fungus was shown to be similar to *F. luteum*, for which the teleomorph, *B. lutea* A.J.L. Phillips, was described. *Botryosphaeria lutea*, thus, seems to be a more important pathogen of fruit and forestry crops than was previously recognized. This pathogen has evidently also been mistaken for *B. dothidea* (Fr.: Moug.) Ces. & De Not. in the past (Phillips et al 2002).

Botryosphaeria rhodina (Berk. & Curt.) Arx and *B. dothidea* have both been reported from Australian native *Acacia* spp., where these trees are planted as exotics (Roux 1998, Roux et al 2001). However, during a recent survey of *Botryosphaeria* spp. on native Australian flora, some cultures from an *Acacia* sp. produced a yellow pigment in culture, similar to that described for *B. lutea*. Initial ITS rDNA sequence data confirmed a close relationship with *B. lutea*, but some sequence divergence was also obvious. The aim of this study was, therefore, to determine the relationship between these isolates from *Acacia* in Australia and *B. lutea* isolates from New Zealand and Portugal. In order to evaluate the phylogenetic significance of the sequence variation seen in the ITS region, sequence data from three gene regions (ITS rDNA, β-tubulin and Elongation factor 1α (EF-1α)) were compared. Teleomorph, anamorph and cultural characters were also considered.

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MATERIALS AND METHODS

Isolates.--Thirteen isolates of a *Botryosphaeria* species resembling *B. lutea* and its anamorph *F. luteum*, were collected from diseased or dying stems of *Acacia* spp. in Australia. Collections were made by J Roux from *A. mearnsii* during 1999 and by the senior author during 2001 (TABLE I). Four similar isolates were also obtained from samples of diseased *Sequoiadendron gigantea* growing as an exotic in Canberra (TABLE I). Two isolates of *B. lutea* (one ex-type) were provided by AJL Phillips (CAP002, CAP037) (TABLE I). Two isolates originating from the original description of *F. luteum* (one ex-type) by Pennycook and Samuels (1985) were also included (TABLE I). Isolates were maintained on malt yeast extract agar (MYA) (2% malt extract, 0.2% yeast extract and 2% agar; Biolab, Johannesburg, S.A.) at 25°C in the dark or under near UV light. Isolates are maintained in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Molecular phylogenetic characterization.--A phenol:chloroform DNA extraction technique was used to isolate the genomic DNA as described in Raeder and Broda (1985), and Smith et al (2001). Four isolates from *Acacia* and *Sequoiadendron* from Australia were used in phylogenetic comparisons with two isolates from each of the studies of Pennycook and Samuels (1985) and Phillips et al (2002), including the ex-type cultures of *B. lutea* and *F. luteum* from these studies (TABLE I). DNA sequences of other *Botryosphaeria* spp. that were included in the analysis for comparative purposes were from Slippers et al (2003) (TABLE I). Furthermore, Smith and Stanosz (2001) and Denman et al (2003) used ITS rDNA sequence comparisons to identify *B. lutea* or *F. luteum* isolates from native *Banksia* spp., *Eucalyptus marginata* and a *Protea* sp. in Australia and South Africa. ITS sequences from these studies were obtained from GenBank and compared with the isolates examined in the present study (TABLE I).

Three housekeeping gene sequences were used for phylogenetic comparisons between isolates. The region spanning the 3' end of the 16S (small subunit) rRNA gene, the first internal transcribed spacer (ITS1), the complete 5.8S rRNA gene, the second ITS (ITS2) and the 5' end of the 26S (large subunit) rRNA gene, was amplified using the primers ITS1 (5' TCCGTAGGTGAACCTGCGG) and ITS4 (5' TCCTCCGCTTA

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TTGATATGC) (White et al 1990). A region of the β-tubulin gene was amplified using the primers Bt2a (5' GGTAACCAAATCGGTGCTGCTTTC) and Bt2b (5' ACCCTCA GTGTAGTGACCCTTGGC) (Glass and Donaldson 1995). In addition, a part of the elongation factor 1-α was amplified using the primers EF1-728F (5' CATCGAGAAGTTCGAGAAGG) and EF1-986R (5' TACTTGAAGGAACCCT ACC) (Carbone et al 1999). PCR reaction mixtures, PCR conditions and visualization of amplicons are as described by Slippers et al (2003). The amplicons of all three DNA regions were also cleaned and sequenced as described by Slippers et al (2003), using the same primers used to generate the amplicons.

Sequence data were analyzed using Sequence Navigator version 1.0.1™ (Perkin Elmer Applied Biosystems, Foster City, CA) and manually aligned by inserting gaps. Gaps were treated as a fifth character and all characters were unordered and of equal weight. A partition homogeneity test was done to determine whether the datasets could be combined (Farris et al 1995, Huelsenbeck et al 1996). Estimated levels of homoplasy and phylogenetic signal (retention and consistency indices and g1-value) (Hillis and Huelsenbeck 1992) were determined. Maximum parsimonious trees were determined using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b8 (Swofford 1999), with heuristic searches of only informative characters and tree bisection and reconstruction (TBR) as branch swapping algorithm (random stepwise addition). Maxtrees were unlimited, branches of zero length were collapsed and all multiple equally parsimonious trees were saved. Branch and branch node supports were determined using 1000 bootstrap replicates (Felsenstein 1985) and decay analysis of the branch nodes using Autodecay (Eriksson 1998). Phylogenetic species hypotheses were also tested using distance analyses with the Neighbor Joining algorithm, using both an uncorrected p-factor and HKY85 parameters alternatively in PAUP.

To test the consistency of branches in the combined dataset, the three partial gene sequence datasets were also analyzed separately, but in the same way as described above. The ITS rDNA dataset was also expanded and analyzed separately to include isolates for which only ITS rDNA data were available from GenBank.

Morphological characterization.--All isolates were grown on sterilized pine needles that were placed on water agar (WA) (2% agar; Biolab Midrand, Johannesburg, S.A.) at 25°C under near UV light, to promote sporulation. Fruiting structures from *in vivo* and *in vitro* collections were sectioned with an American Optical Freezing Microtome or by

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hand, and mounted in clear lactophenol. Morphological observations and photographs were done on an Axiocam digital camera (Carl Zeiss, Germany). The morphology of these samples was compared with material from of *B. lutea* [LISE94070 (holotype) and LISE94073] and *F. luteum* (PDD45400).

Growth rate, colony morphology and color (Rayner 1970) of isolates obtained during this study, as well as of ex-type isolates of *B. lutea* and *F. luteum*, were determined. Two isolates of each species were incubated on potato dextrose agar (PDA) (0.4% potato extract, 2% dextrose, 1.5% agar; Biolab, Midrand, Johannesburg, S.A.) at 5 C intervals ranging from 10 to 35 C in the dark. Growth rate was measured at the leading edge in 24 hr intervals. The experiment was repeated for the new species described below.

RESULTS

Molecular phylogenetic characterization.--PCR products of approximately 580 bp (ITS rDNA), 450 bp (β-tubulin) and 300 bp (EF1-α) were amplified for all the isolates. Sequence data at the 5' and 3' ends were deleted from the dataset if they were doubtful. The partition homogeneity test of the ITS-rDNA, β-tubulin and EF1-α data sets, indicated that they could be combined (P value = 0.53). The total data set consisted of 1324 characters after alignment. A seven base pair region in the EF1-α region was repeated twice in most species. Isolates of *B. ribis* contained two extra of these repeats, which were coded to represent only two evolutionary events and not fourteen as was the case before the coding. Of the remaining characters, 322 were parsimony-informative and used in the analyses. This combined data set contained significant phylogenetic signal compared to random trees ($P < 0.01$; $g1 = -0.97$) (Hillis and Huelsenbeck 1992). After heuristic searches in PAUP, 3 most parsimonious trees of 540 steps were retained (CI = 0.843; RI = 0.909) (FIG. 1). Trees obtained using distance methods were the same as those obtained using parsimony.

