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Botryosphaeria species from *Eucalyptus* in Australia are pleoanamorphic, producing *Dichomera* synanamorphs in culture

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Species within the genus *Botryosphaeria* include some of the most widespread and important pathogens of woody plants, and have been the focus of numerous taxonomic studies in recent years. It is currently accepted that anamorphs of *Botryosphaeria* belong to two distinct genera, *Fusicoccum* and *Diplodia*. Species within the genus *Fusicoccum* commonly produce aseptate, hyaline conidia. In the present study, fungi were isolated from foliage and wood of *Eucalyptus* in native forests and plantations in Australia. Although these fungi produced *Dichomera* anamorphs in culture, they clustered within the *Fusicoccum* clade of *Botryosphaeria* based on their ITS sequence data. Four species, *Botryosphaeria dothidea*, *B. parva*, *B. ribis* and *B. australis* produced *Dichomera* conidia in culture. The *Dichomera* synanamorphs are described for these four species of *Botryosphaeria*. In addition, falling within the *Fusicoccum* clade of *Botryosphaeria*, two species were found to be distinct from previously described *Botryosphaeria* spp. based on their ITS sequences, but synonymous with *D. versiformis* and *D. eucalypti*. These observations are currently unique to isolates from host trees within the genus *Eucalyptus* in Australia, and the pleoanamorphic nature of these species is discussed.

INTRODUCTION

Eighteen anamorph genera have been linked to the ascomycete genus *Botryosphaeria* and this has resulted in a confused taxonomic history of the genus. The best known of these anamorphs are *Botryodiplodia*, *Diplodia*, *Dothiorella*, *Fusicoccum*, *Lasiodiplodia*, *Macrophoma* and *Sphaeropsis* (Sivanesan 1984, Denman *et al*. 2000). Traditionally, substantial emphasis has been placed on the morphological characters of these anamorphs to distinguish between *Botryosphaeria* species (Shoemaker 1964, Pennycook & Samuels 1985, Morgan-Jones & White 1987, Denman *et al*. 2000, Phillips *et al*. 2002, Slippers *et al*. 2004a). Unlike the teleomorphs, these anamorphs are frequently observed in nature, sporulate readily in culture, and have a greater variability in spore morphology, including shape, size, colour, septation and ornamentation.

Classical taxonomic studies have resulted in *Macrophoma* being reduced to synonymy with *Sphaeropsis* (Sutton 1980), *Dothiorella* being reduced to synonymy with *Diplodia*, and *Botryodiplodia* being regarded as a *nomen dubium* (Crous & Palm 1999). Studies of the anamorphs based on morphological characters and phylogenetic analysis of ITS rDNA sequence data have supported these findings and provided evidence for the separation of the anamorphs into two groups, one having *Diplodia*-like, most commonly ellipsoid and broad, thicker-walled and frequently septate and pigmented conidia, and the other with *Fusicoccum*-like, most commonly fusoid and narrow, thinner-walled, rarely septate and pigmented conidia (Jacobs & Rehner 1998, Denman *et al*. 2000, Zhou & Stanosz 2001b). Slippers *et al*. (2004a) sequenced the ITS rDNA, β-tubulin and EF1-α regions to provide a phylogeny supporting the view that *Botryosphaeria* represents two distinct phylogenetic assemblages, corresponding to species with *Diplodia* and *Fusicoccum* anamorphs.

Substantial confusion has surrounded the taxonomy of *Fusicoccum* and whether the type species, *F. aesculi*, should reside in *Fusicoccum* as described and illustrated by Saccardo (1880) or in *Dothiorella* according to

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Petrak (1922). *F. aesculi* has generally been accepted as the anamorph of *B. dothidea sensu* von Arx & Müller (1954). Slippers *et al*. (2004a) characterized *B. dothidea* based on morphology and the ITS rDNA, β-tubulin and EF1-α DNA-sequence data. In their study, epitype, neotype and syntype material was designated and it was shown that the conidia and other morphological structures were consistent with those in the amended description of *F. aesculi* (Crous & Palm 1999). *Fusicoccum aesculi* was thus designated as the anamorph of *B. dothidea*.

Butin (1993) studied oak-inhabiting fungi, and reported *Fusicoccum cfr aesculi* and *Dichomera saubinetii* from the bark and *Camarosporium oreades* and *Dothiorella cfr aesculi* from the leaves. Monosporous isolates of *F. cfr aesculi* and *D. saubinetii* gave rise to pale grey-brown mycelium and produced stromatic structures after six weeks. These structures contained pigmented muriform conidia (*Dichomera* spore-type) or hyaline, aseptate, fusiform conidia (*Fusicoccum* spore-type) and a substantial proportion of the isolates contained both spore forms within the same locules. Similar results were observed when single conidial isolates of *C. oreades* and *D. cfr aesculi* were compared. Sutton (1980) referred to *Dichomera* as the stromatic analogue of *Camarosporium*. Butin (1993) demonstrated a new form-complex comprising *F. cfr aesculi*, *D. saubinetii*, *C. oreades*, and *D. cfr aesculi* occurring on oak, and also recognized pleomorphism in these conidial forms.

In comparision to *Fusicoccum*, *Dichomera* is a relatively poorly studied genus. Sutton (1980) noted that more than 40 species had been described in *Dichomera*. Conidiomata in the genus were described as eustromatic and the distinctive conidia as muriform, brown, euseptate, globose, pyriform or cylindrical, often variable and irregular in shape, constricted or not constricted at the septa and smooth-walled with truncate bases. A considerable number of species residing in other genera have been transferred to *Dichomera*. Sutton (1975) reduced *Camarosporellum eucalypti* (syn. *Camarosporium eucalypti*) and *Coryneum viminale* to synonymy with *Dichomera eucalypti*. In their extensive revision of *Hendersonula*, Sutton & Dyko (1989) reduced *H. botryosphaerioides* to synonymy with *D. rhamnicola*, *H. conglobata* to synonymy with *D. conglobata*, and renamed *Camarosporium rhamni* as *D. neorhamni*.

The majority of *Dichomera* species have been described outside Australia, and from a wide range of hosts other than *Eucalyptus*. The most recently discussed species from outside Australia are *D. gemmicola*, causing bud blight of conifers in western Canada (Funk & Sutton 1972) and China (Yuan & Wang 1995), and *D. saubinetii* associated with cankers on sycamore (*Acer pseudoplatanus*) in the UK (Bevercombe & Rayner 1978) and twig lesions on oak (*Quercus robur*) in Switzerland (Sieber, Kowalski & Holdenrieder 1995).

Three species of *Dichomera*, *D. eucalypti*, *D. macrospora* and *D. versiformis*, and a number of undetermined *Dichomera* spp. have been described from *Eucalyptus* (Sankaran, Sutton & Minter 1995, Yuan, Wardlaw & Mohammed 2000). Relatively little is known of the taxonomy and biology of *Dichomera* spp. occurring in Australia, where most *Eucalyptus* spp. are endemic.

No definitive links to teleomorph states have been made for this genus, although Sutton & Dyko (1989) referred to species of *Cucurbitaria* as possible teleomorphs of *Dichomera*. This assumption was based on species of *Cucurbitaria* being stromatic ascomycetes producing muriform ascospores of similar size and shape to the conidia. However, there have been no phylogenetic studies on species of *Dichomera*, and connections to teleomorph states remain unclear.

This study was initiated to consider the identity of isolates commonly encountered during surveys of *Eucalyptus* in Australia, having cultural morphologies typical of *Botryosphaeria* spp. but producing muriform conidia in culture typical of *Dichomera* spp. Preliminary phylogenetic studies on isolates that have muriform conidia resembling *Dichomera* spp. showed that they were related to *Botryosphaeria* spp. with *Fusicoccum* anamorphs. Previous morphological studies (Butin 1993) have also suggested a close relationship between these genera and understanding their relatedness was another objective of this investigation.

MATERIALS AND METHODS

Fungal isolates

We examined 16 isolates of the 45 considered in this study microscopically (Table 1). Isolates WAC12398, WAC12400 and WAC12399 were from healthy twigs of *Eucalyptus diversicolor* or *E. marginata* near Denmark, Western Australia in 2001. Isolates WAC12396, WAC12395 and WAC12397 were from stem cankers found in eucalypt species trials near Cairns in Far North Queensland in 2003. WAC12404 was isolated from a stem canker of *Eucalyptus calophylla* growing in the native forest of Western Australia in 2003. WAC12401, WAC12402, WAC12403 and VPRI31988 were isolated from leaf lesions on *E. pauciflora* or *E. camaldulensis* growing in native forests of Victoria between 1999 and 2003.

For comparative purposes, species of *Botryosphaeria* with *Fusicoccum* anamorphs as well as a *Dichomera* sp. were obtained from various culture collections (Table 1). Six of the *Fusicoccum* isolates were included in the study by Slippers *et al*. (2004a, c). All isolates considered were subjected to the same cultural conditions for morphological characterisation and comparison.

Different methods were used to isolate fungi from leaf or bark specimens. The method described by Park & Keane (1984) for obtaining conidia of *Colletogloeopsis nubilosum* was modified slightly to

Table 1. Isolates of *Botryosphaeria*, *Dichomera* and *Guignardia* species considered in the phylogenetic study. Those in bold were examined microscopically.

