Phylogenetic lineages in the *Botryosphaeriales*: a systematic and evolutionary framework

B. Slippers[1#*], E. Boissin[1,2#], A.J.L. Phillips[3], J.Z. Groenewald[4], L. Lombard[4], M.J. Wingfield[1], A. Postma[1], T. Burgess[5], and P.W. Crous[1,4]

[1]*Department of Genetics, Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria 0002, South Africa*; [2]*USR3278-Criobe-CNRS-EPHE, Laboratoire d'Excellence "CORAIL", Université de Perpignan-CBETM, 58 rue Paul Alduy, 66860 Perpignan Cedex, France*; [3]*Centro de Recursos Microbiológicos, Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516, Caparica, Portugal*; [4]*CBS-KNAW Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands*; [5]*School of Biological Sciences and Biotechnology, Murdoch University, Perth, Australia*

*Correspondence: B. Slippers, Bernard.Slippers@fabi.up.ac.za
# These authors contributed equally to this paper.

Abstract: The order *Botryosphaeriales* represents several ecologically diverse fungal families that are commonly isolated as endophytes or pathogens from various woody hosts. The taxonomy of members of this order has been strongly influenced by sequence-based phylogenetics, and the abandonment of dual nomenclature. In this study, the phylogenetic relationships of the genera known from culture are evaluated based on DNA sequence data for six loci (SSU, LSU, ITS, EF1, BT, mtSSU). The results make it possible to recognise a total of six families. Other than the *Botryosphaeriaceae* (17 genera), *Phyllostictaceae* (*Phyllosticta*) and *Planistromellaceae* (*Kellermania*), newly introduced families include *Aplosporellaceae* (*Aplosporella* and *Bagnisiella*), *Melanopsaceae* (*Melanops*), and *Saccharataceae* (*Saccharata*). Furthermore, the evolution of morphological characters in the *Botryosphaeriaceae* were investigated via analysis of phylogeny-trait association. None of the traits presented a significant phylogenetic signal, suggesting that conidial and ascospore pigmentation, septation and appendages evolved more than once in the family. Molecular clock dating on radiations within the *Botryosphaeriales* based on estimated mutation rates of the rDNA SSU locus, suggests that the order originated in the Cretaceous period around 103 (45–188) mya, with most of the diversification in the Tertiary period. This coincides with important periods of radiation and spread of the main group of plants that these fungi infect, namely woody Angiosperms. The resulting host-associations and distribution could have influenced the diversification of these fungi.

Key words: *Aplosporellaceae*, *Melanopsaceae*, molecular dating, *Phyllostictaceae*, *Planistromellaceae*, *Saccharataceae*, systematics.
Taxonomic novelties: New families – *Aplosporellaceae* Slippers, Boissin & Crous, *Melanopsaceae* Phillips, Slippers, Boissin & Crous, *Saccharataceae* Slippers, Boissin & Crous.

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INTRODUCTION

DNA sequence-based phylogenetics has dramatically influenced both the taxonomy and systematics of the *Botryosphaeriaceae* during the course of the past decade (Crous *et al*. 2006), as it has done in most other groups of *Fungi* (James *et al*. 2006, Hibbett *et al.* 2007). At a higher taxonomic level, DNA sequence data have led to the recognition that the *Botryosphaeriaceae* represents a distinct order within the *Dothideomycetes*, leading Schoch *et al*. (2006) to introduce the *Botryosphaeriales*. The circumscription of the *Botryosphaeriales* has suffered from insufficient sampling and it was only recently that Minnis *et al*. (2012) provided molecular evidence to show that the *Planistromellaceae* resides in this order. In a subsequent study, Liu *et al*. (2012) provided a comprehensive phylogenetic analysis of genera in the *Botryosphaeriales* and they also concluded that, other than the *Botryosphaeriaceae* and *Planistromellaceae*, a number of clearly defined evolutionary lineages exist.

Apart from the *Planistromellaceae*, the genera traditionally associated with *Botryosphaeria* and *Phyllosticta* have sexual morphs that are clearly distinct phylogenetically, morphologically and ecologically. However, both are still grouped within the *Botryosphaeriaceae*. Members of the *Botryosphaeria* group are common endophytes of leaf and woody tissue of many woody plant species, have hyaline to dark ascospores, multilocular ascomata, and a wide range of asexual morphs that typically lack a mucoid sheath and apical appendage. Species in the *Guignardia* group (= *Phyllosticta*) typically infect leaves and fruit, less commonly wood, have unilocular ascomata with smaller ascospores that typically have mucoid appendages, and *Phyllosticta* asexual morphs. The *Phyllostictaceae* has been resurrected to accommodate this group of taxa (see Wikee *et al*. 2013b, this volume).

Substantial changes to the definition of sexual and asexual genera linked to the *Botryosphaeriaceae* have been made during the past decade (e.g. Crous *et al*. 2006, Phillips *et al*. 2008, Liu *et al.* 2012). Only a selection of the most common examples is discussed here. The first DNA sequence data for the *Botryosphaeriaceae* appeared to reveal a distinction between asexual morphs with hyaline fusicoccum-like conidia and those with pigmented diplodia-like conidia, termed sections *Hyala* and *Brunnea* (Jacobs & Rehner 1998, Denman *et al*. 2000, Zhou & Stanosz 2001). This distinction became increasingly less obvious as sampling increased and it was evident that conidial pigmentation is a feature that evolved more than once. It was, for example, shown that dark, septate and even muriformly septate dichomera-like conidia could be synasexual morphs of well-known genera such as *Fusicoccum*

and *Neofusicoccum* (Barber *et al*. 2005, Phillips *et al.* 2005). Furthermore, dark, septate ascospores were shown to be a polyphyletic character of several genera and more common than previously believed (Phillips *et al.* 2008). As the true phylogenetic diversity within the group emerged, a number of new genera were described (e.g. *Botryobambusa*, *Cophinforma*, *Neofusicoccum*, *Neoscytalydium*, *Pseudofusicoccum*, etc.) or older genera re-defined (e.g. *Auerswaldia*, *Barriopsis*, *Dothiorella*, etc.) (e.g. Crous *et al*. 2006, Phillips *et al*. 2008, Liu *et al.* 2012). The most recent work by Liu *et al*. (2012) reviewed these genera, and this study reflects a growing consensus regarding the circumscription of the majority of the genera (29 in total, of which sequence data are available for 20).