Based on the combined analysis of the sequence data from the three gene regions, *B. lutea* and the *Botryosphaeria* sp. from *Acacia* and *Sequoiadendron* in Australia grouped into two distinct clades (FIG. 1). Although the branches separating these two clades were short, compared to the branches separating other well-defined species in the analysis, they were strongly supported (d5/96% and d5/100% decay values and

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bootstrap support). The separation was also supported in the individual analyses of the sequence datasets of the three gene regions (analyses done using the same parameters as for the combined dataset) (FIG. 2A-C). Analysis of the sequences between these two groups showed that at the alleles were fixed in the two groups at 14 of the 15 polymorphic loci (TABLE II). These data also showed a significant bias towards transitions (only one transversion recorded) in these polymorphic sites. This factor is considered in the distance analysis (HKY85 parameters) and does not change the interpretation of the results. Among the isolates studied here, these two clades are considered phylogenetically separate and the fungus from Australia is described as new.

GenBank sequences of the ITS rDNA region from isolates that were previously identified as *B. lutea* or *F. luteum* (Smith and Stanosz 2001, Denman et al 2003), separated into both the *B. lutea* and *B. australis* clades, although the bootstrap values for both groups were low (63% and 73% respectively) (FIG. 3). Sequences from *Actinidia* (New Zealand) and *Buckinghamia* (Australia) grouped with *B. lutea*. Isolates from Australian native hosts including *Eucalyptus* and *Banksia*, and from a South African native *Protea,* grouped with the newly identified *Botryosphaeria* sp. Three isolates from *Banksia* in Australia (Denman et al 2003) resided in a sister group to the *B. lutea* and *B. australis* clades.

Isolates residing in the clades representing *B. lutea* and the new species described in this study were more closely related to each other (d14/100% bootstrap) than to any other taxon included in the analysis. These two species were also more closely related to *B. ribis*, *B. parva* and *B. eucalyptorum* (d28/100% bootstrap) than to *B. dothidea.* All these species have *Fusicoccum* anamorphs and group together (100% bootstrap), as opposed to isolates of *B. obtusa*, *B. stevensii* and *B. rhodina*, which all have *Diplodia* or *Lasiodiplodia* anamorphs. DNA sequence of the latter species were used as outgroup taxa in the analyses.

Morphological characterization.--The isolates from *Acacia* spp. and *Sequoiadendron giganteum* produced anamorph structures on pine needles on WA within two to three weeks. Teleomorph and anamorph structures of this fungus from field samples and from conidia formed in culture were very similar in morphology to those of *B. lutea* and *F. luteum* (FIGS. 4-12). This new species also produced a yellowish pigment in young cultures, as was true for *B. lutea*. The fungus from *Acacia* and *Sequoiadendron* could, however, be distinguished from *B. lutea* and *F. luteum* by its longer conidia with a

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higher l/w ratio (TABLE III). Isolates of *B. lutea* also produce a much brighter yellow pigment in culture than the Australian isolates, especially at 25 C and higher. Unlike *B. lutea*, no yellow pigment was produced between 25--30 C by the Australian isolates.

TAXONOMY

The fungus collected and isolated from *Acacia* spp. and *S. giganteum* in Australia is phylogenetically and morphologically distinct from *B. lutea*. The currently known host ranges of these taxa also do not overlap. The fungus is thus described as new as follows:

Botryosphaeria australis Slippers, Crous & M.J. Wingf., sp. nov. FIGS. 4-14

Anamorph. ***Fusicoccum australe*** Slippers, Crous & M.J. Wingf., sp. nov.

Etymology. Referring to the origin in the southern hemisphere.

Ascostromata per corticem erumpentia, 1.2 mm diametro. Ascomata pseudothecialia, 2--10 botyroide aggregata, interdum solitaria, globosa ostiolo centrale, papillata vel glabra, inclusa cum solum papillis emergentibus usque ad 2/3 emergentia, nigra, 100--300 µm; paries pseudothecii e 5--8 stratis texturae angularis, extus e cellulis atrobrunneis vel brunneis composita, intus e cellulis hyalinis revestimentum loculi facientes. Asci bitunicati, clavati, 60--125 x 16--25 µm, inter pseudpoaraphyses multas, filiformes, septatas, raro apicem versus ramosas, 3--4 µm latas. Ascosporae fusoideae vel ovoideae, 20--23(--25) x 7--8(--9) µm, unicellulares, hyalinae, laeves, contentis granularibus, in asco biseriatae. Conidiomata (in "WA" in acis pinorum sterilifactis in 7--21 diebus formata) pycnidialia, superficialia, globosa, plerumque solitaria mycelio tectaque. Conidia fusiformia, basibus subtruncatis vel obtuse rotundatis, (18--)23--26(--30) x 5--6(--7.5) µm, hyalina, unicellularia raro septum ante germinationem facientia, laevia contentis granularibus. Cellulae conidiogenae holoblasticae, hyalinae, subcylindricae, 10--14 x 2--3 µm, percurrenter proliferantes cum 1--2 proliferationibus, inspissatione periclinali. Spermatia non visa.

Ascostromata erumpent through the bark, 1.2 mm diam. *Ascomata* pseudothecial, forming botryose aggregates of 2--10, sometimes solitary; globose with a central ostiole, papillate or not, imbedded with only papilla emerging up to 2/3 emergent, black, 100--300 µm; pseudothecial wall comprising 5--8 layers of *textura angularis*, outer region of dark brown or brown cells, inner region 3--6 layers of hyaline cells lining the locules. *Asci* bitunicate, clavate, 60--125 x 16--25 µm, 8-spored, between numerous

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filiform, septate pseudoparaphyses, rarely branched towards the tips, 3--4 µm wide. *Ascospores* fusoid to ovoid, 20--23(--25) x 7--8(--9) µm (average of 50 ascospores 21.9 x 7.6 µm, l/w 2.9), unicellular, hyaline, smooth with granular contents, biseriate in the ascus. *Conidiomata* (formed on WA on sterilized twigs within 7--21 d) pycnidial, superficial, globose, mostly solitary and covered by mycelium. *Conidia* fusiform, base subtruncate to bluntly rounded, (18--)23--26(--30) x 5--6(--7.5) µm (average of 240 conidia 24.7 x 5.1 µm, l/w 4.8), hyaline, unicellular, rarely forming a septum before germination, smooth with granular contents. *Conidiogenous cells* holoblastic, hyaline, subcylindrical, 10--14 x 2--3 µm, phialidic with periclinal thickening, or proliferating percurrently with 1--4 proliferations. *Spermatia* not observed. *Cultures* having buff (19''f) to light primrose (23''b) colonies, light yellowish pigment most noticeable between 15--20 C in the dark, becoming olivaceous buff (21'''d) to olivaceous gray (21'''''i) after 5--6 days, with a sparse to moderately dense, appressed mycelial mat in center with sparse tufts of aerial mycelium around edges, margin smooth. Optimum temperature for growth 25 C, colony reaching 48 mm in diam on PDA after 4 days at 25 C in the dark.