Culture no.	Other no.	Identity	Host	Location	Isolator	GenBank accession no.
CMW7054	CBS121	*B. ribis*	*Ribes rubrum*	New York, USA	*N. E. Stevens*	AF241177
CMW7772	**108**	***B. ribis***	***Ribes* sp.**	**New York, USA**	***B. Slippers/G. Hudler***	**AY236936**
CMW9079	ICMP7933	*B. parva*	*Actinidia deliciosa*	New Zealand	*S. R. Pennycook*	AY236941
CMW9081	**ICMP8003**	***B. parva***	*Populus nigra*	**New Zealand**	***G. J. Samuels***	**AY236943**
CMW6837		*B. australis*	*Acacia* sp.	Batemans Bay, Australia	*M. J. Wingfield*	AY339262
CMW9073		***B. australis***	***Acacia* sp.**	**Victoria, Australia**	***J. Roux/D. Guest***	**AY339261**
CMW992	KJ93.52	*B. lutea*	*Actinidia deliciosa*	New Zealand	*G. J. Samuels*	AF243396
CMW9076	ICMP7818	*B. lutea*	*Malux* × *domestica*	New Zealand	*G. J. Samuels*	AF027745
CMW10126		*B. eucalyptorum*	*Eucalyptus grandis*	Mpumalanga, S. Africa	*H. Smith*	AF283687
CMW10125		*B. eucalyptorum*	*E. grandis*	Mpumalanga, S. Africa	*H. Smith*	AF283686
CMW7024	BRIP24101	*F. mangiferum*	*Mangifera indica*	Australia	*G. I. Johnson*	AY615185
CMW7797	BRIP24083	*F. mangiferum*	*M. indica*	Australia	*G. I. Johnson*	AY615185
CMW7999	119	*B. dothidea*	*Ostrya* sp.	Crocifisso, Switzerland	*B. Slippers*	AY236948
CMW8000	**118**	***B. dothidea***	***Prunus* sp.**	**Crocifisso, Switzerland**	***B. Slippers***	**AY236949**
	ZS97-59	*B. mamane*	*Sophora chrysophylla*	Hawaii	*D. Gardner*	AF246930
	ATCC22929	*B. corticis*	*Vaccinium* sp.	North Carolina, USA	*R. D. Milholland*	AF243397
	KJ93.56	*B. obtusa*	Hardwood Shrub	New York, USA	*G. J. Samuels*	AF243397
CMW7774		*B. obtusa*	*Ribes* sp.	New York, USA	*B. Slippers/G. Hudler*	AY236953
	ZS94-6	*B. stevensii*	*Malus pumila*	New Zealand	*N. Tisserat*	AF243407
CMW7060	CBS431	*B. stevensii*	*Fraxinus excelsior*	Netherlands	*H. A. van der Aa*	AY236995
	CBS112545	*B. corticola*	*Quercus ilex*	Spain	*M. A. Sanchez/A. Trapero*	AY259089
	CBS112551	*B. corticola*	*Q. suber*	Portugal	*A. Alves*	AY259101
	CBS418.64	*B. tsugae*	*Tsuga heterophylla*	Canada	*A. Funk*	AF243405
	KJ94.07	*Diplodia pinea*	*Pinus resinosa*	Winsconsin, USA	*D. R. Smith*	AF027758
	STE-U2269	*B. proteae*	*Protea laurifolia*	Hawaii	*P. W. Crous*	AF452563
	STE-U4378	*B. proteae*	*Protea* sp.	Australia	*M. E. Palm*	AF452560
	STE-U4365	*B. protearum*	*P. mangifica*	South Africa	*S. Denman*	AF452547
	STE-U4368	*B. protearum*	*Protea repens*	South Africa	*S. Denman*	AF452542
CMW9074		*B. rhodina*	*Pinus* sp.	Mexico	*T. Burgess*	AY236952
CMW10130		*B. rhodina*	*Vitex donniana*	Uganda	*J. Roux*	AY236951
CMW17679	CBS447	*Bionectria* sp.	*Taxus baccata*	Netherlands	*H. A. van der Aa*	AF312014
	CBS 990.70	***D. saubinetii***	***Quercus* sp.**	**Baarn, Netherlands**	***H. A. van der Aa***	**AY744379**
WAC 12395	**FNQ58B**	***B. ribis***	***Eucalyptus pellita***	**Queensland, Australia**	***T. Burgess/G. Pegg***	**AY744368**
WAC 12396	**FNQ27C**	***B. ribis***	***E. grandis* × *E. camaldulensis***	**Queensland, Australia**	***T. Burgess/G. Pegg***	**AY744369**
WAC 12397	**FNQ78B**	***B. parva***	***E. pellita***	**Queensland, Australia**	***T. Burgess/G. Pegg***	**AY744370**
WAC 12398	**BOT6**	***D. eucalypti***	***E. diversicolor***	**Western Australia**	***T. Burgess/K.-L. Goei***	**AY744371**
WAC 12399	**BOT15**	***B. australis***	***E. diversicolor***	**Western Australia**	***T. Burgess/K.-L. Goei***	**AY744374**
WAC 12400	**BOT29**	***B. australis***	***E. marginata***	**Western Australia**	***T. Burgess/K.-L. Goei***	**AY744375**
WAC 12401	**VIC1**	***D. eucalypti***	***E. pauciflora***	**Victoria, Australia**	***P. J. Keane***	**AY744372**
WAC 12402	**VIC2**	***D. eucalypti***	***E. camaldulensis***	**Victoria, Australia**	***G. Whyte***	**AY744373**
WAC 12403	**VIC3**	***D. versiformis***	***E. camaldulensis***	**Victoria, Australia**	***P. A. Barber***	**AY744376**
	VPRI31988	***D. versiformis***	***E. pauciflora***	**Victoria, Australia**	***P. J. Keane***	**AY744377**
WAC 12404	**WA7**	***B. dothidea***	***E. calophylla***	**Western Australia**	***T. Paap***	**AY744378**

obtain single conidial isolates from leaves. Sections were made through mature pycnidia embedded within leaves using a fine blade and these were agitated in 1ml DWT20 (40 µl Tween 20/100 ml distilled water v/v) for approx 1 min to release conidia. Aliquots (0.1 ml) of spore suspension were placed onto the surface of 0.5% malt-extract-agar (MEA) containing 50 µg ml^{-1} of chloramphenicol in 90 mm Petri dishes individual germinating conidia were transferred to 0.5% MEA and half-strength potato-dextrose-agar (PDA) in 90 mm Petri dishes and incubated at ~21 °C in the dark for 2 wk. Fungi were isolated from cankers by surface sterilising (in 70% ethanol) species of cankered bark, flaming, and plating onto half-strength PDA plates containing 50 µg ml^{-1} of streptomycin. Mycelia were transferred to tap water agar overlaid with sterile pine needles and incubated under near-UV light for 6 wk at 25 ° to induce sporulation. Single conidial isolates were obtained from pycnidia formed on the pine needles and maintained on half-strength PDA.

DNA isolation and amplification

The ITS regions of the rDNA operon was amplified using the primers ITS1F (5′ CTT GGT CAT TTA GAG GAA GTAA 3′) (Gardes & Bruns 1993) and ITS4 (5′ TCC TCC GCT TAT TGA TAT GC 3′) (White *et al*. 1990). The PCR reaction mixture (25 µl) contained 200 µM of each deoxynucleotide triphosphate, 150 nM of each primer, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 5–10 ng of DNA template and 1 U of Taq polymerase (Fisher Biotech, Perth, WA). The reactions were carried out in a Gene Amp 9600 thermocycler (PE Applied Biosystems, Foster City, CA) programmed for an initial denaturization of 2 min at 95 °, followed by 35 cycles of 30 s at 95 °, 45 s at 56 ° and 60 s at 72 ° and a final elongation step of 5 min at 72 °. PCR products were run on a 1% agarose gel, stained with ethidium bromide and visualized under UV illumination. PCR products were cleaned using Ultrabind® DNA purification kit (MO BIO Laboratories, Solana Beach, CA). Products were sequenced with the BigDye terminator cycle sequencing kit (PE Applied Biosystems) using the same primers that were used in the initial amplification. The products were separated by PAGE on an ABI Prism 377 DNA sequencer (PE Applied Biosystems).

Phylogenetic analysis

In order to compare all isolates collected in this study with *Botryosphaeria* spp. used in previous studies, 31 ITS rDNA sequences obtained from GenBank were included in the analyses. Trees were rooted to *Guignardia philoprina*, a species closely related to *Botryosphaeria*. Sequence data were analysed using Sequence Navigator v. 1.0.1™ (Perkin Elmer, Foster City, CA) and manually aligned by inserting gaps. Gaps were treated as a fifth character, all ambiguous characters and parsimony uninformative characters were excluded prior to analysis. Phylogenetic analysis based on parsimony was performed using PAUP 4.0bl0 (Swofford 2000). The most parsimonious trees were obtained by using heuristic searches with random stepwise addition in 100 replicates, with the tree bisection-reconnection branch-swapping option on and the steepest-descent option off. Maxtrees were unlimited, branches of zero length were collapsed and all multiple equally parsimonious trees were saved. Estimated levels of homoplasy and phylogenetic signal (retention and consistency indices) were determined (Hillis & Huelsenbeck 1992). In the initial analysis, all characters were unweighted and unordered; thereafter characters were reweighted according to the consistency index. Branch and branch node supports were determined using 1000 bootstrap replicates (Felsenstein 1985), and characters were sampled with equal probability, but weights were applied.

Morphological characterisation

Mature pycnidia were removed from cultures mounted in lactoglycerol, and conidia measured under 1000× magnification with a light microscope. Morphological observations for at least 50 conidia were determined for each isolate. Some collections contained spores of two different morphological forms: (1) elongate and somewhat fusiform in shape; and (2) smaller, more globose to obpyriform in shape. 50 conidia of each form were measured and conidia were photographed using an Axiocam digital camera (Carl Zeiss, Iena) and spores drawn under 1000× magnification using a drawing tube.

RESULTS

Phylogenetic sequence analyses

PCR products of approximately 570 bp were amplified in all the isolates from Australia considered in this study. Sequence data at each end were deleted in the aligned data set. The aligned data set consisted of 550 characters, of which 375 uninformative characters were excluded prior to analysis. The data set contained significant phylogenetic signal compared to 1000 random trees ($P < 0.01$, $g1 = -0.83$). Heuristic searches in PAUP resulted in 42 most parsimonious trees of 488 steps (CI = 0.70, RI = 0.88), characters were reweighted according to the consistency index and the subsequent heuristic search resulted in 12 trees of 345 steps (CI = 0.79, RI = 0.91).