DNA-based sequence analyses have also resulted in significant changes to the nomenclature, identification and circumscription of species in the *Botryosphaeriaceae*. These changes have resulted in the implementation of a single nomenclature for all morphs of a species (Crous *et al.* 2006, Hawksworth *et al*. 2011, Wingfield *et al*. 2012). For the *Botryosphaeriaceae*, this has included the description of cryptic species based on DNA sequence data, where morphological characters were not variable enough for this purpose (Pavlic *et al*. 2009a, Sakalidis *et al.* 2011).

Insights gained from contemporary studies on the *Botryosphaeriaceae* have led to uncertainty regarding the application of names published in the older literature. The analyses show for example that morphological characters typically used for species identification (chiefly conidia or ascospore dimensions, shape, septation and pigmentation) are frequently unreliable. Even ecological and geographical data are difficult to interpret, with some species occurring on numerous hosts, and single locations or hosts often yielding numerous co-occurring species (Slippers & Wingfield 2007, Slippers *et al*. 2009). For this reason (together with the significant changes in generic descriptions mentioned above) many, if not most, of the taxa dealt with before the introduction of DNA sequence-based phylogenetic inference will need to be redefined (possibly neo- or epitypified), to allow meaningful comparisons with currently applied names (also see the discussion in Phillips *et al*. 2013, this volume). Where it is not possible to follow this approach, older names may have to be ignored and new species introduced that are supported by DNA data (see Slippers *et al.* 2014).

The *Botryosphaeriales* is an important group of fungi due to the ecological and economic significance of many of its species. All species are plant-associated, and many are classified as pathogens, known to cause disease on a wide range of ecologically and economically important plants (Mehl *et al.* 2012). Some species are also known to cause opportunistic infections in humans (de Hoog *et al.* 2000). Most species exist as endophytes living in healthy plant tissues for extended periods of time (Slippers & Wingfield 2007). Their roles as endophytes or pathogens often overlap, as is for example found in the case of *Diplodia sapinea*. This well-known pathogen of *Pinus* (Swart *et al*. 1991) is also a common endophyte in branches, the trunks and seed cones of these trees. In an extreme example, *D. sapinea* has been isolated from the wood of *Pinus* in South Africa, where it must have existed without causing disease subsequent to the tree being infected as long as a decade previously (Bihon *et al.* 2011).

Unlike the case for *D. sapinea*, the ecological roles for the majority of species of *Botryosphaeriaceae* are unknown. The changes to the taxonomy of the group are already strongly promoting an ability to characterise the diversity in this group. In turn, this is providing an evolutionary framework making it possible to study the ecological role that remains obscure for the majority of these fungi.

In this paper, the phylogenetic relationships of all the genera known from culture and considered to reside in the *Botryosphaeriales* and *Botryosphaeriaceae* are determined based on DNA sequence data for six loci. The *Planistromellaceae* is well defined within the *Botryosphaeriales*. As expected, *Phyllosticta* (= *Guignardia*) also forms a strongly supported monophyletic lineage, recognised as the *Phyllostictaceae* (see Wikee *et al*. 2013b, this volume). *Saccharata*, however, groups separately with respect to all other genera in the *Botryosphaeriales*, as does *Aplosporella, Bagnisiella* and *Melanops*. The nomenclatural changes necessary to reflect these distinctions are considered in this study. With the well-supported phylogeny provided by these analyses, we also test hypotheses regarding the evolution of major morphological features typically used in taxonomy of the *Botryosphaeriaceae*. Finally, we use the nuclear ribosomal subunit data to date the divergence in the major groups of the *Botryosphaeriales*.

MATERIALS AND METHODS

Isolates and DNA extractions

A total of 96 strains corresponding to 85 species were grown on 2 % potato dextrose agar (PDA) plates incubated at 25 ºC. Genomic DNA was extracted from mycelium using the PrepMan™ Ultra protocol (Applied Biosystems). Sequences from additional species were retrieved from GenBank. A total of 140 taxa were included in the ingroup and six taxa in the outgroup (see Table 1 for details).

PCR and sequencing

A total of six partial gene portions were used in this study: the nuclear ribosomal small subunit (SSU), the nuclear ribosomal large subunit (LSU), the intergenic spacer (ITS), the translation elongation factor 1-alpha (EF1), the β-tubulin gene (BT) and the mitochondrial ribosomal small subunit (mtSSU).

The primers used were NS1 and NS4 (White *et al*. 1990) for SSU, LROR and LR5 (Vilgalys Laboratory, Duke university, www.biology.duke.edu/fungi/mycolab/primers.htm) for LSU, ITS-1 and ITS-4 (White *et al*. 1990) for ITS, EF-AF and EF-BR (Sakalidis *et al*. 2011) for EF1, BT2A and BT2B (Glass & Donaldson 1995) for BT and mrSSU1 and mrSSU3R (Zoller *et al.* 1999) for mtSSU. All PCR reactions were conducted in 15 µL containing 1.5 mM of $MgCl_2$, 0.5 mM of dNTP, 1 × final concentration of buffer, 1 µM of each primer, 0.25 U of FastStart *Taq* Polymerase (Roche), 1.5 µL of DNA template and Sabax sterilised water (Adcock Ingram) to complete up to 15 µL. The cycling parameters were as follows: a first step of denaturation at 95 ºC for 5 min followed by 35 cycles of (i) denaturation at 95 ºC for 60 s, (ii) annealing at optimal temperature (55 ºC for ITS, EF1, LSU and 45 ºC for SSU, mtSSU, BT) for 80 s, (iii) elongation at 72 ºC for 90 s, and a final elongation step of 5 min was applied.

Sephadex columns (Sigma-Aldrich) were used to clean the samples both before and after the sequencing reactions. The sequencing PCRs were performed in 10 µL containing 1 µL of PCR product, 0.7 µL of Big Dye Terminator v. 3.1 (Applied Biosystems), 2.5 µL of sequencing buffer (provided with Big Dye), 1 µL of primer (10 µM) and 4.8 µL of Sabax sterilised water. Cycling parameters consisted of 25 cycles with three steps each: 15 s at 95 ºC, 15 s at 55 ºC (for ITS, EF1, LSU) or 45 °C (for SSU, mtSSU, BT) and 4 min at 60 ºC. The sequencing PCR products were sent to a

partner laboratory for sequencing of both strands (Sequencing Facility, University of Pretoria).

