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Multiple gene sequences delimit *Botryosphaeria australis* sp. nov. from *B. lutea*

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Abstract: *Botryosphaeria lutea* (anamorph *Fusicoccum luteum*) most easily is 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 from the one formed by 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 *Acacia* 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, however, was 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* also was 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 been identified previously 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.

Key words: culture, conidia, morphology, multiple genealogy concordance, native hosts, phylogeny, Southern Hemisphere

INTRODUCTION

Various native Australian woody plants, such as *Acacia* spp., *Eucalyptus* spp., species of Proteaceae and others, make up commercial plantations worldwide (Evans 1984, Wingfield et al 2001a, b, Denman et al 2003). To establish and maintain these plantations, germplasm must be introduced into different countries, which 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 is overlooked easily is *Botryosphaeria* spp., which lives as endophytes in healthy plants and seeds for part of its 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). 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 planted commercially (Palm 1999, Wingfield et al 2001b).

Members of the genus *Botryosphaeria* commonly

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are accepted to be difficult 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 found frequently in nature and is produced only rarely in culture. In addition, it currently is 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 often are not distinctive at species level (von Arx and Müller 1954, Slippers et al 2004).

Conidial and cultural morphology often are used to distinguish different *Botryosphaeria* spp. (Shoemaker 1964, Pennycook and Samuels 1985, Denman et al 2000, Slippers et al 2004). The anamorphs of these fungi are encountered commonly, both in nature and in culture. Distinctive features of conidia are the shape, size, length/width ratio, septation, content, color, wall thickness and ornamentation. In addition, the general growth pattern, speed and color of a colony on agar sometimes are informative for species identification (Pennycook and Samuels 1985, Phillips et al 2002, Slippers et al 2004).

In recent years, various DNA based techniques have been used to distinguish between *Botryosphaeria* spp. These techniques include dominant and codominant molecular markers such as RAPDs, ISSRs 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 2004). However, these data have 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 2004). 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 be distinguished most easily 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 Portuguese 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 previously was recognized. This pathogen evidently has been mistaken as well for *B. dothidea* (Fr. : Moug.) Ces. & De Not. (Phillips et al 2002).

Botryosphaeria rhodina (Berk. & Curt.) Arx and *B. dothidea* both have been reported from native Australian *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 *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 also was obvious. The aim of this study, therefore, was to determine the relationship between these isolates from *Acacia* in Australia and *B. lutea* isolates from New Zealand and Portugal. 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 translation elongation factor 1α [EF-1α]) were compared. Teleomorph, anamorph and cultural characters also were considered.

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* in 1999 and by the senior author in 2001 (TABLE I). Four similar isolates also were 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) also were included (TABLE I). Isolates were maintained on malt-yeast extract agar (MYA) (2% malt extract, 0.2% yeast extract and 2% agar; Biolab, Johannesburg, South Africa) 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 pur-