The resultant tree consisted of two distinct clades with 100% bootstrap support separating the *Botryosphaeria* spp. with *Fusicoccum* anamorphs from those with *Diplodia* anamorphs (Fig. 1). The *Fusicoccum* clade was further subdivided into groups that represent discrete species. Isolates from *Eucalyptus* in Australia fell into the *Fusicoccum* clade and were identical in their ITS sequence to *B. ribis*, *B. parva*, *B. dothidea* and

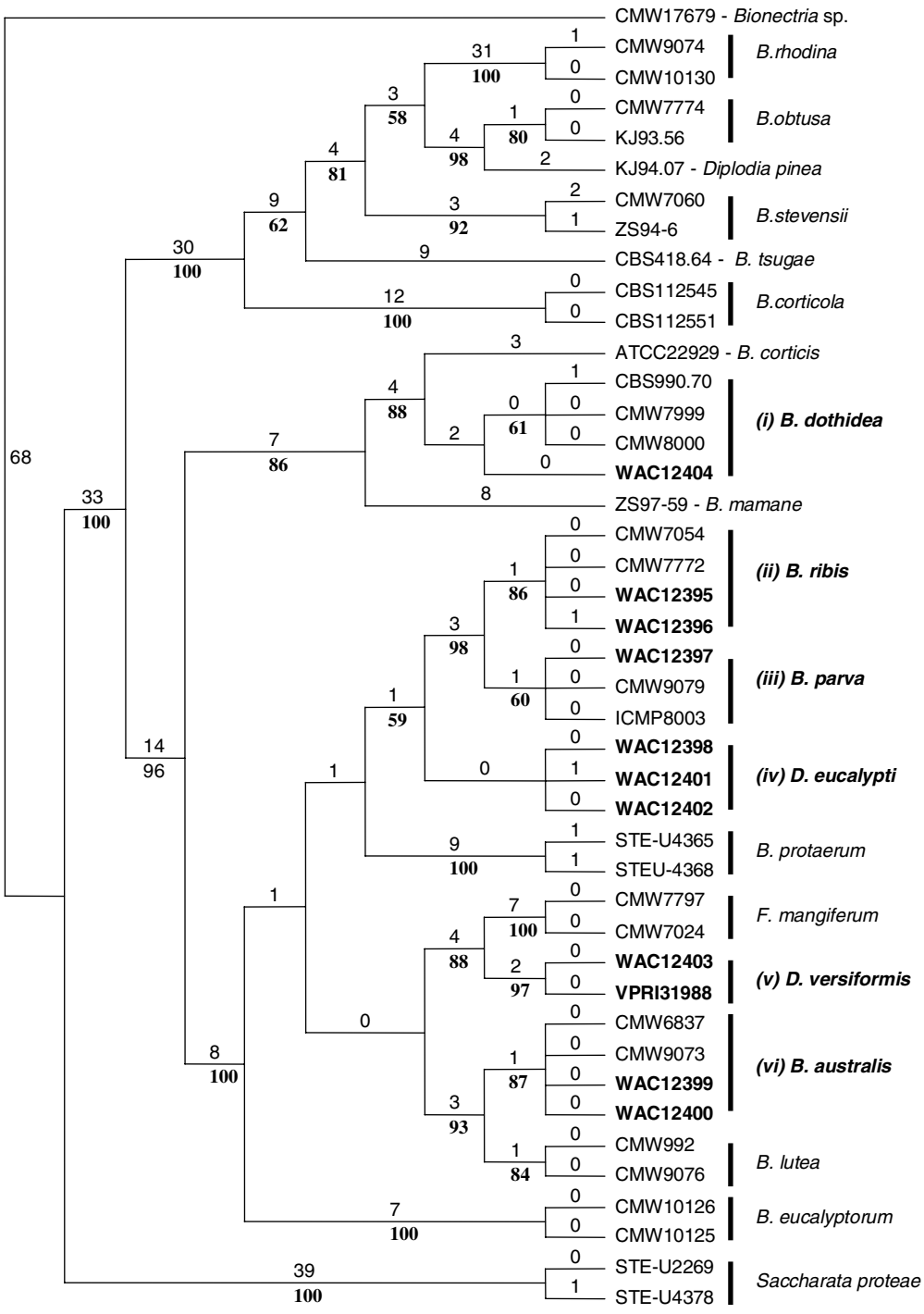


Fig. 1. One of 12 most-parsimonious trees obtained through heuristic searches of the ITS-rDNA regions. Support for branches and nodes are indicated respectively as bootstrap values (1000 replicates) below and branch length above the line. The tree was rooted to *Guignardia philoprina*.

B. australis (Fig. 1). In addition, there were five isolates (WAC12401, WAC12402, WAC12398, WAC12403, and VPRI31988) that resided in the *Fusicoccum* clade but were distinct from any known and sequenced *Botryosphaeria* spp. with *Fusicoccum* anamorphs (Fig. 1).

Morphological characterisation

Morphological comparisons were made between all isolates with muriform spores (resembling *Dichomera* spp.) and isolates of various *Fusicoccum* spp. (Slippers *et al.* 2004a, c) with which they were conspecific based on ITS rDNA sequences. Some isolates collected in this study produced conidia of two different morphological forms. The conidia of one form were dark coloured, rounded or variable in shape, globose, subglobose, obovoid, obpyriform to fusiform with muriform septa. These were typical of the genus *Dichomera*. In contrast, some isolates had conidia that were hyaline, more elongated (ellipsoid or fusiform), and regular

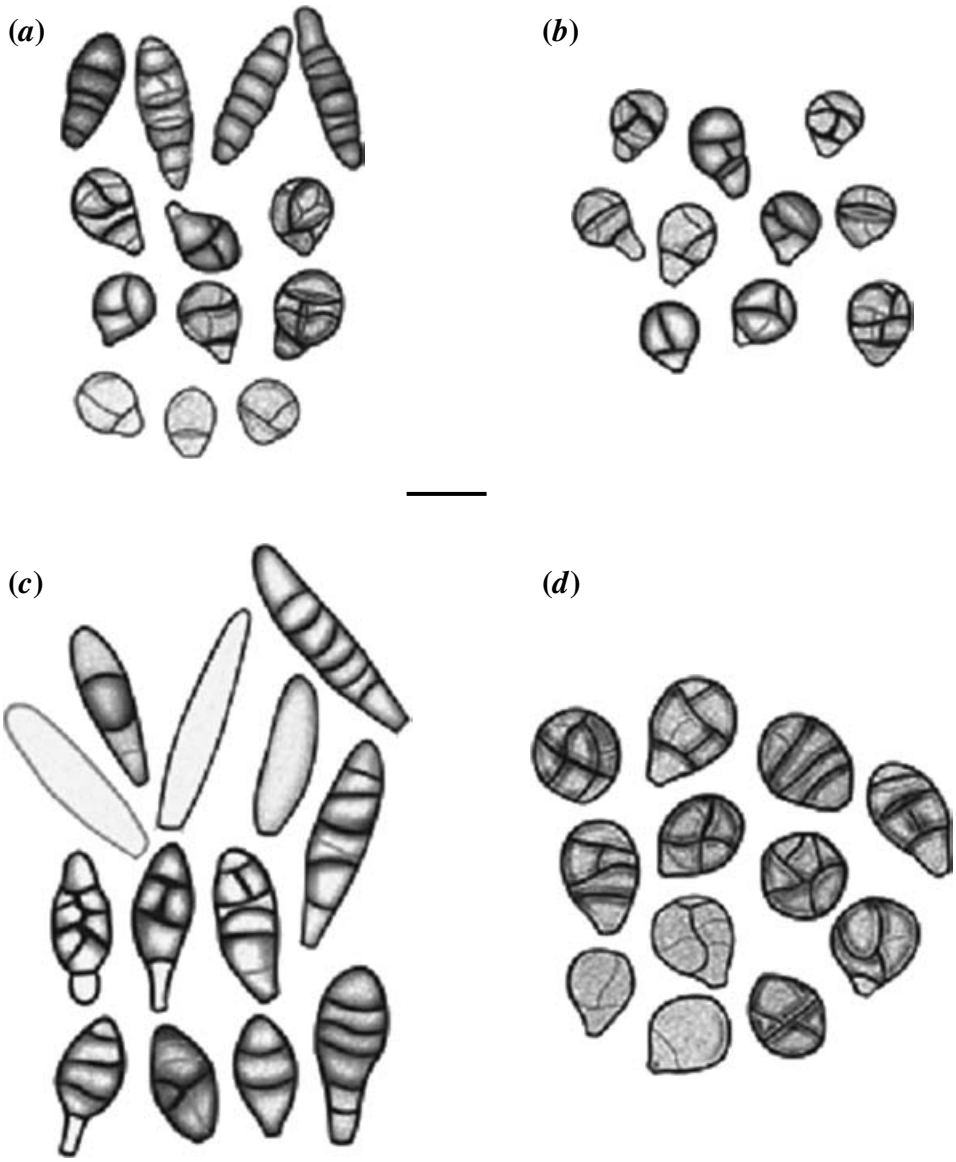


Fig. 2. Conidia of *Botryosphaeria* spp. with *Dichomera* synanamorphs isolated in this study for (*a*) *B. ribis*, (*b*) *B. parva*, (*c*) *B. dothidea*, and (*d*) *B. australis*. Bar = 10 μm.

in shape; these conidia were typical of species of *Fusicoccum*.