Data analyses

Sequences were aligned using MAFFT v. 6 online (Katoh & Toh 2008) and refined visually. In order to retrieve the genetic information from indels, GapCoder (Young & Healy 2003) was used to code the gaps contained in the EF1 and the mSSU alignments. Analyses were run both with gaps coded and not coded.

The datasets were combined because this is believed to increase phylogenetic accuracy (Bull *et al*. 1993, Cunningham 1997). The six phylogenies resulting from the six data sets were first inspected visually to check whether there were conflicts between the histories of the genes that would preclude the combination of data. Additionally, the Incongruence Length Difference (ILD) test (Farris *et al.* 1995a, b) also known as the partition homogeneity test was run using PAUP v. 4.0b10 (Swofford 2003).

MrAIC (Nylander 2004) was used to determine the best fit model of nucleotide substitutions for each gene. Phylogenetic relationships of the samples were investigated using both Maximum Likelihood (ML) and Bayesian Inference (BI). The ML analysis was conducted using PhyML online (Guindon *et al.* 2005). The reliability of each node was assessed using the bootstrap (Felsenstein 1985) resampling procedure (100 replicates).

The BI was conducted using the software BEAST v. 1.7.4 (Drummond *et al*. 2012). The phylogenetic relationships were estimated by running 10 000 000 generations and sampling every 100th generation. Bayes Factors were computed to choose between the different options available in BEAST (four clock models: strict clock, exponential or lognormal uncorrelated relaxed clocks, and random local clock and two tree priors: Yule process or Birth-Death speciation model). The Birth-Death speciation model and a relaxed uncorrelated exponential clock were selected as best fitting our data. Six independent runs were performed and outputs were combined using LogCombiner (in the BEAST package). The programme TRACER v. 1.5 (available on the BEAST website) was used to check that the effective sampling sizes (ESS) were above 200 (as advised by the programmers to ensure an accurate estimation of phylogeny and parameters of interest). The programme Tree Annotator (available in the BEAST package) was used to summarize the resulting trees using the maximum clade credibility option. The final tree was visualised in FigTree v. 1.4.

Molecular clock dating

Using a mean molecular rate of 1–1.25 % per lineage per 100 million years, commonly accepted in fungi for the SSU gene (Berbee & Taylor 2010), a rough estimation of the times to the most recent common ancestors of groups of interest was assessed using BEAST. A normal distribution was used as prior and this was centred on a 95 % interval spanning 1.0–1.25 % (mean = 0.000113; standard deviation = 0.000006). The dating of the distinct groups of interest and their 95 % highest posterior density (HPD) were retrieved using TRACER v. 1.5.

Analysis of phylogeny-trait association

In order to investigate the evolution of morphological characters in the *Botryosphaeriaceae*, ancestral trait reconstructions and tests for phylogenetic signal were conducted in Mesquite v. 2.74 (Maddison & Maddison 2010). The characters considered were 1) ascospore colour; 2) presence or absence of ascospore septa; 3) conidial colour; 4) presence or absence of conidial septa; and 5) presence or absence of a mucus sheath. Both parsimony and ML reconstructions were used in Mesquite to test for phylogenetic signal. The observed distribution of character states at the tips of the phylogeny was compared to null distributions obtained when reshuffling the tip characters on the tree topology (10 000 times). Where the number of steps (or likelihood value) in the observed trait reconstruction fell outside the 95 % range of the null distribution, this was seen to indicate that character states are not distributed randomly on the phylogeny (i.e. there is a phylogenetic component).

RESULTS

Phylogenetic relationships

The alignment included a total of 4498 bp from six gene portions. The results from the ILD test were not significant and supported a decision to combine the 6 gene datasets. The number of polymorphic and parsimony informative sites ranged from 108 for the SSU, 165 for the mtSSU, 170 for the LSU, 222 for BT, 335 for the EF1 to 361 for the ITS.

The Maximum Likelihood (ML) and Bayesian Inference (BI) phylogenetic reconstructions were similar, and the BI tree is shown on Fig. 1. Species residing in the genera *Aplosporella* and *Bagnisiella* (in one clade), *Melanops, Saccharata* and *Kellermania* had a basal position on the tree with respect to other genera in the *Botryosphaeriales*. The remaining genera in the *Botryosphaeriales* clustered together with a bootstrap support value of 94 % and Posterior Probability (PP) value of 1. The first cluster to split from the rest of the main group was formed by species of *Phyllosticta*, hereafter treated as *Phyllostictaceae* (see Wikee *et al*. 2013b, this volume). The main clade below *Phyllostictaceae* was defined as *Botryosphaeriaceae* s. str. (0.99 PP and 89 % bootstrap).

Pseudofusiccocum was basal within the *Botryosphaeriaceae*, followed by *Endomelanconiopsis*. The remaining species formed a clade having strong bootstrap support v (99 %) and could be further subdivided into four sub-clades. Sub-clade 1 encompassed species of the genera *Diplodia*, *Neodeightonia*, *Lasiodiplodia*, *Macrovalsaria*, *Phaeobotryosphaeria*, *Phaeobotryon*, *Barriopsis*, *Botryobambusa* and *Tiarosporella*. The recently described *Auerswaldia lignicola* clustered in sub-clade 1, together with *Lasiodiplodia*. Sub-clade 2 encompassed species of *Neoscytalidium*, *Cophinforma*, *Botryosphaeria* (= *Fusicoccum*) and *Macrophomina* together with *Dichomera saubinetti*. Sub-clade 3 accommodated species in the genera *Dothiorella*, *Spencermartinsia* and the recently described *Auerswaldia dothiorella*. Sub-clade 4 included species of *Neofusicoccum* and *Dichomera.*

Molecular dating

Based on the models used, families split between 57 (28–100) – 103 (45–188) mya. Divergence within some families was also very ancient, such as the split between *Pseudofusicoccum* [65 (28–112) mya] and *Endomelanconiopsis* [52 (27–78) mya] and the rest of the *Botryosphaeriaceae*. The split between sub-clades 1 to 4 within the *Botryosphaeriaceae* was estimated to be between 33 (15–55) and 44 (25–64) mya.