Specimens examined. AUSTRALIA. VICTORIA: Batemans Bay, *Acacia* sp., *M.J. Wingfield* (HOLOTYPE PREM57589) (culture CMW6838); Batemans Bay, *Acacia* sp., *M.J. Wingfield* (PREM57590); Batemans Bay, *Acacia* sp., *M.J. Wingfield* (PREM57592); Batemans Bay, *Acacia* sp., *M.J. Wingfield* (PREM57593); ACT: Canberra, *Sequoiadendron* sp., *M.J. Wingfield* (PREM57594); Canberra, *Sequoiadendron* sp., *M.J. Wingfield* (PREM57595)

DISCUSSION

In this study *Botryosphaeria australis* and its anamorph, *F. australis*, are newly described. This fungus is phylogenetically closely related to *B. lutea.* The branches of the clades representing these two species are short subdivisions of a deeply branched and well-defined clade. This indicates a relatively recent speciation event.

The genetic and subsequent taxonomic separation of the closely related species, *B. lutea* and *B. australis*, was confirmed using the phylogenetic species concept. Sequence variation within the ITS, β-tubulin and EF1-α regions were small, and were considered insignificant if compared to the divergence between other well defined *Botryosphaeria* spp. The alleles were, however, distributed in the same manner, or fixed

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for the two groups, in each of the three gene regions. These fixed alleles over multiple gene regions indicate a barrier to genetic exchange and are considered indicative of phylogenetic species (Taylor et al 2000). This phylogenetic separation confirmed the taxonomic value of small but distinct phenotypic variation that would otherwise have been overlooked. The combination of these DNA based and phenotypic data are considered sufficiently robust evidence to treat these fungi as separate taxa.

The distinction between *B. lutea* and *B. australis* was not recognized in previous studies based only on ITS rDNA sequence data (Smith and Stanosz 2001, Zhou and Stanosz 2001a, Denman et al 2003). This was due to the small ITS sequence divergence between them. The fact that we had access to a much larger collection of isolates also facilitated the discovery of the new taxon. ITS data alone can thus obscure the true diversity in *Botryosphaeria*. This is similar to *B. ribis* and *B. parva* (Slippers et al 2003), or *Diplodia pinea* (Desm.) Kickx. (= *S. sapinea*) and *D. scrobiculata* De Wet, Slippers & M.J. Wingf. (De Wet et al 2003), that were considered to represent single species based on their ITS data alone. Multiple gene genealogies, however, showed that these species are phylogenetically distinct (De Wet et al 2003, Slippers et al 2003). These case studies provide good evidence to show that single gene genealogies are insufficient to distinguish cryptic *Botryosphaeria* spp.

Internal transcribed spacer sequences provided by Smith and Stanosz (2001) and Zhou and Stanosz (2001a) for one isolate from a *Eucalyptus* sp. and one from a *Banksia* sp. were included in this study. Comparisons show that these isolates, previously treated as *B. lutea,* actually represent *B. australis*. In the studies of Smith and Stanosz (2001) and Zhou and Stanosz (2001a), *B. australis* isolates could, however, not be separated from ex-type isolates of *F. luteum* based on RAPD data. These authors were also not able to separate two other cryptic *Botryosphaeria* sp., namely *B. ribis* and *B. parva* using RAPD data. Another similar example where RAPD data have been insufficient to define species, has been with the three recognized "morphotypes" of *D. pinea*. These three groups are distinguished using RAPD data, but correspond to only two phylogenetic species (De Wet et al 2000, 2003).

Botryosphaeria lutea and *B. australis* cannot be distinguished from each other based on teleomorph fruiting structures or ascospores. The conidial dimensions are equally misleading, because they are similar in form and general appearance, and their length and width dimensions overlap. This is not uncommon among *Fusicoccum* spp. Pennycook and Samuels (1985), Phillips et al (2002) and Slippers et al (2003) have all

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reported significant overlap in the sizes of *Fusicoccum* spp. In these cases and in the present study the species could, however, be separated when averages of conidial sizes and septation were considered. Thus, on average, conidia of *F. australis* are longer and appear more slender (higher length/width ratio) than those *F. luteum*.

Culture morphology was useful to distinguish between isolates of *B. lutea* and *B. australis*. *Botryosphaeria australis* produced a distinctly lighter and duller (more cream than yellow) yellow pigment in young cultures than *B. lutea*. In the description of *F. luteum* (Pennycook and Samuels 1985) and *B. lutea* (Phillips et al 2002), the production of a yellow pigment in culture was the easiest way to distinguish this taxon from other species. Prior to these studies, this pigment was not considered taxonomically useful (Witcher and Clayton 1963). Recent studies of other botryosphaeriaceous fungi have also recognized the value of culture morphology as a useful tool to distinguish *Botryosphaeria* spp. (Jacobs 2002, De Wet et al 2003, Slippers et al 2003).

Botryosphaeria australis differs in morphology and etiology from other botryosphaeriaceous fungi described from native Australian *Acacia* spp., *Banksia* spp. and *Eucalyptus* spp. Hansford (1954) described *B. banksiae* Hansford from *Banksia marginata* from Australia. The ascospores of this species are, however, one-seriate in the ascus and significantly different in size (17--20 x 13--15 µm). *Botryosphaeria acaciae* (Hansford) Dingley (= *Physalospora acaciae* Hansford) causes galls and cankers on *Acacia* spp. in Australia and New Zealand (Hansford 1954, Dingley 1970). Both of the latter studies, however, report that the teleomorph structures and galls are also associated with a *Diplodia* sp. Dingley (1970) reports 'small papilla on one end' of the ascospores, which is absent in *B. australis*. This disease was also described from Australian *Acacia* by Scurfield (1966), who identified the causal agent as a fungus in the Sphaeriaceae. Based on the differences in anamorph, the lack of an association with a gall-forming disease and differences in ascospore morphology, we are confident that *B. australis* is not the same fungus as either of those mentioned above.

Botryosphaeria australis appears to be native to the southern hemisphere, and most likely Australia. This hypothesis is based on the current known host and geographic distribution of this taxon. *Botryosphaeria australis* was the only *Botryosphaeria* sp. found on native species of *Acacia*, in the different areas and over the two seasons that our collections were made. Isolates from previous studies are also identified here as *B. australis*, e.g. from *E. marginata* and *Banksia caleyi* in Australia, and *Protea cynaroides* in South Africa (Smith and Stanosz 2001, Zhou and Stanosz

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2001a, Denman et al 2003). Only one isolate from outside of these regions grouped with *B. australis* and this was from *Pistacia* in Italy. Smith and Stanosz (2001) identified *F. luteum* from the Australian native hosts, *Banksia*, *Jacksonia horrida*, *Isopogon tribolus*, *Dryandra tenuifolia* and a *Leucopogon* sp. These identifications were, however, based only on RAPD data, which does not separate *B. lutea* and *B. australis*. We are thus of the opinion that the exotic *S. giganteum* trees in Australia have become infected with a native pathogen.

Botryosphaeria lutea is common throughout the Australasian region and in Portugal, but its origin is unknown. All reports from New Zealand have been from introduced hosts. Pennycook and Samuels (1985) collected this fungus from *Malus*, *Populus* and *Actinidia* species in New Zealand. Hartill (1991) also reported it from avocado from this area. The isolates from a native Australian *Buckinghamia* sp. (Denman et al 2003), however, also group with *B. lutea*. Similarly, in Portugal *B. lutea* occurs on introduced (*Vitis* and *Sophora japonica*) and indigenous (*Fraxinus angustifolia*) hosts (Phillips et al 2002). This fungus, thus, occurs on native and exotic hosts in both Europe and Australasia, which makes it difficult to predict its natural range.