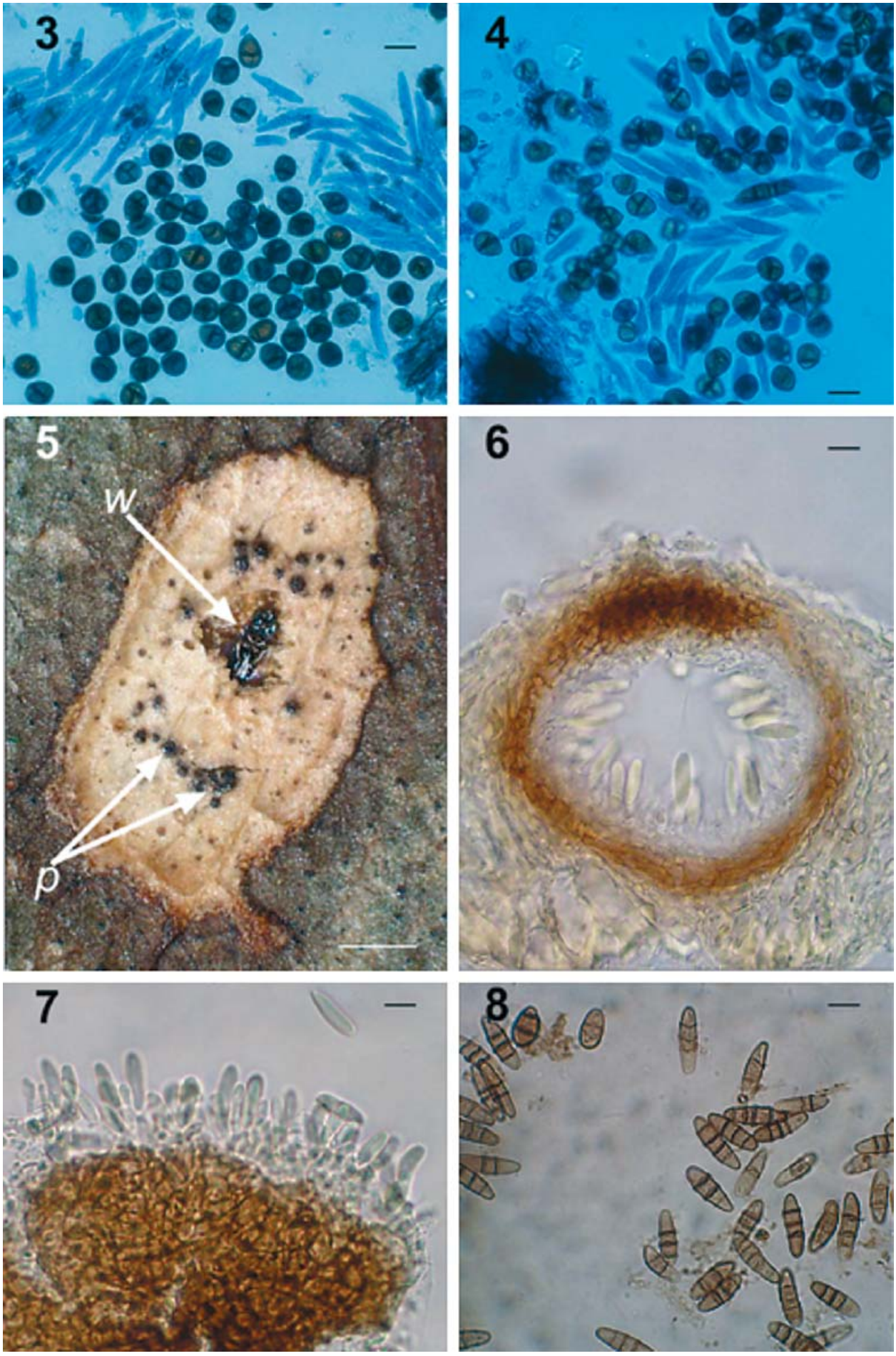
Five isolates: WAC12395, WAC12396, WAC12398, WAC12403, WAC12404, and the type specimen of *D. versiformis*, produced both the hyaline, elongate *Fusicoccum*-like conidia, as well as the dark, variable shaped, muriform conidia typical of species of *Dichomera* spp. (Fig. 2). None of the isolates previously studied by Slippers *et al.* (2004a, c) produced *Dichomera*-like conidia. Rather, they all produced spores typical of their respective *Fusicoccum* spp. as previously described (Slippers *et al.* 2004a, c, Pennycook & Samuels 1985) with dimensions as given in Table 2.

The ex-holotype culture of *F. ribis* (CMW7772) produced aseptate, hyaline conidia as described by Slippers *et al.* (2004a). Two isolates (WAC12395 and WAC12396) collected in this study had ITS sequences identical to that of CMW7772 (Fig. 1). Both of these isolates produced only muriform conidia typical of *Dichomera* in culture. It was impossible to distinguish between the smaller, rounded conidia of the *Dichomera* form of *B. ribis* (isolates WAC12395 and WAC12396) and the conidia of isolate WAC12397 (Fig. 2), which matched the DNA sequence of the *B. parva* ex-type isolate (ICMP8003) (Fig. 1). However, isolates WAC12395 and WAC12396 produced muriform conidia, which were also broadly fusiform to fusiform in shape, whereas WAC12397 did not produce spores with this shape (Fig. 2).

Conidia of the ex-epitype (CMW8000) of *B. dothidea* produced in culture in this study were somewhat shorter and wider on average than those described by Slippers *et al.* (2004a). This resulted in a smaller l:b ratio (4.39), and spores from older cultures occasionally had two and rarely three septa. Conidia of the Western Australian isolate (WAC12404) residing in the same clade as *B. dothidea* (CMW8000) (Fig. 1), were distinctly different in shape to all other cultures with

Table 2. Conidial dimensions of *Botryosphaeria* and *Dichomera* isolates (conidia produced *in vitro*) examined in this study. Isolates in **bold** produced a *Dichomera* synanamorph in culture.

Identity	Culture No.	Spore shape	Range of conidial dimensions (μm)	Mean conidial dimensions (μm)	L:B ratio	Host	Location
B. ribis	CMW 7772 (ex-type)		(13.5–) 15.5–21 (–24) × (4.5–) 5–6.5 (–9.5)	17.5 × 5.5	3.2	*Ribes* sp.	New York, USA
	WAC 12395	Round	8–10.5 (–13.5) × (6.5–) 7–9 (–9.5)	9.4 × 8.0	1.18	*Eucalyptus pellita*	Qld, Australia
		Long	(12–) 13.5–17.5 (–20) × (5–) 5.5–7 (–8)	15.9 × 6.0	2.65		
	WAC 12396	Round	(7–) 8–13.5 (–17) × 7–9.5 (–10.5)	10.8 × 8.5	1.27	*E. grandis* × *E. camaldulensis*	Qld, Australia
		Long	(13.5–) 15.5–22.5 (–24) × 6.5–8	18.2 × 6.9	2.63		
B. parva	**ICMP 8003** (ex-type)		(12–) 13.5–18.5 × 5–5.5 (–6.5)	15.5 × 5.3	2.92	*Populus nigra*	New Zealand
	WAC 12397	Round	8–10.5 (–12) × (6.5–) 7–8 (–9)	9.1 × 7.3	1.25	*E. pellita*	Qld, Australia
D. eucalypti	**WAC 12401**	Round	9.5–13 × (8–) 9–10.5 (–11)	11.2 × 9.4	1.19	*E. pauciflora*	Victoria, Australia
	WAC 12402	Round	(9–) 10.5–14.5 × 8–10.5 (–11)	12.3 × 9.4	1.31	*E. camaldulensis*	Victoria, Australia
	WAC 12398	Round	(9–) 9.5–13 (–14.5) × (6.5–) 8–9 (–9.5)	11.7 × 8.6	1.36	*E. diversicolor*	W.A., Australia
		Long (16 conidia)	12–18.5 × 5–7	15.3 × 6.0	2.55		
B. australis	**WAC 12399**	Round	(9.5–) 10.5–14.5 (–17.5) × (7–) 9–10.5 (–11)	12.5 × 9.5	1.32	*E. diversicolor*	W.A., Australia
	WAC 12400	Round	(9.5–) 10.5–13.5 (–14.5) × 9–11	12.2 × 9.9	1.23	*E. marginata*	W.A., Australia
	CMW 9073		(19–) 22–26 × 5–6 (–7.5)	23.5 × 5.0	4.7	*Acacia* sp.	Victoria, Australia
D. versiformis	**WAC 12403**	Round	10.5–17 (–19) × 7–9.5	13.6 × 8.3	1.64	*E. camaldulensis*	Victoria, Australia
		Long	(17–) 18–24 × 5–8	21.1 × 6.6	3.18		
	VPRI 31988	Round	8–11.5 (–13.5) × 5.5–8 (–9.5)	9.4 × 7.0	1.34	*E. pauciflora*	Victoria, Australia
		Long (15 conidia)	13–19.5 × 5–7	14.9 × 5.5	2.7		
B. dothidea	**WAC 12404**	Round	(13–) 16–21 (–28) × (5.5–) 6.5–9	18.9 × 7.8	2.42	*E. calophylla*	W.A., Australia
		Long (14 conidia)	23.5–28 × 5.5–8	26.3 × 6.8	3.9		
	CBS 990.70					*Quercus* sp.	Netherlands
	CMW 8000 (ex-type)		18.5–26 (–30) × (4–) 5–5.5	22.4 × 5.1	4.39	*Prunus* sp.	Crocifisso, Switzerland



Figs 3–8. For legend see opposite page.

muriform conidia (Fig. 2). Elongate, muriform conidia were observed in the culture of isolate WAC12404, along with a number of hyaline or pale brown, aseptate, narrowly fusiform conidia that possessed a basal frill (Fig. 2) and were somewhat similar in size (23.5–28 × 5.5–8 μm, mean = 26.3 × 6.8 μm, l:b = 3.9) to those of CMW8000 (Table 2). The l:b ratio of these conidia was significantly larger than in all other isolates in this study that produced *Dichomera* conidia in culture (Table 2). The elongate, obpyriform conidia of WAC12404 also had a greater l:b ratio than the shorter conidia of other isolates of *Botryosphaeria* spp. with *Dichomera* conidia such as *B. ribis*, *B. parva* and *B. australis* (Table 2).

An isolate labelled as *D. saubinetii* (CBS 990.70) from *Quercus* sp. by H. A. van der Aa in 1970 was shown to be identical in ITS rDNA sequence to *B. dothidea* (Fig. 1). All attempts to induce this isolate to sporulate on pine needles and eucalypt twigs in culture were unsuccessful. It was thus impossible to compare the morphology of this fungus with CMW8000 or WAC12404. The type specimen of *D. saubinetii* could not be located in various herbaria that might have such a collection (IMI, K, P, BP, BRA), therefore, morphological comparison could not be made.

Two specimens labelled *D. saubinetii* were obtained on loan from the Kew Herbarium. These included specimens collected by M. C. Cooke (K(M) 122468) from *Rhamnus frangula* (date unknown), and F. Petrak (K(M) 122471) from *Quercus pedunculata* in 1918. The specimens collected by Petrak and by Cooke produced both long and short conidia in the same pycnidium (Figs 3–4), typical of a number of isolates observed in the present study. Many of the longer conidia were aseptate and hyaline, typical of *Fusicoccum* spp. (Figs 3–4). Dimensions of conidia in both collections were very similar (Table 3), as was the shape and degree of septation (Figs 3–4). The shorter conidia overlapped in dimensions with most isolates producing *Dichomera* conidia observed in the present study, with the exception of WAC12404. However, the longer conidia were significantly longer than all isolates producing *Dichomera* conidia with the exception of WAC12404 (Tables 2–3). These collections clearly did not match any of the *Dichomera* isolates examined in this study according to the morphology of the conidia.

The anamorph (*F. australe*) of *B. australis* is described as having hyaline, fusiform, aseptate conidia rarely forming septa before germination (Slippers *et al.* 2004c). In contrast, the two isolates collected in this study (WAC12399 & WAC12400) that had the same ITS sequences as *B. australis* (Fig. 1) both formed muriform, brown conidia typical of *Dichomera* in culture (Fig. 2). These *Dichomera* conidia of *B. australis* could be distinguished from the *Dichomera* form of *B. ribis*, *B. parva* and *B. dothidea* based on size or shape or both (Fig. 2) (Table 2). The conidia of the *Dichomera* form of *B. australis* were consistently longer and wider than those of *B. ribis* and *B. parva* (Fig. 2), but all three had similar l:b ratios (Table 2). The *Dichomera* form of *B. australis* also lacked the longer, ellipsoid or fusiform conidia in culture produced by the *Dichomera* form of *B. ribis* (Fig. 2). *Dichomera* conidia of *B. australis* were very different in size and shape from those of the *Dichomera* form of *B. dothidea* (Fig. 2) (Table 2).