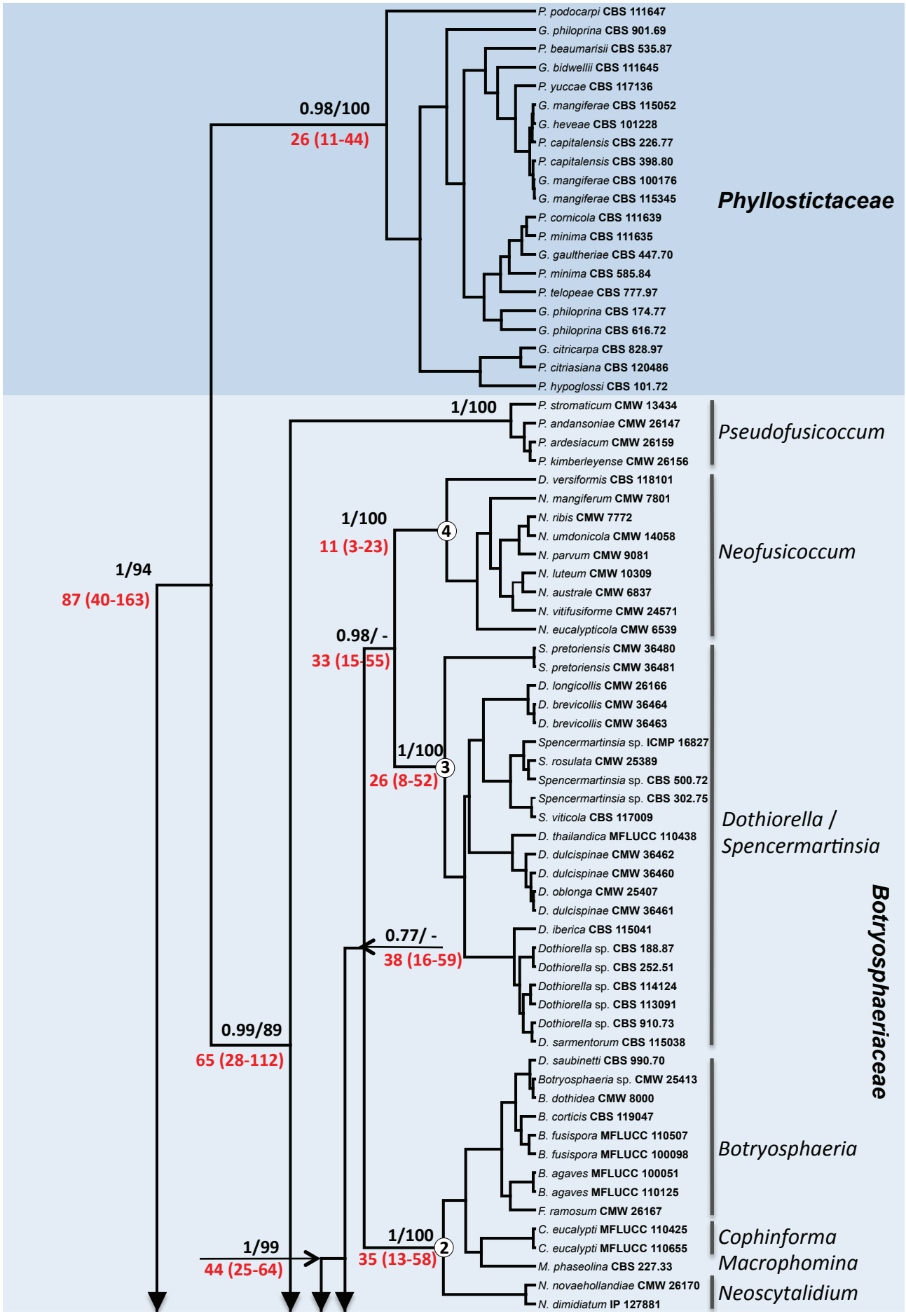

Fig. 1. Phylogenetic relationships of the *Botryosphaeriales* using Bayesian reconstruction and six gene portions (LSU, SSU, ITS, EF1, BT and mtSSU). Numbers above branches indicate bootstrap values/posterior probabilities. Numbers highlighted in red below branches indicate estimated dates in million years with the 95 % Highest Posterior Density interval given in brackets. Clades 1–4 in the *Botryosphaeriaceae* are indicated by a circled number on the corresponding node.