Botryosphaeria. australis and *B. lutea* have been moved between the northern- and southern hemispheres, most likely on germplasm of commercially valuable species. This observation is based on the pattern of distribution and host ranges revealed in this study. It is also clear from the current study that both these fungi can infect native and introduced or cultivated hosts, in both regions. These introductions can, thus, have significant implications for agricultural and forestry industries and conservation of native flora. Population level studies are needed to find the areas of greatest diversity and understand patterns of gene flow between populations of these two fungi. Such information will help to assess the current threat of these pathogens and help curtail their continued spread.

LITERATURE CITED

Arx JA von, Müller E. 1954. Die Gattungen der amerosporen Pyrenomyceten. Beitr Kryptogamenfl Schweiz 11, 2:1--434.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Burgess T, Wingfield MJ, Wingfield BD. 2001. Simple sequence repeat markers distinguish between morphotypes of *Sphaeropsis sapinea*. Appl Environ Microbiol 67:352--362.

---, ---. 2002. Impact of fungal pathogens in natural forest ecosystems: A focus on *Eucalyptus*. In: Sivasithamparam K, Dixon KW, eds. Microorganisms in plant conservation and biodiversity. Dordrecht, The Netherlands: Kluwer Academic Press. p 285-306.

Carbone I, Anderson JB, Kohn LM. 1999. A method for designing primer sets for the speciation studies in filamentous ascomycetes. Mycologia 91:553--556.

Cesati V, De Notaris G. 1863. Schema di classificazione degli sferiacei italici. Comment Soc Crittog Ital 1, 4:177--240.

Denman S, Crous PW, Taylor JE, Kang JC, Pascoe I, Wingfield MJ. 2000. An overview of the taxonomic history of *Botryosphaeria* and a re-evaluation of its anamorphs based on morphology and ITS rDNA phylogeny. Stud Mycol 45:129--140.

---, ---, Groenewald JZ, Slippers B, Wingfield BD, Wingfield MJ. 2003. Circumscription of *Botryosphaeria* species associated with Proteaceae based on morphology and DNA sequence data. Mycologia 95:294--307.

De Wet J, Wingfield MJ, Coutinho TA, Wingfield BD. 2000. Characterization of *Sphaeropsis sapinea* isolates from South Africa, Mexico and Indonesia. Plant Dis 84:151--156.

---, Burgess T, Slippers B, Preisig O, Wingfield BD, Wingfield MJ. 2003. Multiple gene genalogies and microsatellite markers reflect relationships between morphotypes of *Sphaeropsis sapinea* and distinguish a new species of *Diplodia*. Mycol Res 107:557--566.

Dingley JM. 1970. Records of fungi parasitic on plants in New Zealand 1966-1968. NZ J Agric Res 13:325--337.

Eriksson T. 1998. Autodecay ver. 4.0 (program distributed by the author). Department of Botany, Stockholm University, Stockholm.

Evans J. 1984. Plantation forestry in the tropics. Oxford: Clarendon Press. 472 p.

Farris JS, Kallersjo M, Kluge AG, Bult C. 1995. Testing significance of incongruence. Cladistics 10:315--319.

Felsenstein J. 1985. Confidence intervals on phylogenetics: an approach using bootstrap. Evolution 39:783--791.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Glass NL, Donaldson GC. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous Ascomycetes. Appl Environ Microbiol 61:1323--1330.

Hansford CG. 1954. Australian fungi II. New records and revisions. Proc Linn Soc NSW 79:97--144.

Hartill WFT. 1991. Postharvest diseases of avocado fruit in New Zealand. NZ J Crop Hort Sci 19:297--304.

Hillis DM, Huelsenbeck JP. 1992. Signal, noise, and reliability in molecular phylogenetic analyses. J Hered 83:189--195.

Huelsenbeck JP, Bull JJ, Cunningham CW. 1996. Combining data in phylogenetic analysis. TREE 11:152--158.

Jacobs R. 2002. Characterization of *Botryosphaeria* species from mango in South Africa. [M.Sc. thesis]. Pretoria, South Africa: University of Pretoria. 162 p.

Jacobs KA, Rehner SA. 1998. Comparison of cultural and morphological characters and ITS sequences in anamorphs of *Botryosphaeria* and related taxa. Mycologia 90:601--610.

Palm ME. 1999. Mycology and world trade: a view from the front line. Mycologia 91:1--12.

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

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

Punithalingam E, Holliday P. 1973. *Botryosphaeria ribis.* CMI descriptions of pathogenic fungi and bacteria, No. 395. Kew, Surrey, England: Commonwealth Mycological Institute. 2 p.

Punithalingam E, Waller JM. 1973. *Botryosphaeria obtusa.* CMI descriptions of pathogenic fungi and bacteria, No. 394. Kew, Surrey, England: Commonwealth Mycological Institute. 2 p.

Raeder U, Broda P. 1985. Rapid preparation of DNA from filamentous fungi. Lett Appl Microbiol 1:17--20.

Rayner RW. 1970. A mycological colour chart. Kew, Surrey, UK: CMI and British Mycological Society. 34 p.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Roux J. 1998. Diseases of *Acacia mearnsii* in South Africa, with particular reference to Ceratocystis wilt. [PhD thesis]. Pretoria, South Africa: University of Pretoria. 238 p.

Roux J, Coutinho TA, Mujuni Byabashaija D, Wingfield MJ. 2001. Diseases of plantation *Eucalyptus* in Uganda. S Afr J Sci 97:16--18.

Saccardo PA. 1877. Fungi Veneti novi vel critici. Michelia 1:1--72.

Scurfield G. 1966. The cankers of *Acacia mucronata* Willd. Ex H. Windl. Aust J Bot 14:293--302.

Shoemaker RA. 1964. Conidial states of some *Botryosphaeria* species on *Vitis* and *Quercus*. Can J Bot 42:1297--1301.

Slippers B, Crous PW, Denman S, Coutinho TA, Wingfield BD, Wingfield MJ. 2003. Combined multiple gene genealogies and phenotypic characters differentiate several species previously identified as *Botryosphaeria dothidea*. Mycologia (In press).

Smith H, Wingfield MJ, Crous PW, Coutinho TA. 1996. *Sphaeropsis sapinea* and *Botryosphaeria dothidea* endophytic in *Pinus* spp. and *Eucalyptus* spp. in South Africa. S Afr J Bot 62:86--88.

---, Crous PW, Wingfield MJ, Coutinho TA, Wingfield BD. 2001. *Botryosphaeria eucalyptorum* sp. nov., a new species in the *B. dothidea*-complex on *Eucalyptus* in South Africa. Mycologia 93:277--284.

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

Stevens NE, Jenkins AE. 1924. Occurrence of the current cane blight fungus on other hosts. J Agric Res 27:837--844.

Swofford DL. 1999. PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. Sunderland, Massachusetts: Sinauer Associates.

Taylor JW, Jacobson DJ, Kroken S, Kasuga T, Geiser DM, Hibbett DS, Fisher MC. 2000. Phylogenetic species recognition and species concepts in fungi. Fungal Genet Biol 31:21--32.

Trotter A. 1928. Sylloge fungorum omnium hucusque cognitorum 24(2):810--815.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH,

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Snisky JJ, White TJ, eds. PCR protocols: a guide to methods and applications. San Diego: Academic Press. p 315--322.

Wingfield MJ, Slippers B, Roux J, Wingfield BD. 2001a. Worldwide movement of exotic forest fungi, especially in the tropics and the southern hemisphere. BioScience 51:134--140.

---, Roux J, Coutinho T, Govender P, Wingfield BD. 2001b. Plantation disease and pest management in the next century. S Afr For J 190:67--71.