Based on ITS sequence data, three isolates (WAC12401, WAC12402, WAC12398) with muriform conidia in culture grouped together as a distinct species (Fig. 1). This species was most closely related to *B. australis*, *B. parva*, and *B. ribis*. Two isolates (WAC12401, WAC12402) produced pycnidia on eucalypt leaf tissue and these contained conidia of a *Dichomera* sp. These were identified as *D. eucalypti* based on morphology. Isolate WAC12398 obtained from woody tissue produced a limited number of subcylindrical, long obovoid or broadly fusiform conidia not seen in isolates WAC12401 and WAC12402. The elongate conidia produced by WAC12398 differed in shape to those produced by the *Dichomera* form of *B. parva* (Figs 2, 9). Comparison of the morphological characteristics (Figs 2, 9) (Table 3) of the type specimen of *D. eucalypti* (IMI 75054) and a specimen labelled *D. eucalypti* collected by M. C. Cooke from *Eucalyptus* sp. in New Zealand in 1886 (K(M) 122474), with isolates observed in this study enabled us to conclude that the species represented by WAC12401, WAC12402 and WAC12398 was synonymous with *D. eucalypti sensu* Sutton (1975).

DNA sequence comparisons showed that an isolate (WAC12403) from *Eucalyptus* leaves (VPRI 31989) in south-eastern Australia resided in the *Fusicoccum* clade of *Botryosphaeria*, but it was distinct from other species (Fig. 1). This isolate produced abundant pycnidia within lesions typically associated with eulophid wasps (*Hymenoptera*: *Eulophidae*) (Fig. 5). These pycnidia contained aseptate, hyaline conidia typical of *Fusicoccum* (Figs 6–7). Cultures produced from single conidia of this isolate formed muriform, brown conidia (Figs 8–9). These conidia were morphologically similar to *D. versiformis* (Fig. 9) described from foliage of

Figs 3–8. Fig. 3. Aseptate, hyaline conidia and dematiaceous, muriform conidia of K(M) 122468 *Dichomera saubinetii sensu* Cooke stained in lactocotton blue. **Fig. 4.** Aseptate, hyaline conidia and dematiaceous, muriform conidia of K(M) 122471 *Dichomera saubinetii sensu* Petrak stained in lactocotton blue. **Fig. 5.** Lesion of VPRI 31989 *Dichomera versiformis* collected in this study showing presence of wasp within the lesion (*w*) and abundant pycnidia (*p*). **Fig. 6.** Transverse section of pycnidium of VPRI 31989 showing aseptate, hyaline conidia. **Fig. 7.** Squash mount of pycnidium of VPRI 31989 showing aseptate, hyaline conidia. **Fig. 8.** Conidia produced in culture from single conidium isolate of VPRI 31989 after 4 wk on half strength MEA. Bar Figs 3–4, 6–8 = 10 μm; Fig. 5 = 1 mm.

Table 3. Conidial dimensions of *Botryosphaeria* and *Dichomera* specimens (conidia produced *in vivo*) examined in this study.

Identity	Specimen No.	Spore shape	Range of conidial dimensions (µm)	Mean conidial dimensions (µm)	L:B ratio	Host	Location
D. eucalypti	VPRI 31987	Round	(9–) 10–12 (–15) × (7.5–) 8–10 (–13)	11.2 × 8.4	1.33	*E. pauciflora*	Victoria, Australia
	MURU 406	Round	(9–) 11–13.5 (–16) × (7–) 8.5–10.5 (–13)	12.1 × 9.3	1.30	*E. camaldulensis*	Victoria, Australia
	IMI 75054 (TYPE)	Round	9.5–12 (–14.5) × (6.5–) 7–9	11.0 × 8.0	1.38	*Eucalyptus* sp.	Victoria, Australia
	K(M) 122474	Round	(9–) 10.5–13 (–15) × (7–) 8–9.5	11.1 × 8.6	1.29	*Eucalyptus* sp.	St. Arnaud, New Zealand
D. versiformis	VPRI 31989	Long	16–22 (–25) × (5–) 5.5–6.5 (–8)	19.3 × 5.9	3.27	*E. camaldulensis*	Victoria, Australia
	VPRI 31988	Round	11–14 (–16) × 8.5–11	12.7 × 9.6	1.32	*E. pauciflora*	Victoria, Australia
	VPRI 22038a (TYPE)	Round	(9.5–) 11–16 (–17) × 8–11	14.5 × 10.5	1.4	*E. nitens*	Tasmania, Australia
		Long	18.5–26 (–27.5) × (5.5–) 6.5–9	22.0 × 7.3	3.0		
D. saubinetii sensu Petrak	K(M) 122471	Round	9.5–14 (–16) × 7–9	11.6 × 8.6	1.34	*Quercus pedunculata*	Rybno, Poland
		Long	(17.5–) 20–24.5 (–25.5) × 4.5–6	23.9 × 5.4	4.43		
D. saubinetii sensu Cooke	K(M) 122468	Round	9–14 (–16) × (6–) 7–9	11.2 × 7.9	1.41	*Rhamnus frangula*	London, England
		Long	17.5–26.5 (–29) × 5.5–7	21.9 × 6.0	3.65		

E. nitens in Tasmania (Yuan *et al*. 2000) and this was confirmed in a comparision with the type specimen (VPRI 22038a) of *D. versiformis* (Fig. 9) (Table 3). Conidia were also morphologically distinct from other species examined in this study. Thus, numerous ellipsoid and broadly fusiform conidia, considerably longer than those observed for *D. eucalypti* and the *Dichomera* form of *B. ribis* were produced in culture (Figs 2, 9) (Table 2). Obovoid and obpyriform conidia were produced less commonly and these were also longer than those produced by *D. eucalypti* and the *Dichomera* forms of *B. ribis, B. parva* and *B. australis* (Figs 2, 9). No elongate, obpyriform conidia characteristic of the *Dichomera* form of *B. dothidea* (WAC12404) were produced. A second isolate (VPRI31988) residing in the same clade (*D. versiformis*) (Fig. 1) produced mainly muriform conidia more characteristic of *D. eucalypti* with some elongate conidia more typical of *D. versiformis*. The *Fusicoccum*-like conidia seen in isolate WAC12403 were not seen in isolate VPRI31988.

The mode of conidiogenesis was not observed for most isolates producing muriform conidia in culture. However, where conidium development could be observed (WAC12404), conidia were produced holoblastically on hyaline, ampulliform to cylindrical conidiogenous cells, proliferating percurrently with up to one annellation. This is in agreement with the mode of conidiogenesis described for *Fusicoccum* by Crous & Palm (1999) and Slippers *et al*. (2004a, c). The conidiogenous cells of WAC12404 (*B. dothidea*) were considerably longer (8–40.5 –52.5) × 3–5 µm; (mean = 22.8 × 4.1 µm) than those observed for other isolates with muriform conidia and also from the description of *B. dothidea* provided by Slippers *et al*. (2004a).

TAXONOMY

A number of species of *Fusicoccum* have recently been described or reassessed (Phillips *et al*. 2002, Slippers *et al*. 2004a, c) as anamorphs of *Botryosphaeria*. All these *Fusicoccum* species are described as having hyaline, aseptate, thin-walled conidia that with age become olivaceous and sometimes up to 2-septate. Results of the present study have shown that numerous isolates collected from eucalypts in Australia are identical in their ITS sequence to well-known *Botryosphaeria* spp. with clearly defined *Fusicoccum* anamorphs. However, these Australian isolates have muriform conidia typical of *Dichomera* spp. We, therefore, provide descriptions for these species to include the *Dichomera* synanamorphs observed in this study.

Five isolates collected in this study with irregularly shaped, muriform conidia had ITS sequences different from any known *Botryosphaeria* spp. These fungi resided in two distinct clades in the greater *Fusicoccum* clade (Fig. 1). Conidial morphology of isolates residing in these two clades was the same as that of the type specimens of *D. eucalypti* and *D. versiformis*. Based on