TABLE I. Isolates considered in the phylogenetic study

Culture No.[1]	Other No.[1]	Identity	Host	Location	Collector	GenBank		
						ITS	β-tubulin	EF 1-α
CMW7772		*Botryosphaeria ribis*	*Ribes* sp.	New York, USA	B. Slippers/G. Hudler	AY236925,	AY236906,	AY236877
CMW7054	CBS121	*B. ribis*	*R. rubrum*	New York, USA	N.E. Stevens	AF241177,	AY236908,	AY236879
CMW9078	ICMP7925	*B. parva*	*Actinidia deliciosa*	New Zealand	S.R. Pennycook	AY236940,	AY236914,	AY236885
CMW9081	ICMP8003	*B. parva*	*Populus nigra*	New Zealand	G.J. Samuels	AY236943,	AY236917,	AY236888
CMW10125	BOT24	*B. eucalyptorum*	*Eucalyptus grandis*	Mpumalanga, S Africa	H. Smith	AF283686,	AY236920,	AY236891
CMW11705		*B. eucalyptorum*	*E. nitens*	S Africa	B. Slippers	AY339248,	AY339256,	AY339264
CMW992/3	KJ93.52	*B. lutea*	*A. deliciosa*	New Zealand	G.J. Samuels	AF027745,	AY236923,	AY236894
CMW9076	ICMP7818	*B. lutea*	*Malus* × *domestica*	New Zealand	S.R. Pennycook	AY236946,	AY236922,	AY236893
CMW10309	CAP002	*B. lutea*	*Vitis vinifera*	Portugal	A.J.L. Phillips	AY339258,	AY339250,	AY339266
CMW10310	CAP037	*B. lutea*	*V. vinifera*	Portugal	A.J.L. Phillips	AY339259,	AY339251,	AY339267
CMW9072		*B. australis*	*Acacia* sp.	Melbourne, Australia	J. Roux/D. Guest	AY339260,	AY339252,	AY339268
CMW9073		*B. australis*	*Acacia* sp.	Melbourne, Australia	J. Roux/D. Guest	AY339261,	AY339253,	AY339269
CMW6837		*B. australis*	*Acacia* sp.	Batemans Bay, Australia	M.J. Wingfield	AY339262,	AY339254,	AY339270
CMW6853		*B. australis*	*Sequoiadendron giganteum*	Canberra, Australia	M.J. Wingfield	AY339263,	AY339255,	AY339271
CMW9075		*B. dothidea*	*Populus* sp.	New Zealand	G.J. Samuels	AY236950,	AY236928,	AY236899
CMW8000		*B. dothidea*	*Prunus* sp.	Crocifisso, Switzerland	B. Slippers	AY236949,	AY236927,	AY236898
CMW7060	CBS 431	*B. stevensii*	*Fraxinus excelsior*	Netherlands	H.A. van der Aa	AY236955,	AY236933,	AY236904
CMW7774		*B. obtusa*	*Ribes* sp.	New York, USA	B. Slippers/G. Hudler	AY236953,	AY236931,	AY236902
CMW10130	BOT977	*B. rhodina*	*Vitex* sp.	Uganda	J. Roux	AY236951,	AY236929,	AY236900

[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] Three GenBank accession numbers are given for each isolate representing its ITS rDNA, β-tubulin and EF1-α sequences, in that order.

poses were from Slippers et al (2004) (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).

Sequences of three nuclear loci 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 and ITS4 (White et al 1990). A region of the β-tubulin gene was amplified using the primers Bt2a and Bt2b (Glass and Donaldson 1995). In addition, a part of the EF1-α was amplified using the primers EF1-728F and EF1-986R (Carbone et al 1999). PCR reaction mixtures, PCR conditions and visualization of amplicons are as described by Slippers et al (2004). The amplicons of all three DNA regions were purified and sequenced as described by Slippers et al (2004), using the same primers used to generate the amplicons.

Sequence data were analyzed with Sequence Navigator version 1.0.1™ (Perkin Elmer Applied Biosystems, Foster City, California) 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 were congruent (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 with PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 (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 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 analyzed separately but in the same way as described above. The ITS rDNA dataset was 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, South Africa) 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 hand and mounted in clear lactophenol. Morphological observations were made and images were recorded on a light microscope and Axiocam digital camera (Carl Zeiss, Germany). The morphology of these samples was compared with material 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 extype 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, South Africa) at 5 C intervals ranging from 10 to 35 C in the dark. Growth rate was measured at the leading edge in 24 h 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 isolates. Sequence data at the 5′ and 3′ ends were deleted from the dataset if they were doubtful (GenBank AY339248–AY339271). The partition homogeneity test of the ITS-rDNA, β-tubulin and EF1-α datasets, indicated that they were congruent (*P* value = 0.53). The data from the three regions were combined and the total dataset consisted of 1324 characters after alignment (TreeBASE S1008 [study]; M1704 [matrix]). 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 14 as was the case before the coding. Of the remaining characters, 322 were parsimony informative and used in the analyses. This combined dataset contained significant phylogenetic signal compared to random trees ($P < 0.01$; g1 = −0.97) (Hillis and Huelsenbeck 1992). After heuristic searches in PAUP, three equally 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 bootstrap support). The separation also was supported in the individual analyses of the sequence