Witcher W, Clayton CN. 1963. Blueberry stem blight caused by *Botryosphaeria dothidea* (*B. ribis*). Phytopathology 42:521--525.

Zhou S, Smith DR, Stanosz GR. 2001. Differentiation of *Botryosphaeria* species and related anamorphic fungi using Inter Simple or Short Sequence Repeat (ISSR) fingerprinting. Mycol Res 105:919--926.

---, Stanosz GR. 2001a. Relationships among *Botryosphaeria* species and associated anamorphic fungi inferred from the analyses of ITS and 5.8S rDNA sequences. Mycologia 93:516--527.

---, ---. 2001b. Primers for amplification of mt SSU rDNA, and a phylogenetic study of *Botryosphaeria* and associated anamorphic fungi. Mycol Res 105:1033--1044.

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TABLE I. Isolates considered in the phylogenetic study.

Culture no.[1]	Other no.[1]	Identity	Host	Location	Collector
CMW7772		*Botryosphaeria ribis*	*Ribes* sp.	New York, USA	B. Slippers / G. Hudler
[2]CMW7054	CBS121	*B. ribis*	*R. rubrum*	New York, USA	N.E. Stevens
CMW9078	ICMP7925	*B. parva*	*Actinidia deliciosa*	New Zealand	S.R. Pennycook
CMW9081	ICMP8003	*B. parva*	*Populus nigra*	New Zealand	G.J. Samuels
[2]CMW10125	BOT24	*B. eucalyptorum*	*Eucalyptus grandis*	Mpumalanga, S Africa	H. Smith
CMW11705		*B. eucalyptorum*	*E. nitens*	S Africa	B. Slippers
[2]CMW992/3	KJ93.52	*B. lutea*	*A. deliciosa*	New Zealand	G.J. Samuels
CMW9076	ICMP7818	*B. lutea*	*Malus X domestica*	New Zealand	S.R. Pennycook
CMW10309	CAP0002	*B. lutea*	*Vitis vinifera*	Portugal	A.J.L. Phillips
CMW10310	CAP037	*B. lutea*	*V. vinifera*	Portugal	A.J.L. Phillips
CMW9072		*B. australis*	*Acacia* sp.	Melbourne, Australia	J. Roux / D. Guest
CMW9073		*B. australis*	*Acacia* sp.	Melbourne, Australia	J. Roux / D. Guest
CMW6837		*B. australis*	*Acacia* sp.	Batemans Bay, Australia	M.J. Wingfield
CMW6853		*B. australis*	*Sequoiadendron giganteum*	Canberra, Australia	M.J. Wingfield
CMW9075		*B. dothidea*	*Populus* sp.	New Zealand	G.J. Samuels
CMW8000		*B. dothidea*	*Prunus* sp.	Crocifisso, Switzerland	B. Slippers
CMW7060	CBS 431	*B. stevensii*	*Fraxinus excelsior*	Netherlands	H.A. van der Aa
CMW7774		*B. obtusa*	*Ribes* sp.	New York, USA	B. Slippers / G. Hudler
CMW10130	BOT977	*B. rhodina*	*Vitex* sp.	Uganda	J. Roux

[1] Designation of isolates and culture collections: 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).
[2] ITS sequences for these isolates were obtained from GenBank (from the top down: AF241177, AF283686 and AF027745)

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TABLE II. Polymorphic nucleotides (or alleles) from sequence data of three gene regions for isolates of *Botryosphaeria lutea* and *B. australis*. Positions of polymorphisms are as in the combined and aligned dataset. Alleles that are fixed in these species are indicated in upper case.

Species	**Isolate number**	**β-tubulin**				**ITS**			**EF1-α**								
		40	98	270	584	873	878	958	1032	1112	1226	1246	1253	1283	1292	1323
B. lutea	CMW9076	T	C	G	G	T	T	C	a	C	-	C	A	-	-	C
	CMW992	T	C	G	G	T	T	C	a	C	-	C	A	-	-	C
	CMW10309	T	C	G	G	T	T	C	g	C	-	C	A	-	-	C
	CMW10310	T	C	G	G	T	T	C	g	C	-	C	A	-	-	C
B. australis	CMW9072	C	T	A	-	C	C	T	g	T	T	A	G	A	T	T
	CMW9073	C	T	A	-	C	C	T	g	T	T	A	G	A	T	T
	CMW6837	C	T	A	-	C	C	T	g	T	T	A	G	A	T	T
	CMW6853	C	T	A	-	C	C	T	g	T	T	A	G	A	T	T

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TABLE III. Measurements of ascospores and conidia of *Botryosphaeria lutea* and *B. australis* and their *Fusicoccum* anamorphs.

Identity	Ascospore size (µm)	Conidial size[1] (µm)	L/W	Source of data
F. luteum	Not seen	(14--)20--24(--32) x (5--)6--7(--9) [Ave. 21.7 x 6.7] – *in vitro*	3.2	Pennycook and Samuels 1985
B. lutea / *F. luteum*	18--22.5(--24) x 7.5--12	(12--)16.5--22.5(--24) x 4.5--6(--7.5) [Ave. 17.2 x 4.5--6] – *in vivo*	2.8	Phillips et al 2002
		(15--)18--22.5(--24) x 4.5--6(--7.5) [Ave. 19.7 x 5.6] – *in vitro*	3.6	
B. australis / *F. australe*	20--22(--23.5) x 7--8	(17.5--)24.5(--30) x 5(--7.5) – *in vitro*	4.8	This study

[1] *In vitro* (in culture) and *in vivo* (field collected samples) conidial measurements are given separately, as they differ from each other.

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FIG. 1. One of the most parsimonious trees obtained through heuristic searches of the combined dataset of the ITS rDNA, β-tubulin and EF1-α regions. Support for branches and nodes are indicated as bootstrap values (1000 replicates) below and decay values above the branches, respectively. The *Botryosphaeria* spp. with *Diplodia*-like anamorphs (*B. rhodina, B. obtusa* and *B. stevensii*) are treated as outgroup taxa to which the tree is rooted. The remaining *Botryosphaeria* spp. that form the ingroup all have *Fusicoccum* anamorphs. Isolates' number, host and origin (Aust = Australia, Neth = Netherlands, NY = New York, USA, NZ = New Zealand, SA = South Africa, Swit = Switzerland, Ug = Uganda) are indicated, as well as the taxonomic identities of the clades.