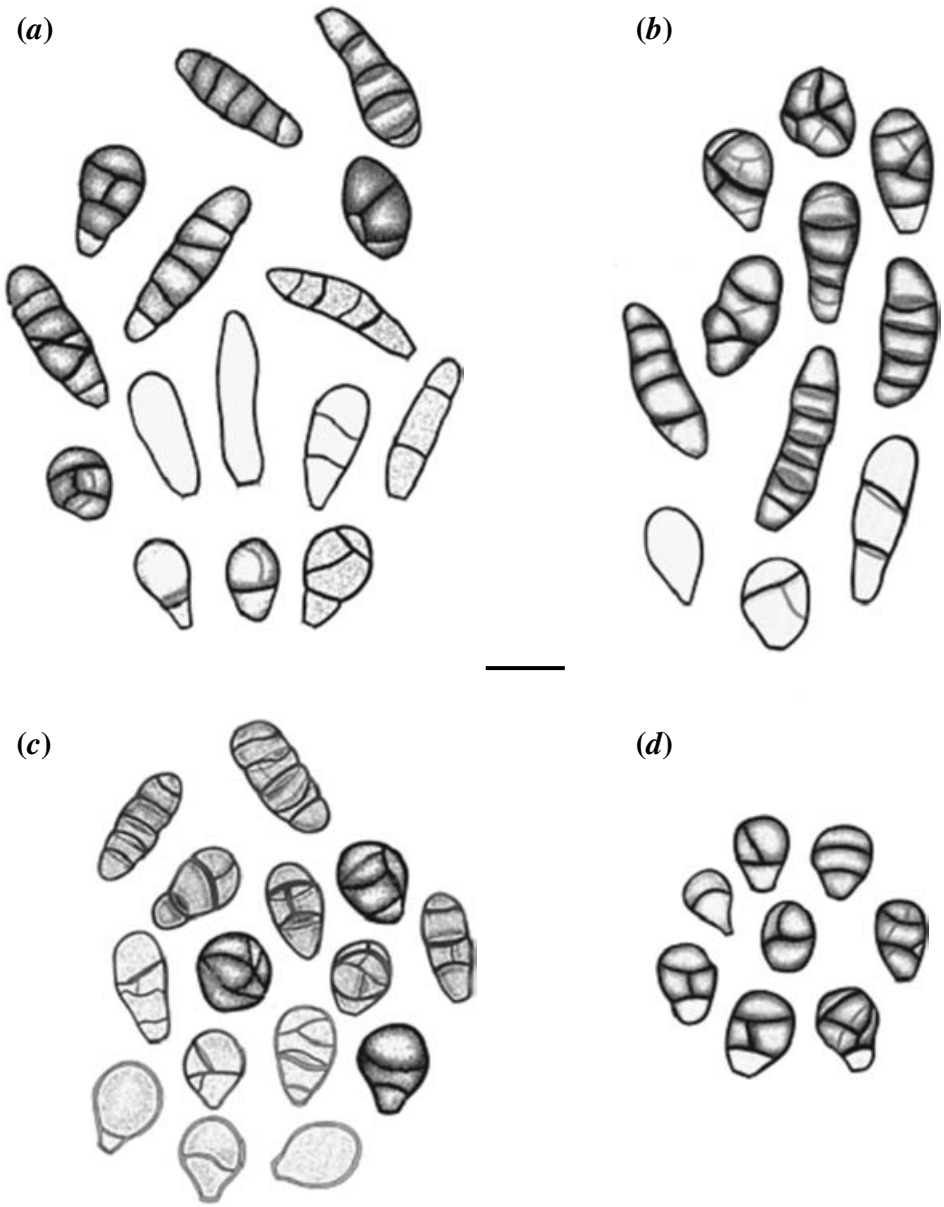


Fig. 9. *Dichomera* spp. falling into the *Fusicoccum* clade of *Botryosphaeria*. (*a*) *Dichomera versiformis* isolated in the present study (*b*) *Dichomera versiformis* holotype (*c*) *D. eucalypti* isolated in the present study and (d) *D. eucalypti* holotype. Bar = 10 μm.

this morphological similarity, as well as the overlap in hosts and geographic occurrence, we propose that the isolates collected here (Clades iv and v) are conspecific with *D. eucalypti* and *D. versiformis*.

Botryosphaeria dothidea (Moug. ex Fr.) Ces. & De Not., *Comm. Soc. Crittog. Ital.* **1**: 212 (1863).

Sphaeria dothidea Moug. ex Fr., *Syst. Mycol.* **2**: 423 (1823); as 'Moug.! *in litt.*'

Botryosphaeria berengeriana De Not., *Sferiacei Itali.*: **82** (1863).

Anamorph: **Fusicoccum aesculi** Corda, in Sturm *Deutschl. Fl.* **3**: 111 (1829).
Synanamorph: **Dichomera** (Fig. 2).

Conidiogenous cells holoblastic, hyaline, subcylindrical to cylindrical, 8–40.5 (–52.5) × 3–5 μm (mean = 22.8 × 4.1 μm) proliferating percurrently with 0–1 proliferations. *Conidia* variable and irregular in shape, mostly elongate obpyriform, less commonly obovoid to ellipsoidal or narrowly fusiform, apex subobtuse to obtuse, base truncate to bluntly rounded, hyaline to pale brown and aseptate when immature becoming darker brown and muriform when mature with 1 to 6 transverse septa, 0 to 1 longitudinal septa and 0 to 4 oblique septa, some cells more darkly pigmented than others, (13–) 16–21 (–28) × (5.5–) 6.5–9 μm (mean = 18.9 × 7.8 μm, l: b 2.42).

Cultures examined: **Australia**: *Western Australia*: Bedfordale: Camms Rd, ex *Eucalyptus calophylla*, Mar. 2003, *T. Paap* (WAC12404). – **Switzerland**: Ticino: Crocifisso,

ex *Prunus* sp., Oct. 2000, *B. Slippers* (CMW 8000 – epitype of *Botryosphaeria dothidea*). – **The Netherlands: Baarn: Maarschalksbos,** ex *Quercus* sp., Dec. 1970, *H. A. van der Aa* (CBS 990.70).

Botryosphaeria ribis Grossenb. & Duggar, *Tech. Bull. N.Y. Agric. Exp. Stn.* **18**: 128 (1911).

Anamorph: **Fusicoccum ribis** Slippers, Crous & M. J. Wingf. 2004
Synanamorph: **Dichomera** (Fig. 2).

Conidia multi-cellular, variable in shape from subglobose, obpyriform or rarely obovoid to broadly fusiform or fusiform, apex sub-obtuse to obtuse, base truncate to bluntly rounded. Subglobose, obpyriform or obovoid conidia (7–) 8–13.5 (–17) × (6.5–) 7–9.5 (–10.5) μm (mean = 10.1 × 8.3 μm, l:b 1.2), hyaline to pale brown when immature with one transverse septum and 0–2 longitudinal septa becoming brown and muriform when mature with 1–4 transverse septa, 0–3 longitudinal septa and 0–4 oblique septa. Broadly fusiform to fusiform conidia (12–) 13.5–22.5 (–24) × (5–) 5.5–8 μm (mean = 17.8 × 6.5 μm, l:b 2.74) brown, muriform with 2–7 transverse septa, and 0–2 oblique septa.

Cultures examined: **Australia**: *Queensland*: Ingam, ex *Eucalyptus pellita*, May 2003, *T. Burgess/G. Pegg* (WAC12395); Ingam, *Eucalyptus grandis* × *Eucalyptus camaldulensis*, May 2003, *T. Burgess/G. Pegg* (WAC12396) – **USA**: *New York*: Ithaca, ex *Ribes* sp., 2000, *G. Hudler* (CMW 7772 – holotype of *Botryosphaeria ribis*).

Botryosphaeria parva Pennycook & Samuels, *Mycotaxon* **24**: 455 (1985).

Anamorph: **Fusicoccum parvum** Pennycook & Samuels 1985
Synanamorph: **Dichomera** (Fig. 2).

Conidia variable in shape, from subglobose to obpyriform and rarely obovoid, brown, apex obtuse, base truncate to bluntly rounded, 8–10.5 (–12) × (6.5–) 7–8 (–9) μm (mean = 9.1 × 7.3 μm, l:b 1.25), muriform, 1–3 transverse septa, 1–2 longitudinal septa, and 1–2 oblique septa.

Cultures examined: **Australia**: *Queensland*: Karunda: Atherton tablelands, ex *Eucalyptus pellita*, 2003, *T. Burgess/G. Pegg* (WAC12397) – **New Zealand**: *Bay of Plenty*: Te Puke: No. 3 Road, Baldwin orchard, ex *Populus nigra*, 17 Dec. 1981, *G. S. Samuels* (ICMP 8003 – holotype of *Botryosphaeria parva*).

Botryosphaeria australis Slippers, Crous & M. J. Wingf., *Mycologia* **96**: 1037 (2004).

Anamorph: **Fusicoccum australe** Slippers, Crous & M. J. Wingf. 2004.
Synanamorph: **Dichomera** (Fig. 2).

Conidia irregular in shape, subglobose, obpyriform or obovoid, apex obtuse, base truncate to bluntly rounded, (9.5–) 10.5–14.5 (–17.5) × (7–) 9–11 (mean = 12.4 × 9.7 μm, l:b 1.3), pale brown when immature with 1–2 transverse septa, 0–1 longitudinal septa, and 0–2 oblique septa, becoming darker brown and muriform when mature with 1–3 transverse septa, 1–4 longitudinal septa, and 0–3 oblique septa.

Cultures examined: **Australia**: *Western Australia*: Pemberton, Big Brook, ex *Eucalyptus diversicolor*, 2001, T. *Burgess/K.-L. Goei* (WAC12399); Denmark, ex *Eucalyptus marginata*, 2001, *T. Burgess/K.-L. Goei* (WAC12400); *New South Wales*: Batemans Bay, ex *Acacia* sp., 2001, *M. J. Wingfield* (CMW6837).

Dichomera eucalypti (G. Winter) B. Sutton, *Mycol. Pap.* **138**: 182 (1975). (Fig. 3)

Camarosporium eucalypti G. Winter, *Rev. mycol.* **8**: 212 (1886).
Coryneum viminale Cooke & Massee, *Grevillea* **20**: 36 (1891).
Camarosporellum eucalypti (G. Winter) Tassi, *Bull. Lab. Ort. Bot. Siena* **5**: 62 (1902).

Conidia in vivo variable in shape from globose, subglobose, obpyriform or obovoid, apex obtuse, base truncate to bluntly rounded, (9–) 10.5–13 (–16) × (7–) 8–10.5 (–13) (mean = 12.0 × 9.0, l:b 1.3), hyaline to pale brown when immature with 0–2 transverse septa, and 0–1 longitudinal septa becoming brown and muriform when mature with 1–2 transverse septa, 0–2 longitudinal septa, and 0–3 oblique septa. *Conidia in vitro* variable in shape from globose, subglobose, obpyriform or obovoid to rarely subcylindrical, long obovoid or broadly fusiform, apex obtuse, base truncate to bluntly rounded. Globose, subglobose, obpyriform or obovoid conidia (9–) 9.5–13.5 (–14.5) × (6.5–) 8–10.5 (–11) (mean = 11.7 × 9.1 μm, l:b 1.3), hyaline to pale brown when immature with 0–3 transverse septa, 0–2 longitudinal septa and 0–2 oblique septa, becoming brown and muriform when mature with 1–3 transverse septa, 0–3 longitudinal septa and 0–2 oblique septa. Subcylindrical, long obovoid or broadly fusiform conidia 12–18.5 × 5–7 μm (mean 16 conidia = 15.3 × 6.0 μm) hyaline to pale brown when immature with three transverse septa, 0–1 longitudinal septa, and 0–2 oblique septa, becoming brown and muriform when mature, with 3–5 transverse septa and 0–1 longitudinal septum.