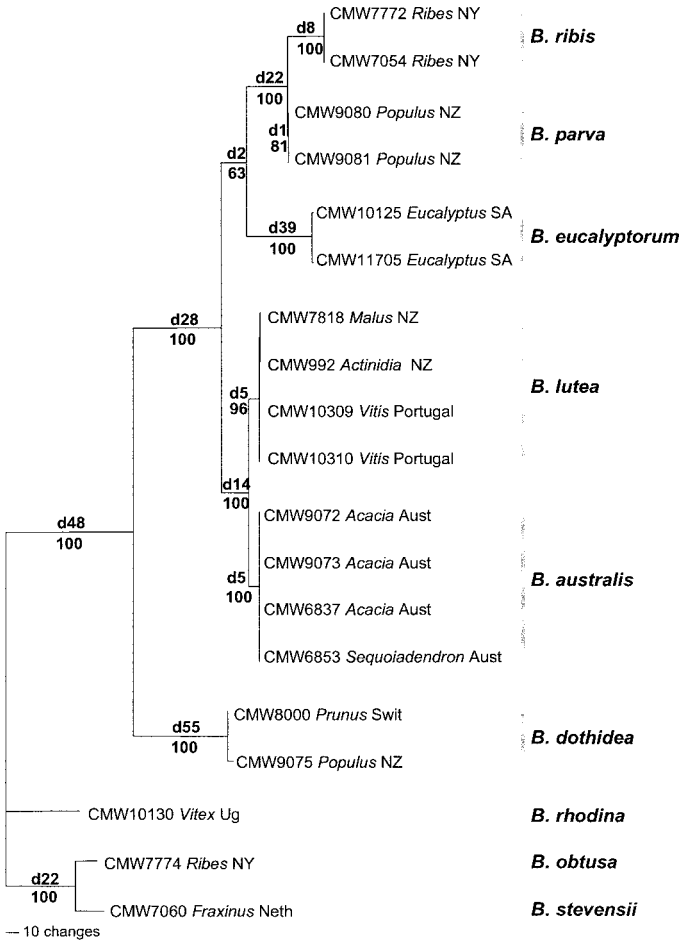


FIG. 1. One of three most-parsimonious trees obtained through heuristic searches of the combined dataset of the ITS-rDNA, β-tubulin and EF1-α regions. Support for branches and nodes are indicated respectively as bootstrap values (1000 replicates) below and decay values above the branches. *Botryosphaeria* 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 ingroup have *Fusicoccum* anamorphs. Isolate numbers, hosts and origins (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.

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 the alleles were fixed in the two groups at 14 of the 15 polymorphic loci. Of these 15 polymorphic sites, four were indels, 10 transitions and a transversion. The bias toward transitions 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 here as new.

GenBank sequences of the ITS rDNA region from isolates that previously were identified as *B. lutea* or *F. luteum* (Smith and Stanosz 2001, Denman et al 2003) separated into both *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 of the *B. lutea* and *B. australis* clades.

Isolates residing in the clades representing *B. lutea* and the new species described in this study were related more closely to each other (d14/100% bootstrap) than to any other taxon included in the analysis. In addition, these two species were related more closely to *B. ribis, B. parva* and *B. eucalyptorum* (d28/100% bootstrap) than to *B. dothidea*. All these species