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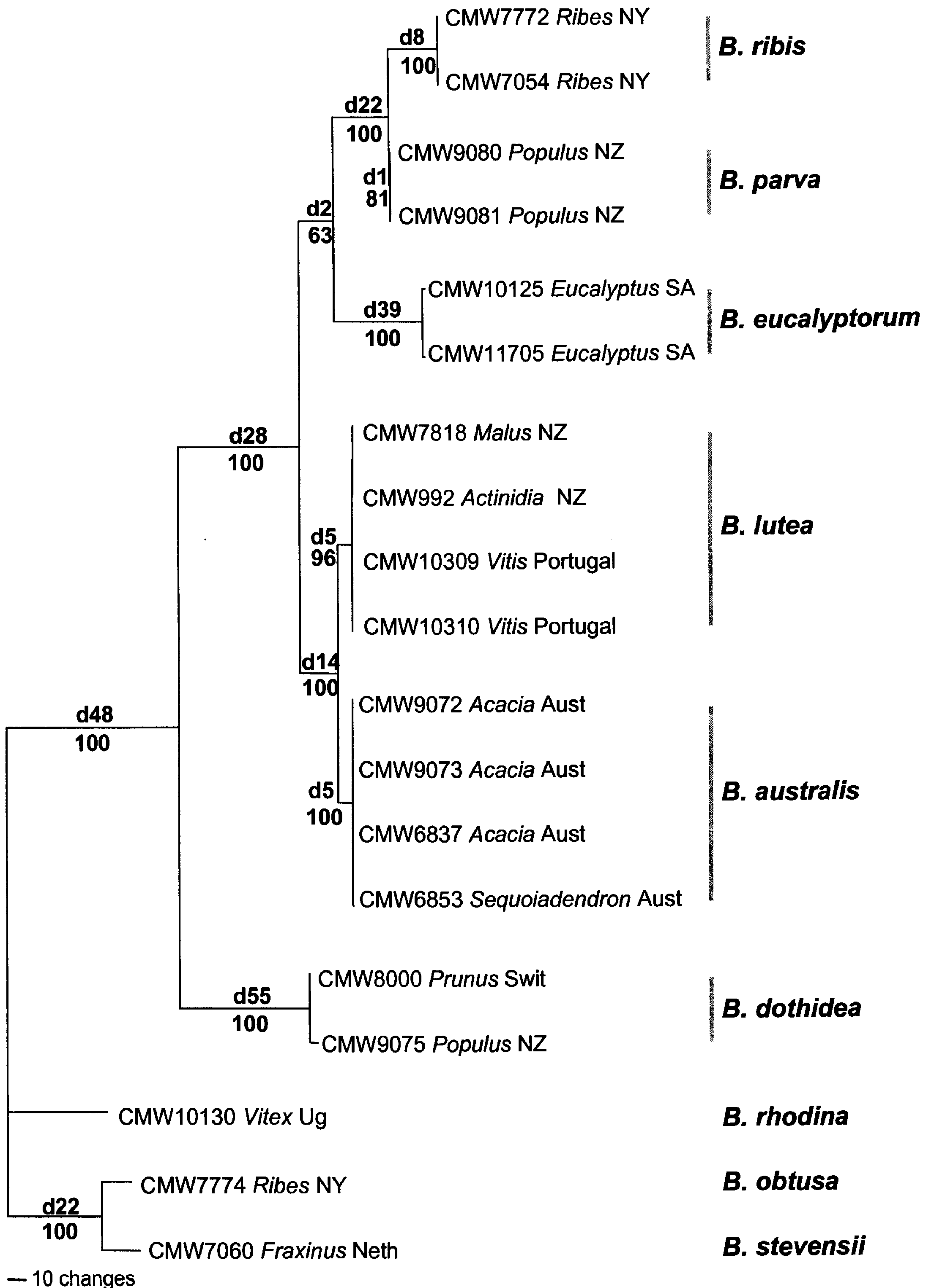

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FIG. 2. Representative most parsimonious (MP) trees of individual analyses of (A) the ITS-rDNA, (B) β-tubulin and (C) EF1-α regions. To avoid repetition, only the branches carrying isolates of the species in question in this study, *Botryosphaeria lutea* and *B. australis*, are shown. The values of tree length, phylogenetic signal (g1), consistency index (CI) and retention index (RI) are given for each tree. The lengths of the branches are indicated above the branches and the bootstrap values (1000 replicates) below the branches.

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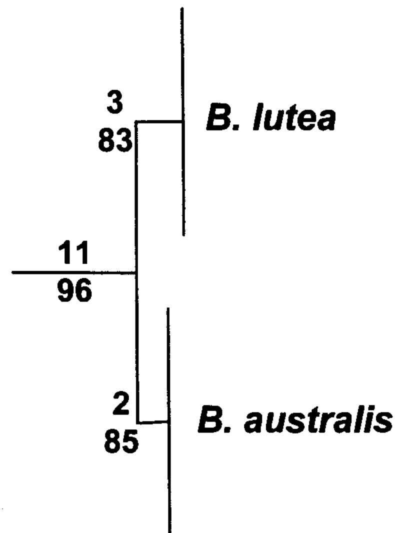
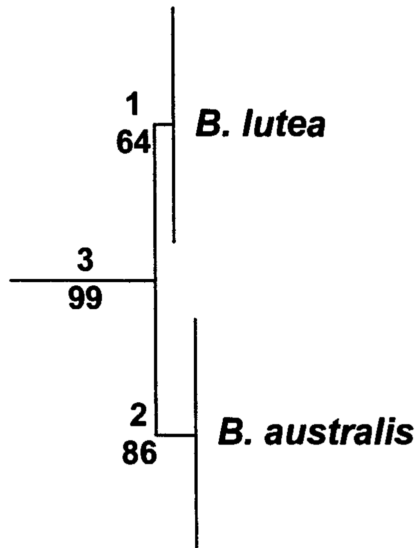
A. ITS rDNA



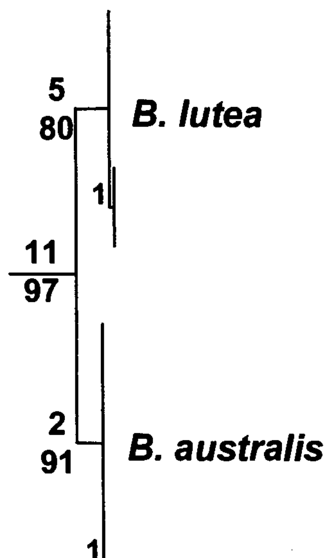
MP scores of 2 trees
Tree length = 141
g1 = -1.41
CI = 0.851
RI = 0.923

B. β-tubulin



MP scores of 3 trees
Tree length = 114
g1 = -0.96
CI = 0.816
RI = 0.896

C. Ef1-α



MP scores of 3 trees
Tree length = 283
g1 = -1.23
CI = 0.855
RI = 0.912

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FIG. 3. The representative branch of the *Botryosphaeria lutea* and *B. australis* clade identified using parsimony and based on ITS-rDNA sequence data. This branch contains all available sequences from this study and GenBank that group with these taxa. The tree length, phylogenetic signal (g1), consistency index (CI) and retention index (RI) are given for the tree. The lengths of the branches are indicated above the branches and the bootstrap values (1000 replicates) below the branches. GenBank sequence- and isolate numbers, host and origin (Aust = Australia, Ital = Italy, NZ = New Zealand, Port = Portugal, SA = South Africa) are indicated, as well as the taxonomic identities of the clades.