Specimens and cultures examined: **Australia**: *Victoria: Eucalyptus* sp., *sine dato*, *Watts* (ex type slide, IMI 59162); Mt Buffalo, on leaf of *Eucalyptus pauciflora*, 8 May 2000, *P. J. Keane* (VPRI 31987 – ***epitypus hic designatus***; culture WAC12401); Bundoora, Gresswell Reserve, on leaf of *Eucalyptus camaldulensis*, Oct. 2003, *G. Whyte* (MURU 406 – culture WAC12402): Melbourne, *Eucalyptus viminalis*, Apr. 1886, *F.R.* (K(M) 122472 – holotype); near Melbourne, *Eucalyptus viminalis*, Apr. 1850, *F.R.* (ex TYPE slide IMI 75054); *New South Wales*: Waste Point, Kosciusko National Park, on leaf of *Eucalyptus rubida*, Feb. 1972, *Y. I. Fripp* (K(M) 122473); *Western Australia*: Denmark, ex *Eucalyptus*

Key to* Botryosphaeria *spp. producing* Dichomera *synanamorphs in culture

1	Conidia subglobose, obpyriform or rarely obovoid, on average <11 μm long, 1:b $\leqslant$1.3, and (or) broadly fusiform to fusiform on average <19 μm long, 1:b = 2.7	2
	Conidia in culture either globose, subglobose, obpyriform, elongate obpyriform, obovoid, on average $\geqslant$11 μm long, 1:b 1.2–2.4, and/or ellipsoidal, broadly fusiform, fusiform or narrowly fusiform, on average 15–26 μm long, 1:b $\geqslant$2.6	3
2(1)	Conidia 8–12 × 6.5–9 μm, never broadly fusiform to fusiform	**B. parva**
	Conidia 7–17 × 6.5–10.5 μm, broadly fusiform to fusiform conidia when present, 12–24 × 5–8 μm	**B. ribis**
3(1)	Conidia predominantly elongate obpyriform or less commonly obovoid, 13–28 × 5.5–9 μm, occasionally ellipsoidal or narrowly fusiform, 23.5–28 × 5.5–8 μm	**B. dothidea**
	Conidia not predominantly elongate obpyriform, less than 25 μm long	4
4(3)	Conidia subglobose, obpyriform or obovoid, 8–19 × 6.5–9.5 μm, commonly ellipsoidal and broadly fusiform, 16–25 × 5–8 μm	**D. versiformis**
	Conidia occasionally or never ellipsoidal or broadly fusiform	5
5(4)	Conidia subglobose, obpyriform or obovoid, 9.5–17.5 × 7–11 μm, never subcylindrical, long obovoid or broadly fusiform	**B. australis**
	Conidia globose, subglobose, obpyriform or obovoid, 9–14.5 × 6.5–11 μm, occasionally subcylindrical, long obovoid or broadly fusiform, 12–18.5 × 5–7	**D. eucalypti**

diversicolor, 2001, *T. Burgess/K.-L. Goei* (WAC12398). – **New Zealand**: St. Arnaud, *Eucalyptus* sp., Aug. 1886, *M. C. Cooke* (K(M) 122474).

Dichomera versiformis Z. Q. Yuan, T. Wardlaw & C. Mohammed, *Nova Hedwigia* **70**: 140 (2000). (Figs 6–9)

Conidia in vivo either regular in shape, broadly fusiform, hyaline and aseptate or irregular in shape from subglobose, obovoid or obpyriform to ellipsoidal or broadly fusiform, aseptate or muriform, hyaline or brown, apex subobtuse to obtuse, base truncate to bluntly rounded. Subglobose, obovoid or obpyriform conidia (9.5–) 11–16 (–17) × 8–11 μm (mean = 14.5 × 10.5 μm, 1:b 1.4), hyaline to pale brown when immature with 0–4 transverse septa and 0–1 oblique septa becoming brown and muriform when mature with 1–3 transverse septa and 0–1 longitudinal septa and 0–3 oblique septa. Ellipsoidal or broadly fusiform conidia (17–) 18–24 (–27.5) × (5–) 5.5–8 (–9) μm (mean = 21.4 × 6.9 μm, 1:b 3.1), hyaline to pale brown, aseptate to muriform with 0–6 transverse septa and 0–1 oblique septa. *Conidia in vitro* variable in shape from subglobose, obovoid or obpyriform to ellipsoidal or broadly fusiform, apex subobtuse to obtuse, base truncate to bluntly rounded. Subglobose, obovoid or obpyriform conidia 8–17 (–19) × 6.5–9.5 μm (mean = 13.6 × 9.3 μm, 1:b 1.5), hyaline to pale brown when immature becoming brown when mature, with 0–3 transverse septa, 0–1 longitudinal and 0–1 oblique septa. Ellipsoidal and broadly fusiform conidia 16–22 (–25) × (5–) 5.5–6.5 (–8) μm (mean = 19.3 × 5.9 μm, 1:b 3.3), hyaline to pale brown when immature with 0–4 transverse septa and no longitudinal or oblique septa becoming brown and muriform when mature with 4–5 transverse septa and 0–2 oblique septa.

Specimens and cultures examined: **Australia**: Victoria: Nareen, *Eucalyptus camaldulensis*, 7 Sep. 1999, *P. A. Barber* (VPRI 31989 – ***epitypus hic designatus***; *culture* WAC12403); Mt Buffalo, 'The Hump', *Eucalyptus pauciflora*, 8 May 2000, *P. J. Keane* (VPRI 31988 – culture VPRI 31988). *Tasmania*: Smithton, *Eucalyptus nitens*, 27 Aug.1998, *Z. Q. Yuan & T. Wardlaw* (VPRI 22038a – holotype of *Dichomera versiformis*).

DISCUSSION

In this study, isolates representing *B. dothidea*, *B. ribis*, *B. parva* and *B. australis*, from eucalypts in Australia, were shown to have synanamorphs with muriform, irregular shaped conidia typical of the genus *Dichomera*. These conidia are formed in addition to their well recognised hyaline, regular-shaped *Fusicoccum* anamorphs, which have previously been described. Two other *Dichomera* spp. (*D. eucalypti* and *D. versiformis*) were found to group with *Botryosphaeria* with *Fusicoccum* anamorphs. Based on the DNA sequence analyses and morphological examination of freshly collected isolates and type specimens, descriptions have been provided for the *Dichomera* synanamorphs of the respective *Botryosphaeria* spp., and for *D. eucalypti* and *D. versiformis*.

Taxonomy

Our observations of the ex-type cultures of *Botryosphaeria ribis* and *B. parva* confirm the finding by Slippers *et al.* (2004a) that conidia of both species can become septate with age. Results of the current study show that certain isolates of *B. ribis* (WAC12395, WAC12396) and *B. parva* (WAC12397) from Australia can also produce a *Dichomera* synanamorph. Isolates of these two species collected in this study could be distinguished from each other based on the conidia of their *Dichomera* synanamorph. However, care should be taken when considering these differences as only one isolate of *B. ribis* with a *Dichomera* synanamorph was examined and additional isolates may show that *B. ribis* also has the ability to produce fusiform or broadly fusiform, muriform conidia in culture.

Our observations based on ITS sequence comparisons, as well as the morphology of conidia of the *Dichomera* synanamorph indicate *B. parva* and *B. ribis* are more closely related to each other than either of them is to *B. dothidea*. Zhou & Stanosz (2001b) reported similar results after comparing ITS sequences and this was confirmed more recently by Slippers *et al.* (2004a) after characterising each species using morphological, cultural and multi-allelic DNA sequence datasets from the rDNA (ITS 1, 5.8S, and ITS 2), β-tubulin and EF1-α genes. Our observations show that the variability in size and shape of conidia produced in culture is consistent with the separation of *B. ribis*, *B. parva* and *B. dothidea* based on sequence data.

Analysis of the ITS sequences of two isolates (WAC12399 and WAC12400) collected from healthy stems of *Eucalyptus diversicolor* and *E. marginata*, respectively, in native forests in Western Australia, and which produced *Dichomera* synanamorphs *in vitro*, showed that they are identical to *B. australis* described by Slippers *et al.* (2004c). Slippers *et al.* (2004c) recently described *B. australis* and its anamorph, *Fusicoccum australe*, from native *Acacia* and exotic *Sequoiadendron* trees in eastern Australia. They found this species was closely related to, but taxonomically distinct from *B. lutea*, based on morphology and sequence data within the ITS, β-tubulin and EF1-α regions. The collection of additional isolates of *B. australis* in this study and their separation from *B. lutea* based on sequence data for the ITS region support the findings by Slippers *et al.* (2004c).

An isolate of *F. aesculi* (WAC12404) collected from stem cankers of *E. calophylla* in Western Australia and forming a *Dichomera* synanamorph in culture, had an ITS DNA sequence identical to *B. dothidea*. Furthermore, an isolate labelled *D. saubinetii* (CBS 990.90) was also identical in ITS sequence to *B. dothidea*. This raises the question whether *D. saubinetti*, the type species of *Dichomera*, is a synanamorph of *B. dothidea* along with *F. aesculi*. Butin (1993) presented a synonymy for *D. saubinetii* and a fungus closely related to *F. aesculi*, suggesting a single biological species had the ability to produce two different spore types with intermediate forms in different pycnidia, as well as within the same pycnidium in culture. The observations of Butin (1993) were made prior to the clarification of the identity of *F. aesculi* (Smith, Michailides & Stanosz 2001, Smith & Stanosz 2001, Slippers *et al.* 2004a). They also came before the publication of significant DNA-based phylogenetic studies on the relationships between species of *Botryosphaeria* and *Fusicoccum*. Sutton (1980) described specimens of *D. saubinetii* from *Rhamnus frangula*, the same host referred to for *B. berengeriana* (asynonym *of B. dothidea*) in the original description by De Notaris (1863). There are, however, no cultures linked to the type of *D. saubinetii*, which precludes us from critically testing the relatedness of *F. aesculi* and *D. saubinetti* on morphological and molecular bases.

The original description of *D. saubinetii* (as *Hendersonia saubinetii*; Montagne 1856) describes conidia as pleomorphic, cellular, brown and pedicillate without giving dimensions. von Höhnel (1918) later designated *D. saubinetii* as the lectotype of the genus. His description did not refer to the pleomorphic and pedicellate nature of conidia, although it gave dimensions of conidia (20×11 μm). Sutton (1980) described *D. saubinetii* as having determinate, holoblastic conidiogenous cells, $7.5–14 \times 3–4$ μm, and conidia which were globose, subglobose, clavate or fusiform, $11–13 \times 7–10$ μm. The type specimen was not included amongst the specimens examined by Sutton. The *Dichomera* synanamorph produced by *B. dothidea* collected in our study differs from this description in having significantly longer conidiogenous cells and conidia. Sutton's description includes fusiform conidia, however, none are illustrated and the conidia that are illustrated do not resemble those observed for the *Dichomera* synanamorph of *B. dothidea* in the present study.