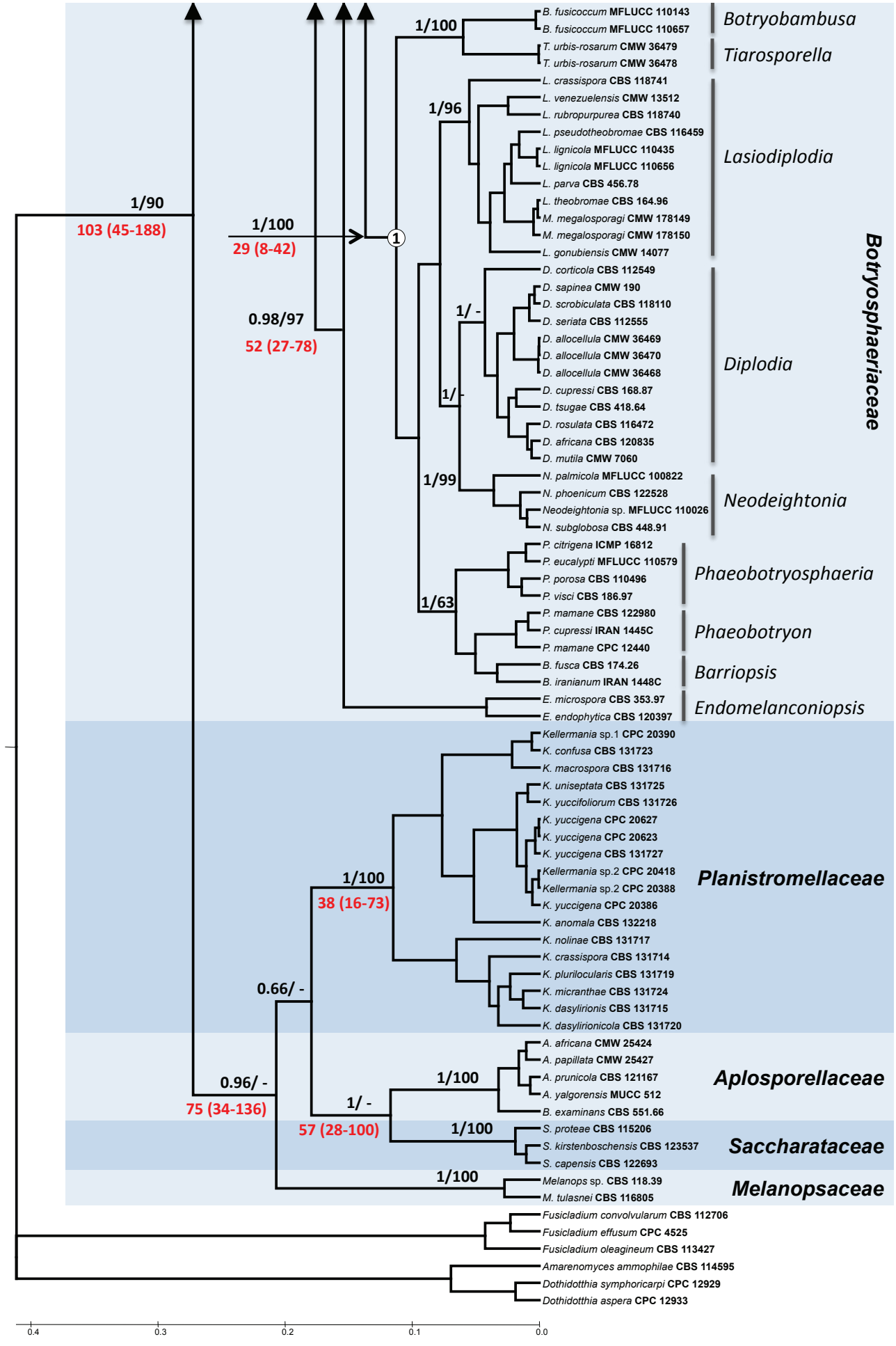

Fig. 1. (Continued).

Table 1. Isolates subjected to DNA analysis in this study.

Species	Isolate No.[1]	GenBank Accession No.					
		SSU	LSU	ITS	EF1	BT	mtSSU
Amarenomyces ammophilae	CBS 114595	N/A	KF766314	KF766146	KF766394	N/A	N/A
Aplosporella africana	CMW 25424, CBS 121777	KF766283	KF766366	KF766196	N/A	N/A	KF766475
Aplosporella papilata	CMW 25427, CBS 121780	KF766284	N/A	KF766197	N/A	N/A	KF766476
Aplosporella prunicola	CBS 121167	KF766229	KF766315	KF766147	N/A	N/A	KF766440
Aplosporella yalgorensis	MUCC512	N/A	EF591944	EF591927	EF591978	EF591961	
							N/A
Bagnisiella examinans	CBS 551.66	EU167562	KF766316	KF766148	GU349056	KF766126	KF766441
Barriopsis fusca	CBS 174.26	KF766230	KF766317	KF766149	KF766395	EU673109	N/A
Barriopsis iranianum	IRAN 1448C	KF766231	KF766318	KF766150	FJ919652	KF766127	N/A
Botryobambusa fusicoccum	MFLUCC110143	JX646826	JX646809	JX646792	JX646857	N/A	N/A
	MFLUCC110657	JX646827	JX646810	JX646793	JX646858	N/A	N/A
Botryosphaeria agaves	MFLUCC100051	JX646824	JX646807	JX646790	JX646855	JX646840	N/A
	MFLUCC110125	JX646825	JX646808	JX646791	JX646856	JX646841	N/A
Botryosphaeria corticis	CBS 119047	KF766232	EU673244	DQ299245	EU017539	EU673107	N/A
Botryosphaeria dothidea	CMW 8000, CBS 115476	KF766233	KF766319	KF766151	AY236898	AY236927	FJ190612
Botryosphaeria fusispora	MFLUCC100098	JX646823	JX646806	JX646789	JX646854	JX646839	N/A
	MFLUCC110507	JX646822	JX646805	JX646788	JX646853	JX646838	N/A
Botryosphaeria ramosa	CMW 26167	KF766253	KF766333	KF766168	EU144070	N/A	N/A
Botryosphaeria sp.	CMW 25413	KF766252	KF766332	KF766167	N/A	N/A	N/A
Cophinforma eucalypti	MFLUCC110425	JX646833	JX646817	JX646800	JX646865	JX646848	N/A
Dichomera saubinetii	CBS 990.70	KF766236	DQ377888	KF766153	KF766396	N/A	N/A
	MFLUCC110655	JX646834	JX646818	JX646801	JX646866	JX646849	N/A
Dichomera versiformis	CMW 15210, CBS 118101	KF766237	KF766321	KF766154	N/A	KF766128	N/A
Diplodia africana	CBS 120835	KF766238	KF766322	KF766155	KF766397	KF766129	KF766442
Diplodia allocellula	CBS 36468	N/A	JQ239410	JQ239397	JQ239384	JQ239378	N/A
	CBS 36469	N/A	JQ239411	JQ239398	JQ239385	JQ239379	N/A
	CBS 36470	N/A	JQ239412	JQ239399	JQ239386	JQ239380	N/A
Diplodia corticola	CBS 112549	KF766239	KF766323	KF766156	AY573227	DQ458853	KF766443
					KF766398		
Diplodia cupressi	CBS 168.87	KF766240	EU673263	KF766157	DQ458878	DQ458861	KF766444
Diplodia mutila	CMW 7060	KF766241	KF766324	KF766158	AY236904	AY236933	N/A
Diplodia rosulata	CBS 116472	EU673212	DQ377897	EU430266	EU430268	EU673131	N/A
Diplodia sapinea	CMW 109	KF766242	KF766325	KF766159	AY624251	AY624256	N/A
Diplodia scrobiculata	CMW 189, CBS 118110	KF766243	KF766326	KF766160	N/A	N/A	KF766445
Diplodia seriata	CBS 112555	KF766244	KF766327	KF766161	AY573220	DQ458856	N/A
Diplodia tsugae	CMW 100325, CBS 418.64	KF766234	DQ377867	DQ458888	DQ458873	DQ458855	N/A
Dothidotthia aspera	CPC 12933	EU673228	EU673276	N/A	N/A	N/A	N/A
Dothidotthia symphoricarpi	CPC 12929	EU673224	EU673273	N/A	N/A	N/A	N/A
Dothiorella brevicollis	CMW 36463	N/A	JQ239416	JQ239403	JQ239390	JQ239371	N/A
	CMW 36464	N/A	JQ239417	JQ239404	JQ239391	JQ239372	N/A
Dothiorella dulcispinae	CMW 36460	N/A	JQ239413	JQ239400	JQ239387	JQ239373	N/A
	CMW 36461	N/A	JQ239414	JQ239401	JQ239388	JQ239374	N/A
	CMW 36462	N/A	JQ239415	JQ239402	JQ239389	JQ239375	N/A
Dothiorella iberica	CBS 115041	KF766245	AY928053	AY573202	AY573222	EU673096	N/A
Dothiorella longicollis	CMW 26166, CBS 122068	KF766246	KF766328	KF766162	EU144069	KF766130	KF766447
Dothiorella oblonga	CMW 25407, CBS 121765	KF766247	KF766329	KF766163	N/A	N/A	KF766448
Dothiorella sarmentorum	CBS 115038	KF766248	DQ377860	AY573206	AY573223	EU673101	KF766446
Dothiorella sp.	CBS 114124	EF204515'	EF204498'	N/A	N/A	N/A	N/A
	CBS 113091	EF204516'	EF204499'	N/A	N/A	N/A	N/A
Dothiorella sp. (=*Diplodia acerina*)	CBS 910.73	EU673160	EU673234	EU673315	EU673282	N/A	N/A