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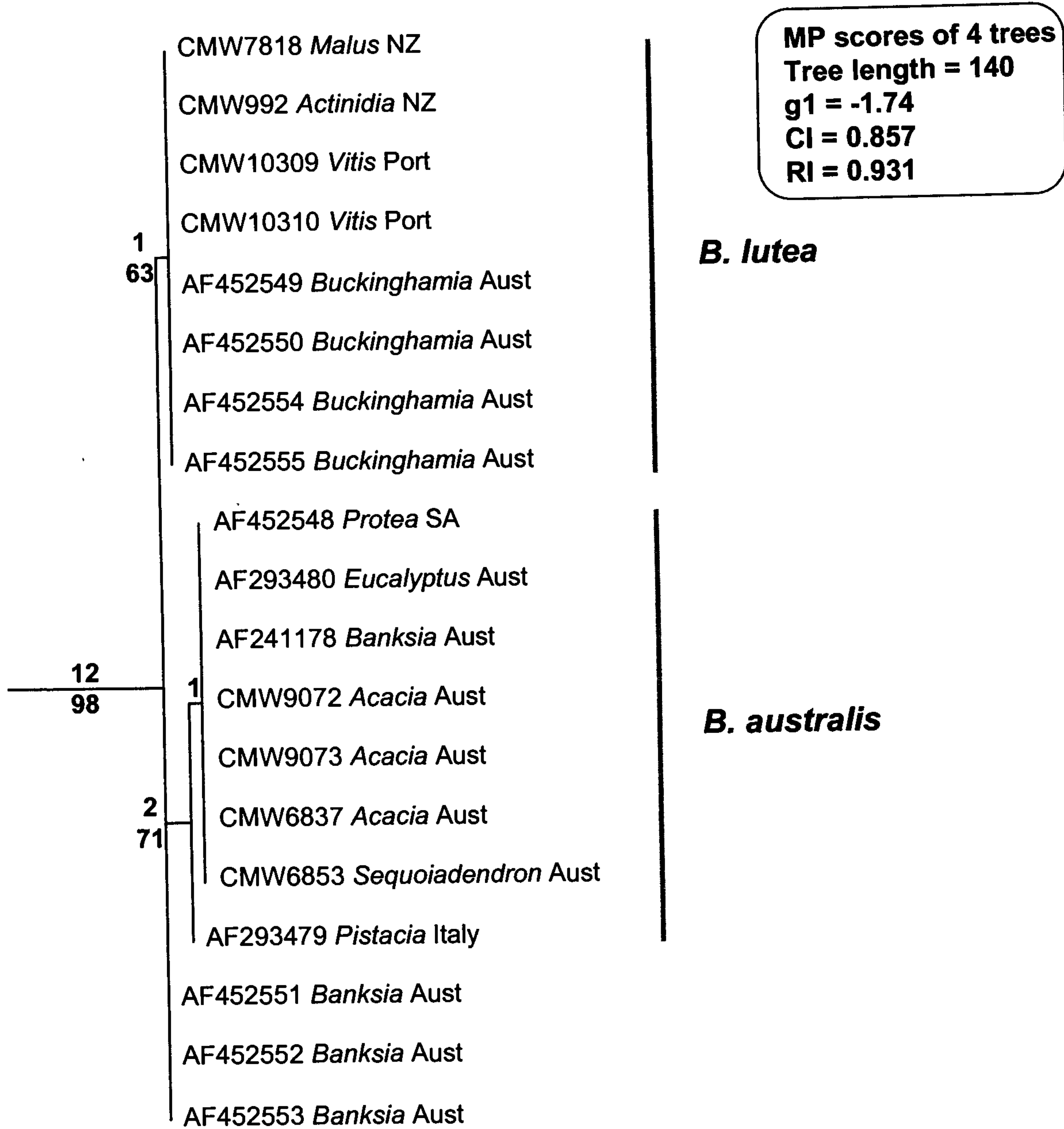

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FIG. 4. Asci, ascospores and pseudoparaphyses of *Botryosphaeria australis.* Bar = 10 μm. (Sketch by PW Crous).

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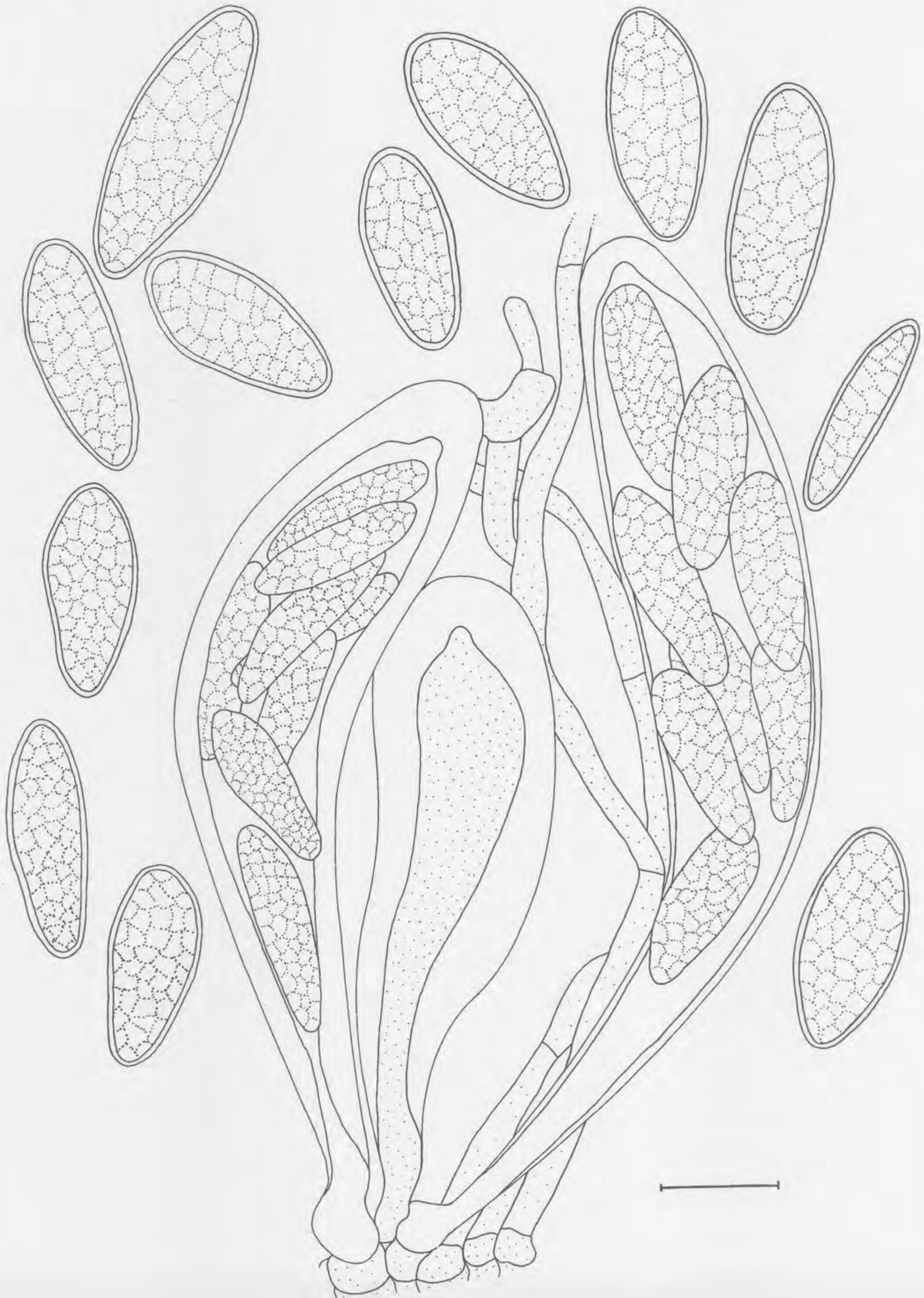

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FIG. 5. Conidia and conidiogenous cells of *Fusicoccum australe*. Bar = 10 µm. (Sketch by PW Crous).