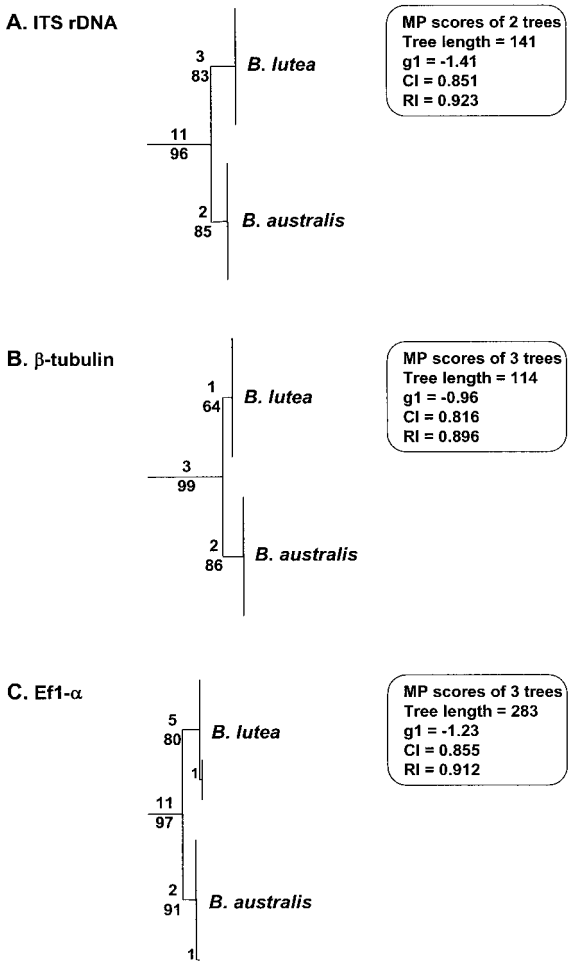


FIG. 2. Representative most parsimonious (MP) trees of individual analyses of (A) the ITS-rDNA, (B) β-tubulin and (C) EF1-α regions. To avoid repetition, only the branches carrying isolates of the species in question in this study, *Botryosphaeria lutea* and *B. australis,* are shown. The values of tree 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.

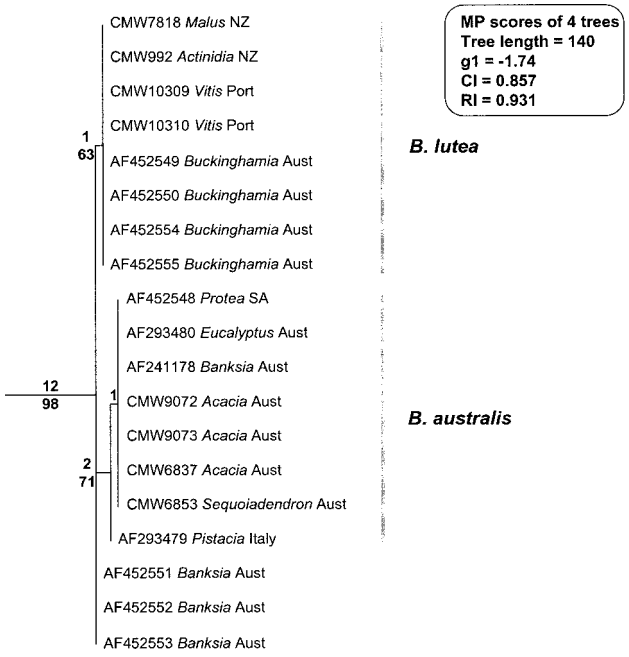


FIG. 3. The representative branch of the *Botryosphaeria lutea* and *B. australis* clade identified using parsimony and based on ITS-rDNA sequence data. This branch contains all available sequences from this study and GenBank that group with these taxa. The tree length, phylogenetic signal (g1), consistency 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.

have *Fusicoccum* anamorphs and group together (100% bootstrap), as opposed to isolates of *B. obtusa, B. stevensii* and *B. rhodina,* which have *Diplodia* or *Lasiodiplodia* anamorphs. DNA sequences from 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 2–3 wk. Teleomorph and anamorph structures of this fungus from field samples and from conidia formed in culture were similar in morphology to those of *B. lutea* and *F. luteum* (FIGS. 4–12). This new species produced a yellowish pigment in young cultures, as also was true for *B. lutea.* The fungus from *Acacia* and *Sequoiadendron,* however, could be distinguished from *B. lutea* and *F. luteum* by its longer conidia with a higher l/w ratio (TABLE II). 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 thus is described as new:

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

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

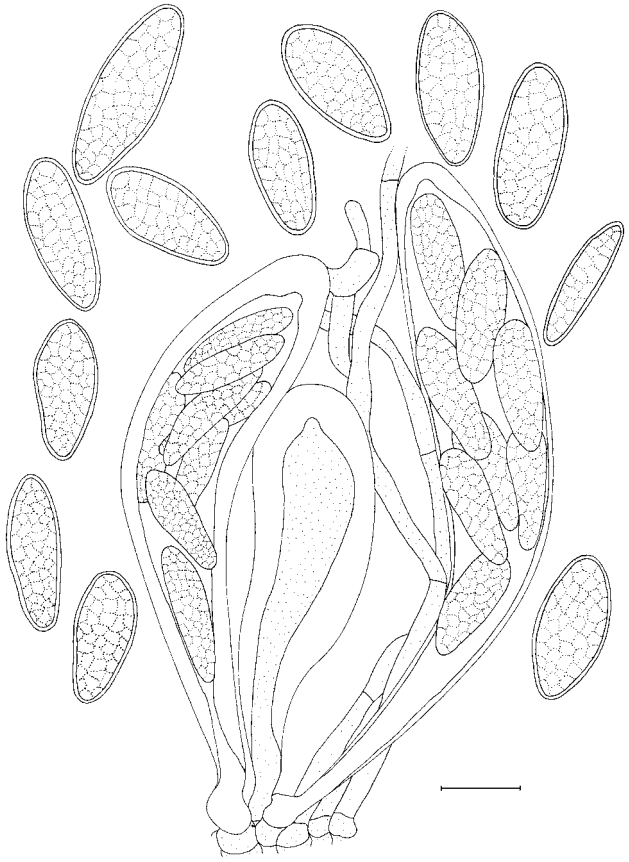


FIG. 4. Asci, ascospores and pseudoparaphyses of *Botryosphaeria australis*. Bar = 10 μm.