Table 1. (Continued).

Species	Isolate No.[1]	GenBank Accession No.					
		SSU	LSU	ITS	EF1	BT	mtSSU
Dothiorella sp. (=*Diplodia coryli*)	CBS 252.51	EU673162	EU673235	EU673317	EU673284	EU673105	N/A
Dothiorella sp. (=*Diplodia juglandis*)	CBS 188.87	EU673161	DQ377891	EU673316	EU673283	EU673119	N/A
Dothiorella thailandica	MFLUCC110438	JX646829	JX646813	JX646796	JX646861	JX646844	N/A
Endomelanconiopsis endophytica	CBS 120397	KF766249	EU683629	KF766164	EU683637	KF766131	KF766449
Endomelanconiopsis microspora	CBS 353.97	KF766250	KF766330	KF766165	EU683636	N/A	KF766450
Fusicladium convolvularum	CBS 112706	AY251124	N/A	AY251082	N/A	N/A	N/A
Fusicladium effusum	CPC 4525	N/A	EU035430'	AY251085	KF766428	N/A	N/A
Fusicladium oleagineum	CBS 113427	KF766251	KF766331	KF766166	N/A	N/A	N/A
Guignardia bidwellii (= *Phyllosticta parthenocissi*)	CBS 111645	EU673223	DQ377876	EU683672	EU683653	FJ824777	N/A
Guignardia citricarpa (= *Phyllosticta citricarpa*)	CBS 828.97	KF766254	KF766334	FJ538318	FJ538376	N/A	N/A
Guignardia gaultheriae	CBS 447.70	N/A	KF766335	KF766169	KF766400	N/A	FJ190646
Guignardia heveae (= *Phyllosticta capitalensis*)	CBS 101228	KF766255	KF766336	FJ538319	FJ538377	N/A	KF766452
Guignardia mangiferae (= *Phyllosticta capitalensis*)	CBS 100176	N/A	KF766337	FJ538321	FJ538379	N/A	N/A
	CBS 115052	N/A	KF766338	FJ538321	FJ538379	N/A	N/A
	CBS 115345	N/A	KF766339	FJ538331	FJ538389	N/A	N/A
Guignardia philoprina (= *Guignardia rhodorae*)	CBS 901.69	KF766258	KF766342	KF766172	KF766403	N/A	N/A
Guignardia philoprina (= *Phyllosticta foliorum*)	CBS 174.77	KF766256	KF766340	KF766170	KF766401	N/A	KF766453
Guignardia philoprina (= *Phyllosticta philoprina*)	CBS 616.72	KF766257	KF766341	KF766171	KF766402	N/A	N/A
Kellermania anomala	CBS 132218	KF766259	KF766343	KF766173	KF766404	KF766133	KF766454
Kellermania confusa	CBS 131723	KF766260	KF766344	KF766174	KF766405	KF766134	KF766455
Kellermania crassispora	CBS 131714	KF766261	KF766345	KF766175	KF766406	KF766135	KF766456
Kellermania dasylirionicola	CBS 131720	KF766262	KF766346	KF766176	KF766407	KF766136	KF766457
Kellermania dasylirionis	CBS 131715	KF766263	KF766347	KF766177	KF766408	KF766137	KF766458
Kellermania macrospora	CBS 131716	KF766264	KF766348	KF766178	KF766409	KF766138	KF766459
Kellermania micranthae	CBS 131724	KF766265	KF766349	KF766179	KF766410	KF766139	KF766460
Kellermania nolinae	CBS 131717	KF766266	KF766350	KF766180	KF766411	KF766140	KF766461
Kellermania plurilocularis	CBS 131719	KF766267	KF766351	KF766181	KF766412	KF766141	KF766462
Kellermania sp. 1	CPC 20390	KF766268	KF766352	KF766182	KF766413	KF766142	KF766463
Kellermania sp. 2	CPC 20418	KF766269	KF766353	KF766183	KF766414	N/A	KF766464
	CPC 20386	KF766273	KF766357	KF766187	KF766418	N/A	KF766467
	CPC 20388	KF766274	KF766358	KF766188	KF766419	N/A	KF766468
Kellermania uniseptata	CBS 131725	KF766270	KF766354	KF766184	KF766415	KF766143	KF766465
Kellermania yuccifoliorum	CBS 131726	KF766271	KF766355	KF766185	KF766416	KF766144	N/A
Kellermania yuccigena	CBS 131727	KF766272	KF766356	KF766186	KF766417	KF766144	KF766466
	CPC 20623	KF766275	KF766359	KF766189	KF766420	N/A	KF766469
	CPC 20627	KF766276	KF766360	KF766190	KF766421	N/A	KF766470
Lasiodiplodia crassispora	CBS 118741	EU673190	DQ377901	DQ103550	EU673303	EU673133	N/A
Lasiodiplodia gonubiensis	CMW 14077, CBS 115812	KF766277	KF766361	KF766191	DQ458877	DQ458860	N/A
Lasiodiplodia lignicola	MFLUCC110435	JX646830	JX646814	JX646797	JX646862	JX646845	N/A
	MFLUCC110656	JX646831	JX646815	JX646798	JX646863	JX646846	N/A
Lasiodiplodia parva	CBS 456.78	KF766278	KF766362	KF766192	EF622063	N/A	KF766471
Lasiodiplodia pseudotheobromae	CBS 116459	KF766279	EU673256	KF766193	EF622057	EU673111	KF766481
Lasiodiplodia rubropurpurea	CBS 118740	EU673191	DQ377903	DQ103553	EU673304	EU673136	N/A
Lasiodiplodia theobromae (*Botryosphaeria rhodina* in CBS)	CBS 164.96	EU673196	EU673253	AY640255	AY640258	EU673110	N/A

Table 1. (Continued).