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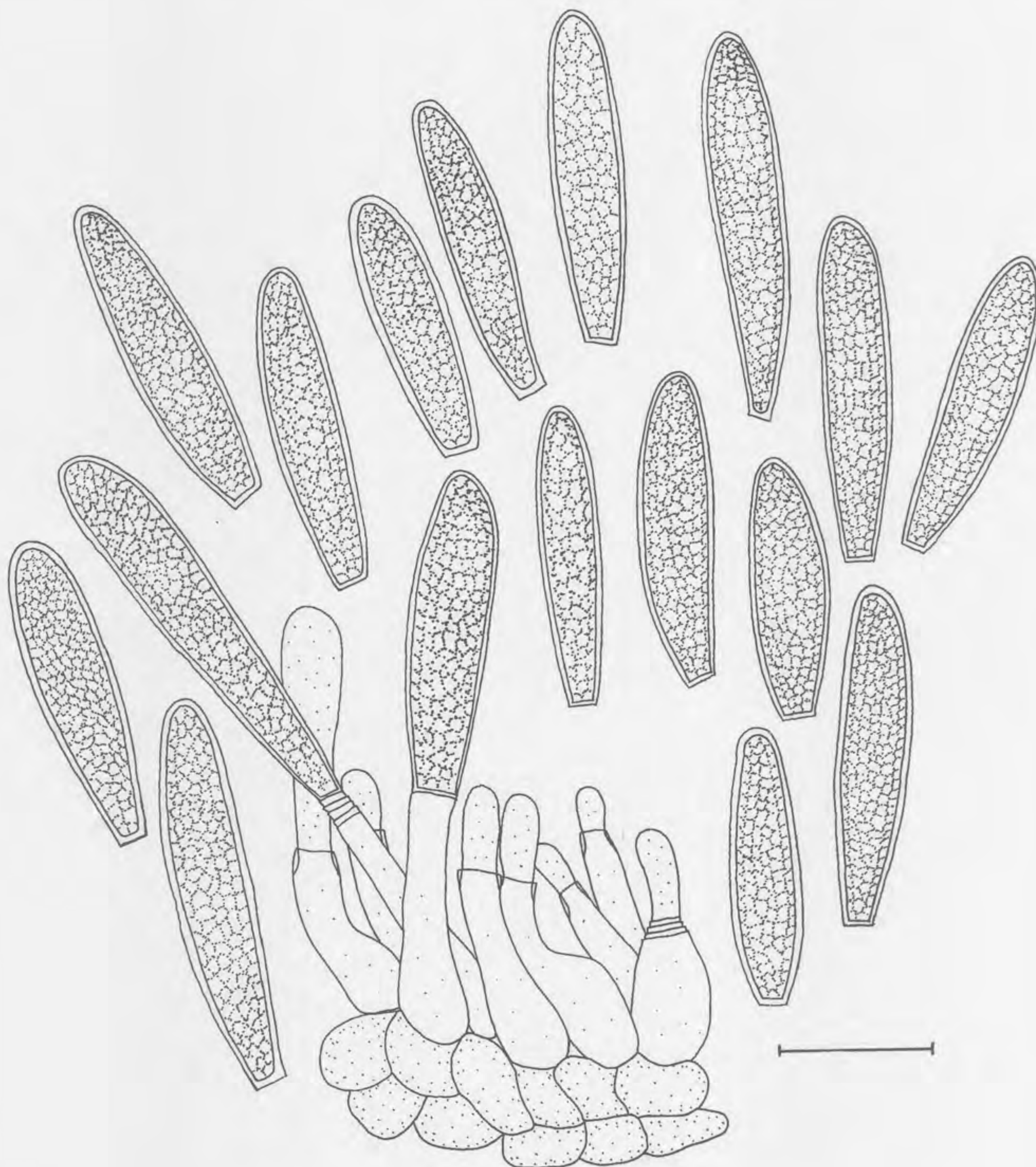

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FIGS. 6-14. *Botryosphaeria australis* dissecting microscope and DIC compound-microscope micrographs. 6. Ascomata in botryose clusters that erupt through the bark. When dissected, the contents are conspicuously white (arrows). 7, 8. Sections through ascomata and ascomatal neck. Bars = 100 μm. 9. Bitunicate asci and immature ascospores. 10, 11. Mature ascospores. 12, 13. Conidiogenous cells with periclinal thickening and percurrent proliferation (arrows). 14. Fusiform conidia. Bars = 10 μm.

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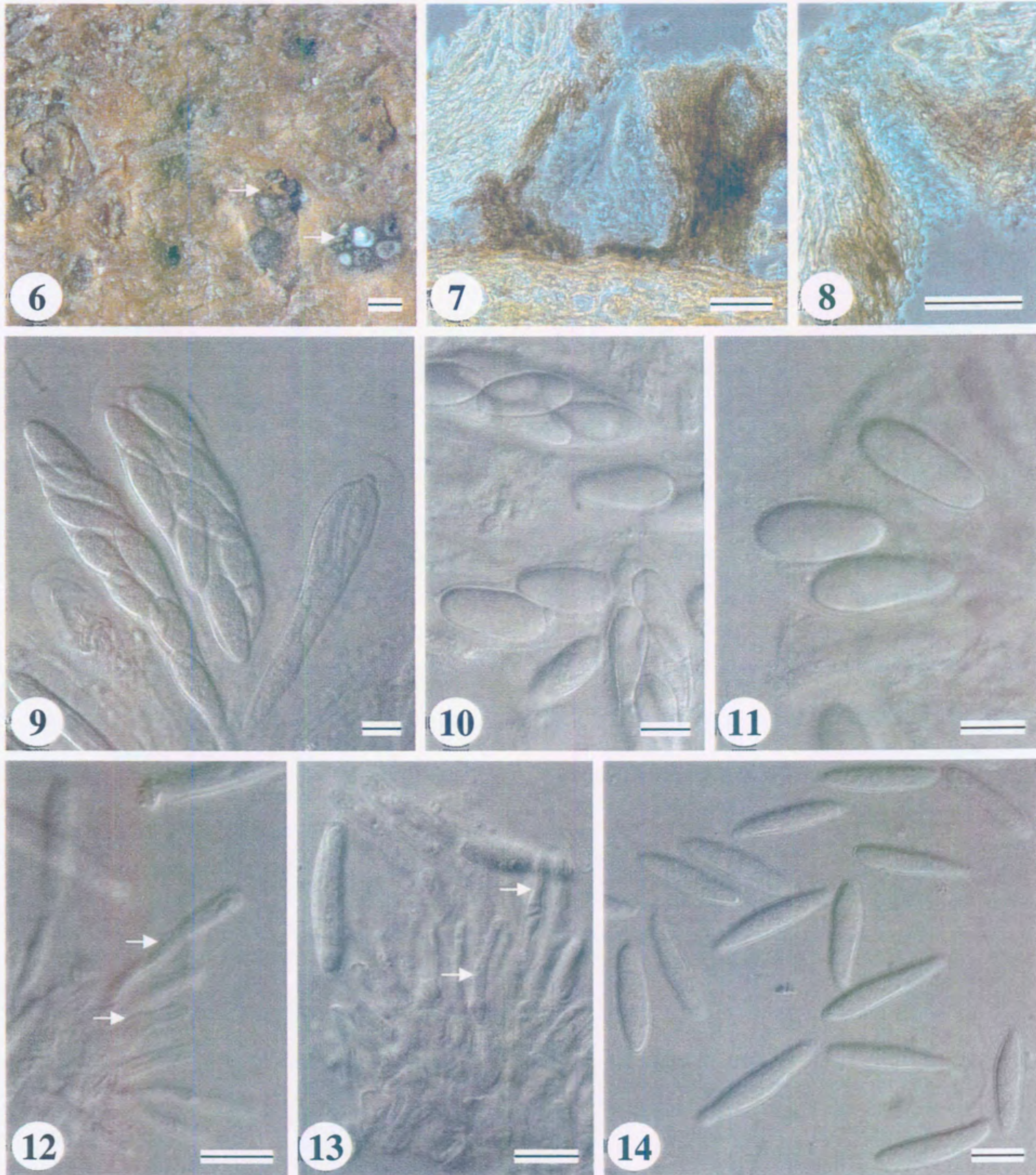

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