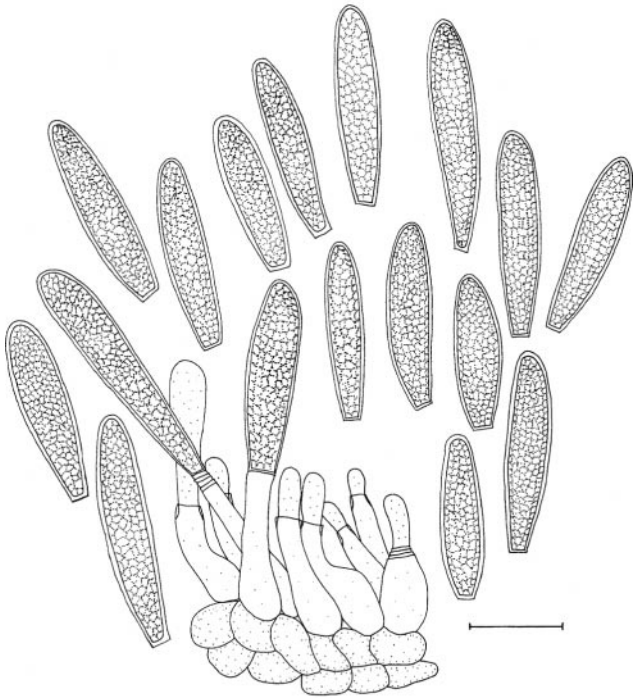


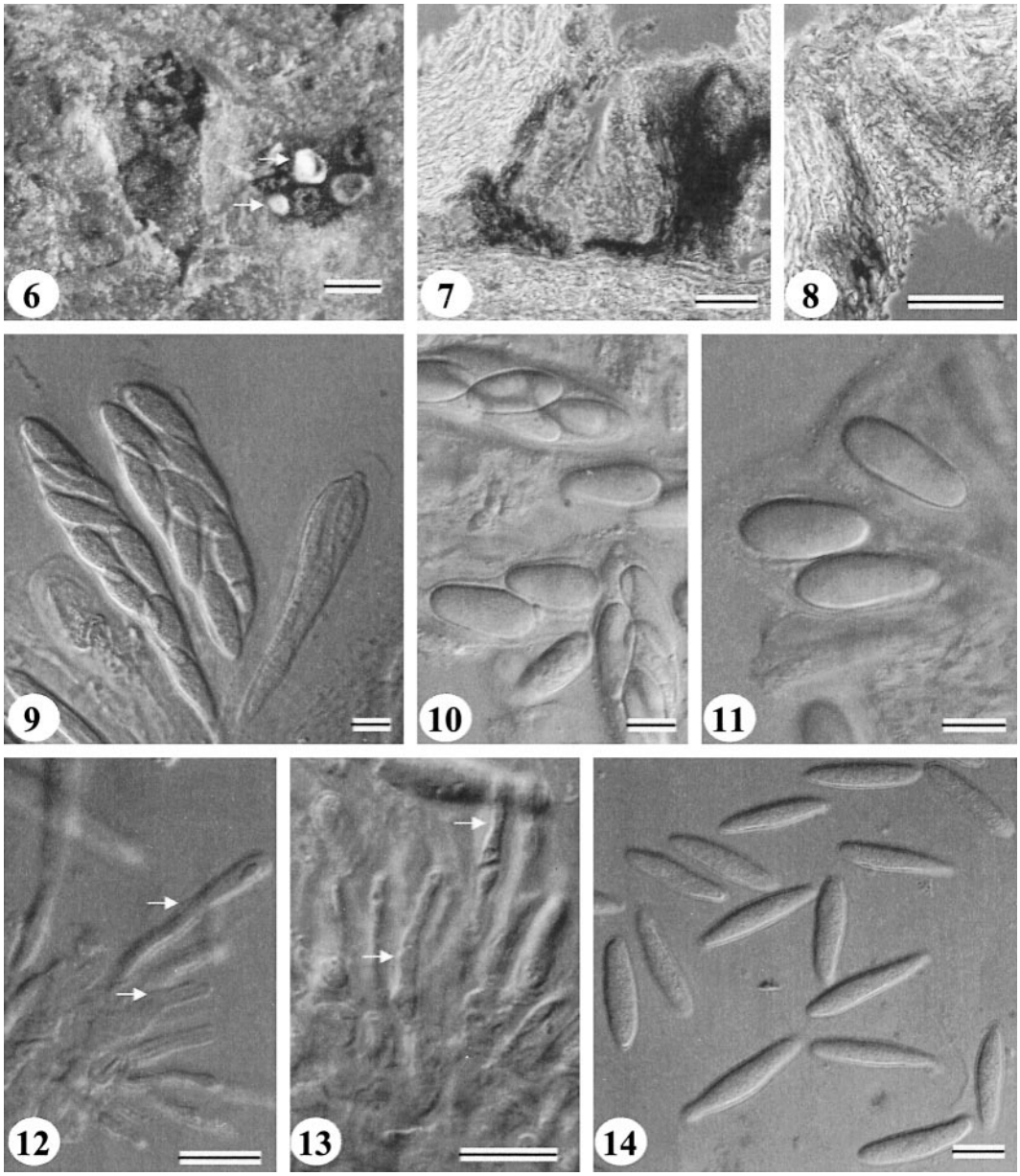
FIG. 5. Conidia and conidiogenous cells of *Fusicoccum australe*. Bar = 10 μm.

Etymology. Referring to the origin in the Southern Hemisphere.

Note. The name *B. australis* (Cooke) Petrak is reported by Gibson (1975) in a checklist of fungi from *Eucalyptus*. A literature survey, however, revealed that Petrak did not describe such a taxon. The listing in Gibson, therefore, is incorrect and the name is available for use.

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 ⅔ 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 × 16–25 μm, inter pseudoparaphyses multas, filiformes, septatas, raro apicem versus ramosas, 3–4 μm latas. Ascosporae fusoideae vel ovoideae, 20–23(–25) × 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) × 5–6(–7.5) μm, hyalina, unicellularia raro septum ante germinationem facientia, laevia contentis granularibus. Cellulae conidiogenae holoblasticae, hyalinae, subcylindricae, 10–14 × 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 ⅔ 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 × 16–25 μm, 8-spored, between numerous filiform, septate pseudoparaphyses, rarely branched in the upper parts, 3–4 μm wide. *Ascospores* fusoid to ovoid, 20–23(–25) × 7–8(–9) μm (average of 50 ascospores 21.9 × 7.6 μm, l/w 2.9), unicellular, hyaline, smooth with granular contents, biseriate in the ascus. *Conidiomata* (formed on WA on sterilized pine needles within 7–21 d) pycnidial, superficial, globose, mostly solitary and covered by mycelium. *Conidia* fusiform, base subtruncate, (18–)23–26(–30) × 5–6(–7.5) μm (average of 240 conidia 24.7 × 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 × 2–3 μm, phialidic with periclinal thickening or proliferating percurrently with



FIGS. 6–14. *Botryosphaeria australis* (anamorph *Fusicoccum australe*) 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 (arrows) with periclinal thickening and percurrent proliferation. 14. Fusiform conidia. Bars = 10 μm.