Species	Isolate No.[1]	GenBank Accession No.					
		SSU	LSU	ITS	EF1	BT	mtSSU
Lasiodiplodia venezuelensis	CMW 13512	KF766280	KF766363	KF766194	EU673305	N/A	N/A
Macrophomina phaseolina	CBS 227.33	KF766281	KF766364	KF766195	KF766422	N/A	KF766473
Macrovalsaria megalosporagi	CMW 178150	FJ215707	FJ215701	N/A	KF766399	N/A	N/A
	CMW 178149	FJ215706	FJ215700	N/A	N/A	N/A	N/A
Melanops sp. (*Botryosphaeria quercuum* in CBS)	CBS 118.39	FJ824763	DQ377856	FJ824771	FJ824776	FJ824782	N/A
Melanops tulasnei	CBS 116805	KF766282	KF766365	FJ824769	KF766423	FJ824780	KF766474
Neodeightonia palmicola	MFLUCC100822	HQ199223	HQ199222	HQ199221	N/A	N/A	N/A
Neodeightonia phoenicum	CBS 122528	KF766285	EU673261	KF766198	EU673309	EU673116	N/A
Neodeightonia sp.	MFLUCC110026	JX646837	JX646821	JX646804	JX646869	JX646852	N/A
Neodeightonia subglobosa	CBS 448.91	KF766286	DQ377866	KF766199	EU673306	EU673137	N/A
Neofusicoccum australe	CMW 6837	KF766287	KF766367	KF766200	AY339270	AY339254	KF766477
Neofusicoccum eucalypticola	CMW 6539, CBS 115679	KF766288	KF766368	KF766201	AY615133	AY615125	N/A
Neofusicoccum lutea	CMW 10309	KF766289	KF766369	KF766202	KF766424	DQ458848	N/A
Neofusicoccum mangiferum	CMW 7801	KF766290	KF766370	KF766203	KF766425	AY615174	KF766479
Neofusicoccum parvum	CMW 9081	KF766291	KF766371	KF766204	KF766426	AY236917	KF766480
Neofusicoccum ribis	CMW 7772, CBS 115475	KF766292	KF766372	KF766205	DQ677893	AY236906	KF766481
Neofusicoccum umdonicola	CMW 14058, CBS 123645	KF766293	KF766373	KF766206	KF766427	KF766145	KF766482
Neofusicoccum vitifusiforme	CMW 24571	KF766235	KF766320	KF766152	FJ752707	N/A	N/A
Neoscytalidium dimidiatum	IP127881	AF258603	DQ377925'	AY819727	EU144063	FM211167	N/A
Neoscytalidium novaehollandiae	CMW 26170, CBS 122071	KF766294	KF766374	KF766207	EF585580	N/A	N/A
Phaeobotryon cupressi	IRAN 1445C	KF766295	N/A	KF766208	N/A	N/A	N/A
Phaeobotryon mamane	CPC 12440	EU673184	EU673248	EU673332	EU673298	EU673121	KF766483
				KF766209			
	CBS 398.80	KF766301	KF766378	KF766213	KF766430	N/A	KF766486
Phaeobotryosphaeria citrigena (*Botryosphaeria fusca* in CBS)	ICMP 16812	EU673180	EU673246	EU673328	EU673294	EU673140	N/A
Phaeobotryosphaeria eucalypti	MFLUCC110579	JX646835	JX646819	JX646802	JX646867	JX646850	N/A
Phaeobotryosphaeria porosa	CBS 110496	KF766297	KF766375	KF766210	N/A	EU673130	N/A
Phaeobotryosphaeria visci	CBS 186.97	KF766298	KF766393	KF766211	EU673293	EU673128	N/A
Phyllosticta beaumarisii	CBS 535.87	KF766299	KF766376	KF766212	KF766429	N/A	KF766484
Phyllosticta capitalensis	CBS 226.77	KF766300	KF766377	FJ538336	FJ538394	N/A	KF766485
	CBS 398.80	N/A	N/A	N/A	N/A	N/A	N/A
Phyllosticta citriasiana	CBS 120486	KF766302	KF766379	FJ538360	FJ538418	N/A	N/A
Phyllosticta cornicola	CBS 111639	N/A	KF766380	KF766214	KF766431	N/A	KF766487
Phyllosticta hypoglossi	CBS 101.72	KF766303	KF766381	FJ538365	FJ538423	N/A	N/A
Phyllosticta minima	CBS 585.84	N/A	KF766382	KF766216	KF766433	N/A	N/A
Phyllosticta minima (= *Phyllosticta rubrum*)	CBS 111635	N/A	EU754194	KF766215	KF766432	N/A	N/A
Phyllosticta podocarpi	CBS 111647	KF766304	KF766383	KF766217	KF766434	N/A	N/A
Phyllosticta telopeae	CBS 777.97	N/A	KF766384	KF766218	KF766435	N/A	N/A
Phyllosticta yuccae	CBS 117136	KF766305	KF766385	KF766219	KF766436	N/A	N/A
Pseudofusicoccum adansoniae	CMW 26147, CBS 122055	KF766306	KF766386	KF766220	EF585571	N/A	KF766488
Pseudofusicoccum ardesiacum	CMW 26159, CBS 122062	KF766307	KF766387	KF766221	EU144075	N/A	KF766489
Pseudofusicoccum kimberleyense	CMW 26156, CBS 122058	KF766308	KF766388	KF766222	EU144072	N/A	KF766490
Pseudofusicoccum stromaticum	CMW 13434, CBS117448	KF766309	KF766389	KF766223	KF766437	EU673094	N/A
Saccharata capensis	CMW 22200, CBS 122693	N/A	KF766390	KF766224	EU552095	N/A	KF766491
Saccharata kirstenboschensis	CBS 123537	KF766310	FJ372409	KF766225	N/A	N/A	KF766492
Saccharata proteae	CBS 115206	KF766311	DQ377882	KF766226	KF766438	N/A	KF766493
Spencermartinsia pretoriensis	CMW 36480	N/A	JQ239418	JQ239405	JQ239392	JQ239376	N/A
	CMW 36481	N/A	JQ239419	JQ239406	JQ239393	JQ239377	N/A

Table 1. (Continued).