Attempts to locate the type specimen of *D. saubinetii* in the present study were unsuccessful. Two specimens labelled *D. saubinetii* (K(M) 122471 and K(M) 122468) had conidia that were morphologically similar, yet still distinct from all other species examined here. The short conidia observed in these herbarium specimens were similar in size to those previously described by Sutton (1980) and fell within the range of those described by von Höhnel (1918); however, the longer conidia were substantially different from any previously described for *D. saubinetii*. Hyaline, aseptate conidia typical of *Fusicoccum* were also observed in these specimens, suggesting a link to *Botryosphaeria*.

Conidia of some isolates collected in this study showed close similarities to those of two *Dichomera* spp. previously described from eucalypts in Australia. Sutton (1975) combined two taxa, *Camarosporellum eucalypti* and *Coryneum viminale*, to describe *D. eucalypti*. There has been no published account of this fungus occurring on eucalypts subsequent to its first description. We examined the type specimen of *D. eucalypti* and an additional specimen labelled *D. eucalypti* by Cooke (K(M) 122474), and found the morphological characteristics to be the same as those of some isolates collected in this study. This fungus was commonly associated with lesions containing eulophid wasps. This association was observed in the foliage specimens collected in this study, as well as the Cooke specimen (K(M) 122474). The ITS sequence data produced in this study show that *D. eucalypti* is a *Botryosphaeria* species grouping in the *Fusicoccum* clade, with the teleomorph and synanamorph forms in these genera still awaiting collection and description.

Two isolates (WAC12403 and VPRI31988) residing in the *Fusicoccum* clade had morphological characteristics indistinguishable from those of the type specimen of *D. versiformis*. *Dichomera versiformis* was described

from a single collection of foliage from *E. nitens* in Tasmania, Australia (Yuan *et al*. 2000). Barber, Smith & Keane (2003) recorded *D. versiformis* from leaf lesions on an additional five eucalypt species and noted that the fungus was relatively common on eucalypts in Victoria. One specimen (WAC12403) produced *in vivo* aseptate, hyaline spores typical of a *Fusicoccum* sp. These spores remained hyaline and aseptate during germination. Muriform, brown conidia were only discovered some weeks later in cultures. In the current study, *D. versiformis* was found on *E. pauciflora* and *E. camaldulensis* for the first time, increasing the host range described by Barber *et al*. (2003). As with *D. eucalypti*, this study shows that it is likely that this species has a *Botryosphaeria* teleomorph and *Fusicoccum* synanamorph yet to be collected and described.

The muriform conidia of the type of *D. versiformis*, as well as each of the *Dichomera* synanamorphs of *B. ribis*, *B. australis*, and *B. dothidea*, could be grouped into two general categories. These include those that are globose, sub-globose, obovoid, obpyriform or elongate obpyriform and those that are ellipsoid or somewhat fusiform. This feature of having conidia with variable shape has been previously considered unique to *D. versiformis* in the genus *Dichomera* (Yuan *et al*. 2000). Both the type of *D. eucalypti* and the anamorph of *B. parva* (WAC12397) have muriform *Dichomera* conidia, but lack the ellipsoid or somewhat fusiform conidia for this anamorph.

The discovery of *Dichomera* synanamorphs with distinctly muriform conidia has provided additional features for morphological comparison between taxa of *Botryosphaeria*. There has been a great deal of research carried out in recent years to clarify the taxonomic confusion surrounding *Botryosphaeria* and its anamorphs (Palmer, Stewart & Wingfield 1987, Jacobs & Rehner 1998, Denman *et al*. 2000, Smith & Stanosz 2001, Smith *et al*. 2001, Zhou & Stanosz 2001a, Phillips *et al*. 2002, de Wet *et al*. 2003, Alves *et al*. 2004, Slippers *et al*. 2004a, c). Considerable debate has surrounded the correct identification of species such as *B. dothidea*, *B. parva*, *B. ribis* and *B. lutea*, which have been regarded as either synonyms or closely related due to the overlap in morphological features. Results of this study have shown that these four *Botryosphaeria* spp. can clearly be separated based on morphology of the *Dichomera* spore form, when this synanamorph is present.

Pleoanamorphy of Botryosphaeria

Pleoanamorphy is the term applied when two or more anamorphs (synanamorphs) are characterised based on morphology, but are identical according to DNA sequence data. The issue of classification and nomenclature of pleoanamorphic fungi has been discussed in detail (Hennebert 1971, 1987, Carmichael 1981, Gams 1982, Seifert & Samuels 2000), with opinions differing on what constitutes a synanamorph, and whether they should be grouped under a single anamorphic name or given their own unique identity. For well known genera with synanamorphs (e.g. *Fusarium*, *Phoma*), the difference in morphology of the synanamorphs and (or) the mode of conidiogenesis is usually very obvious. The application to *Dichomera* and *Fusicoccum* is more complicated as the mode of conidiogenesis is similar in both, and there is considerable overlap in conidial morphology between conidia of *Fusicoccum* and the more fusiform and elongate *Dichomera* spore types (e.g. *B. dothidea*, *D. versiformis*). Despite these similarities, the *Dichomera* and *Fusicoccum* spore-types are clearly different, and are consistently distinguishable from each other and mostly occur independently of each other (hence the association escaping notice before). The decision was thus made to retain *Dichomera* as a synanamorph name rather than to synonymise it with the older generic name *Fusicoccum*.

Ecological consideration

Our results show that *Botryosphaeria dothidea*, *B. parva*, *B. ribis*, and *B. australis*, as defined by Slippers *et al*. (2004a, c) have a wider host and geographical distribution than previously thought. Despite previous reports of *B. dothidea* (Slippers *et al*. 2004b) and *B. ribis* from *Eucalyptus* (Webb 1983, Shearer, Tippett & Bartle 1987, Crous, Knox Davies & Wingfield 1989, Old *et al*. 1990) a recent survey showed that *B. dothidea* was rare on this host and failed to identify *B. ribis* (Slippers *et al*. 2004b), adding support to the contention that *B. ribis* is limited to *Ribes* sp. in the USA (Slippers *et al*. 2004a). Confusion may have arisen because a large number of species were synonymised with *B. dothidea* (including *B. ribis*) (von Arx & Müller 1954), a situation which was not universally accepted and has been discounted in recent studies. We have shown that *B. dothidea* occurs on *Eucalyptus* in Western Australia, where there is a well-established eucalypt plantation industry. Furthermore, the anamorph of *B. ribis*, *F. ribis sensu* Slippers *et al*. (2004a), was shown to occur on *E. pellita* and *E. grandis* × *E. camaldulensis* plantations in Queensland, Australia. This is, however, only based on ITS data and requires further investigation using sequence data of more gene regions for this cryptic species (Slippers *et al*. 2004a).

Botryosphaeria parva sensu Slippers *et al*. (2004a) has been isolated from *Eucalyptus* and other hosts outside Australia (Slippers *et al*. 2004a) and recently from *Tibouchina* in Australia (Slippers *et al*. 2004b). Our findings show that *B. parva* also occurs on *E. pellita* plantations in Queensland, Australia. Similarly, *B. australis* was found for the first time on *E. globulus* plantations in Western Australia, possibly originating from nearby native forests. Slippers *et al*. (2004a, b) concluded that *B. australis* was probably native to the southern hemisphere, most likely Australia, but it has not been commonly found on *Eucalyptus*.

Results of the present study have shown that *D. eucalypti* occurs on both woody tissue and leaves of

several species of eucalypt in southern Australia. The three host species, *E. pauciflora*, *E. camaldulensis* and *E. viminalis*, were situated in native forests or woodlands in south-eastern Australia. The remaining isolate was collected from woody tissue of *E. globulus* growing in plantations in south-western Australia. The fungus could have been transferred to Western Australia from the eastern states in infected tissue, or it may have moved into the plantations from surrounding native eucalypt forests. Further collections will be required to resolve this question.

All the species of *Botryosphaeria* that produced a *Dichomera* synanamorph in culture were collected from eucalypts in Australia. Ex-type cultures of *B. dothidea*, *B. parva*, *B. ribis* and *B. australis* used in this study were from hosts other than eucalypts, and, with the exception of *B. australis*, countries other than Australia. These ex-type isolates produced only hyaline, fusiform, aseptate conidia typical of *Fusicoccum*, despite being subjected to the same cultural conditions as the isolates collected from eucalypts in this study. This raises the question: Is the *Dichomera* synanamorph a characteristic restricted to *Botyryosphaeria* spp. occurring on eucalypts in Australia? Morphological comparisons of *D. saubinetii* specimens from stems of deciduous trees including *Quercus* and *Rhamnus* in Europe, and those previously made by Butin (1993) suggest not. Whilst documenting the fungal assemblages in stem and twig lesions of *Quercus robur* in Switzerland, Sieber *et al.* (1995) noted that *D. saubinetii* was found during their study, but had never been mentioned in other studies of fungi associated with oak decline (Dellavalle-Fedi, Moricca & Ragazzi 1991, Kowalski 1991, Kehr & Wulf 1993). However, these same studies had recorded species such as *Diplodia mutila* and *D. quercina* (syn. *F. quercus*) but not *D. saubinetii*, and these fungi might have been confused with each other. It thus appears that the link between *Botryosphaeria* and *Dichomera* may have been overlooked, partly because of the lack of cultural and molecular studies of *Dichomera* and Australian isolates of *Botryosphaeria*, and needs careful attention in future.

CONCLUSION

Important questions arising from the current study include the abundance, distribution, ecological role (including endophytic status, role in survival of spores, and pathogenicity) and genetic mechanism behind the expression of these distinct phenotypes. A recent study carried out by Whyte (2003) on *Dichomera eucalypti* has suggested that there is a close relationship between eulophid wasps and the fungus. It was hypothesised that the lesions were initially caused by the wasp and that the fungus, which is endophytic, subsequently developed in these lesions. Further work is required to confirm this, but detailed studies like this will help us to understand the biology of these fungi and, therefore, their ability to cause disease.

It is also clear that the genus *Dichomera* and its so called 'stromatic analogue', *Camarosporium*, require extensive revision. This will be a difficult task considering that there are over 400 taxa in these combined genera. Other anamorphic genera which appear to be somewhat related to *Dichomera* and *Camarosporium*, such as *Hendersonula* and *Camarosporellum*, should also be reconsidered. Such a revision would require a combination of morphological and DNA sequence comparisons as well as the examination of types. The effort will clearly be frustrated by the lack of cultures for many species and significant new collections will be required.

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