1–4 proliferations. *Spermatia* not observed. *Cultures* having buff (19″f) to light primrose (23″b) colonies, light yellowish pigment diffusing into the medium most noticeably between 15–20 C in the dark, becoming olivaceous buff (21‴d) to olivaceous gray (21‴‴i) after 5–6 d, 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 diam on PDA after 4 d 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).

TABLE II. 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) × (5–)6–7(–9) [Ave. 21.7 × 6.7]—*in vitro*	3.2	Pennycook and Samuels 1985
B. lutea/F. luteum	18–22.5(–24) × 7.5–12	(12–)16.5–22.5(–24) × 4.5–6(–7.5) [Ave. 17.2 × 4.5–6]—*in vivo*	2.8	Phillips et al 2002
		(15–)18–22.5(–24) × 4.5–6(–7.5) [Ave. 19.7 × 5.6]—*in vitro*	3.6	
B. australis/F. australe	20–22(–23.5) × 7–8	(18–)23–26(–30) × 5–6(–7.5) [Ave. 24.7 × 5.1]—*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.

DISCUSSION

In this study *Botryosphaeria australis* and its anamorph *F. australe* are newly described. This fungus phylogenetically is related closely 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, however, were distributed in the same manner or fixed 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 otherwise would 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 thus can obscure the true species diversity in *Botryosphaeria*. This is similar to *B. ribis* and *B. parva* (Slippers et al 2004), 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 2004). These case studies provide good evidence to show that single gene genealogies are insufficient to distinguish cryptic *Botryosphaeria* spp.

Based on ITS rDNA sequences from GenBank, three isolates grouped closer to *B. lutea* and *B. australis* than any other *Botryosphaeria* spp. included in the study, but was separate from both of them. Due to the small differences between the species based on ITS data, it is not clear whether these isolates represent a further closely related species or an ITS variation of either *B. lutea* or *B. australis*. More gene

sequence data is needed to clarify the true identity of these isolates. Similarly the small ITS sequence difference between the isolate from *Pistacia* in Italy and other *B. australis* isolates warrants further investigation.

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, however, could not be separated from ex-type isolates of *F. luteum* based on RAPD data. These authors also were not able to separate two other cryptic *Botryosphaeria* sp., namely *B. ribis* and *B. parva,* using RAPD data. Another similar example, in which 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 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 (2004) all have reported significant overlap in the sizes of *Fusicoccum* spp. In these cases and in our study the species, however, could be separated when averages of conidial sizes and septation were considered. Thus, on average, conidia of *F. australe* are longer and appear more slender (higher length/width ratio) than those of *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) 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. Before these studies, this pigment was not considered taxonomically useful (Witcher and Clayton 1963). Recent studies of other botryosphaeriaceous fungi also have recognized the value of culture morphology as a useful tool to distinguish *Botryosphaeria* spp. (Jacobs 2002, de Wet et al 2003, Slippers et al 2004).

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. However, the ascospores of this species are 1-seriate in the ascus and significantly different in size (17–20 × 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 also are associated with a *Diplodia* sp. Dingley (1970) reports ''small papilla on one end'' of the ascospores, which is absent in *B. australis*. This disease also was 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, 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 also are 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 2001a, Denman et al 2003). Only one isolate from outside these regions grouped with *B. australis*; this was from *Pistacia* in Italy. Thus we are of the opinion that the exotic *S. giganteum* trees sampled during this study in Australia have become infected with a native pathogen.

Botryosphaeria lutea is common throughout Australasia and 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*. 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, however, were based only on RAPD data, which did not separate *B. lutea* and *B. australis*. In Portugal *B. lutea* also 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 also is clear from our study that both these fungi can infect native and introduced or cultivated hosts in both regions. These introductions, thus, can have significant implications for agricultural and forestry industries and conservation of native flora. Population 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.

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