Species	Isolate No.[1]	GenBank Accession No.					
		SSU	LSU	ITS	EF1	BT	mtSSU
Spencermartinsia rosulata	CMW 25389, CBS 121760	KF766312	KF766391	KF766227	KF766439	N/A	KF766494
Spencermartinsia sp. (*Botryosphaeria* sp. in ICMP)	ICMP 16827	EU673171	EU673241	EU673322	EU673289	EU673144	N/A
Spencermartinsia sp. (*Diplodia medicaginis* in CBS)	CBS 500.72	EU673167	EU673237	EU673318	EU673285	EU673118	N/A
Spencermartinsia sp. (*Diplodia spegazziniana* in CBS)	CBS 302.75	N/A	EU673238	EU673319	EU673286	EU673135	N/A
Spencermartinsia vitícola	CBS 117009	KF766313	KF766392	KF766228	AY905559	EU673104	N/A
Tiarosporella urbis-rosarum	CMW 36478	N/A	JQ239421	JQ239408	JQ239395	JQ239382	N/A
	CMW 36479	N/A	JQ239422	JQ239409	JQ239396	JQ239383	N/A

[1]CBS: CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands; CMW: Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; IRAN: Iranian Fungal Culture Collection, Iranian Research Institute of Plant Protection, Iran; MFLUCC: Mae Fah Luang University Culture Collection, Chiang Mai, Thailand; MUCC: Culture Collection, Laboratory of Plant Pathology, Mie University, Tsu, Mie prefecture, Japan.

Table 2. Test of phylogenetic signal for each of the 5 traits investigated using Parsimony and Maximum Likelihood reconstructions.

Phylogenetic Signal	Parsimony (number of steps)			Maximum Likelihood (-log likelihood values)		
	Observed	Expected Mean (Range)	P-value	Observed	Expected Mean (Range)	P-value
Ascospore colour	3	5,89 (3-7)	ns	11,35	12,87 (10,6-13,7)	ns
Ascospore septation	4	5,29 (3-6)	ns	11,7	12,79 (8,9-13,7)	ns
Conidial colour	6	8,99 (5-13)	ns	15,51	17,76 (15,1-18,4)	ns
Conidial septation	8	8,90 (5-12)	ns	18,02	17,77 (15,8-18,6)	ns
Mucus	4	3,98 (2-4)	ns	13,59	14,09 (8,0-18,1)	ns

Analysis of phylogeny-trait association

None of the five morphological traits that were investigated had a significant phylogenetic signal (Table 2; Fig. 2A–E).

Taxonomy

Based on the phylogenetic distinctions found in this study, as well as the morphological and in some cases ecological distinction between the major groups in the *Botryosphaeriales*, six families are recognised. Of these, the *Planistromellaceae* (accommodating *Kellermania*) and *Phyllostictaceae* (accommodating *Phyllosticta*) are accepted as previously described (Minnis *et al.* 2012, Wikee *et al.* 2013b, this volume). The *Botryosphaeriaceae* is redefined, while the *Aplosporellaceae*, *Saccharataceae* and *Melanopsaceae* are newly described.

Botryosphaeriaceae Theis. & P. Syd., Ann. Mycol. 16: 16. 1918.

Type genus: *Botryosphaeria* Ces. & De Not., Comment. Soc. Crittog. Ital. 1: 211. 1863.

Type species: *B. dothidea* (Moug. : Fr.) Ces. & De Not., Comment. Soc. Crittog. Ital. 1: 212. 1863.

Genera included based on support by DNA sequence data: *Barriopsis, Botryobambusa, Botryosphaeria* (= *Fusicocccum*, incl. *Dichomera pro parte*), *Cophinforma, Dothiorella, Diplodia, Endomelanconiopsis, Lasiodiplodia* (incl. *Auerswaldia, Macrovalsaria*), *Macrophomina, Neodeightonia, Neofusicoccum* (incl. *Dichomera pro parte*), *Neoscytalidium, Phaeobotryon, Phaeobotryosphaeria, Pseudofusicoccum, Spencermartinsia, Tiarosporella.*

Genera lacking DNA sequence data: *Auerswaldiella, Leptoguignardia, Microdiplodia, Phyllachorella, Pyrenostigma, Septorioides, Sivanesania, Thyrostroma, Vestergrenia* (Liu *et al*. 2012).

Ascostromata uni- to multilocular, solitary or in clusters, fully or partially erumpent at maturity, with multi-layered, dark brown walls, infrequently embedded in stromatic tissue. *Asci* bitunicate, fissitunicate, chiefly 8-spored, with a thick endotunica and well-developed apical chamber, short stipitate, clavate. *Pseudoparaphyses* intermixed with asci, hyaline, septate, frequently constricted at septa, hyphae-like, branched or not, frequently deliquescing at maturity. *Ascospores* 2–3 seriate, hyaline to pigmented, smooth to verruculose, septate or not, fusoid to ellipsoid or ovoid, with or without a mucoid sheath or rarely with appendages. *Asexual morphs* mostly have uni-, rarely multilocular pycnidial *conidiomata*, infrequently embedded in stromatic tissue. *Conidiophores* mostly reduced to conidiogenous cells. *Conidiogenous cells* hyaline, phialidic, proliferating percurrently or via periclinal thickening, with or without collarettes. *Conidia* hyaline to pigmented, aseptate, one or multi-septate, sometimes muriform, smooth or striate, thin to thick-walled, and sometimes with mucoid sheaths or appendages. *Synasexual morphs* coelomycetous or hyphomycetous (see Crous *et al*. 2006). *Spermatogonia* similar to conidiomata in anatomy. *Spermatogenous cells* ampulliform to lageniform or subcylindrical, hyaline smooth, phialidic. *Spermatia* developing in conidiomata or spermatogonia, hyaline, smooth, granular, subcylindrical or dumbbell-shaped, with rounded ends.

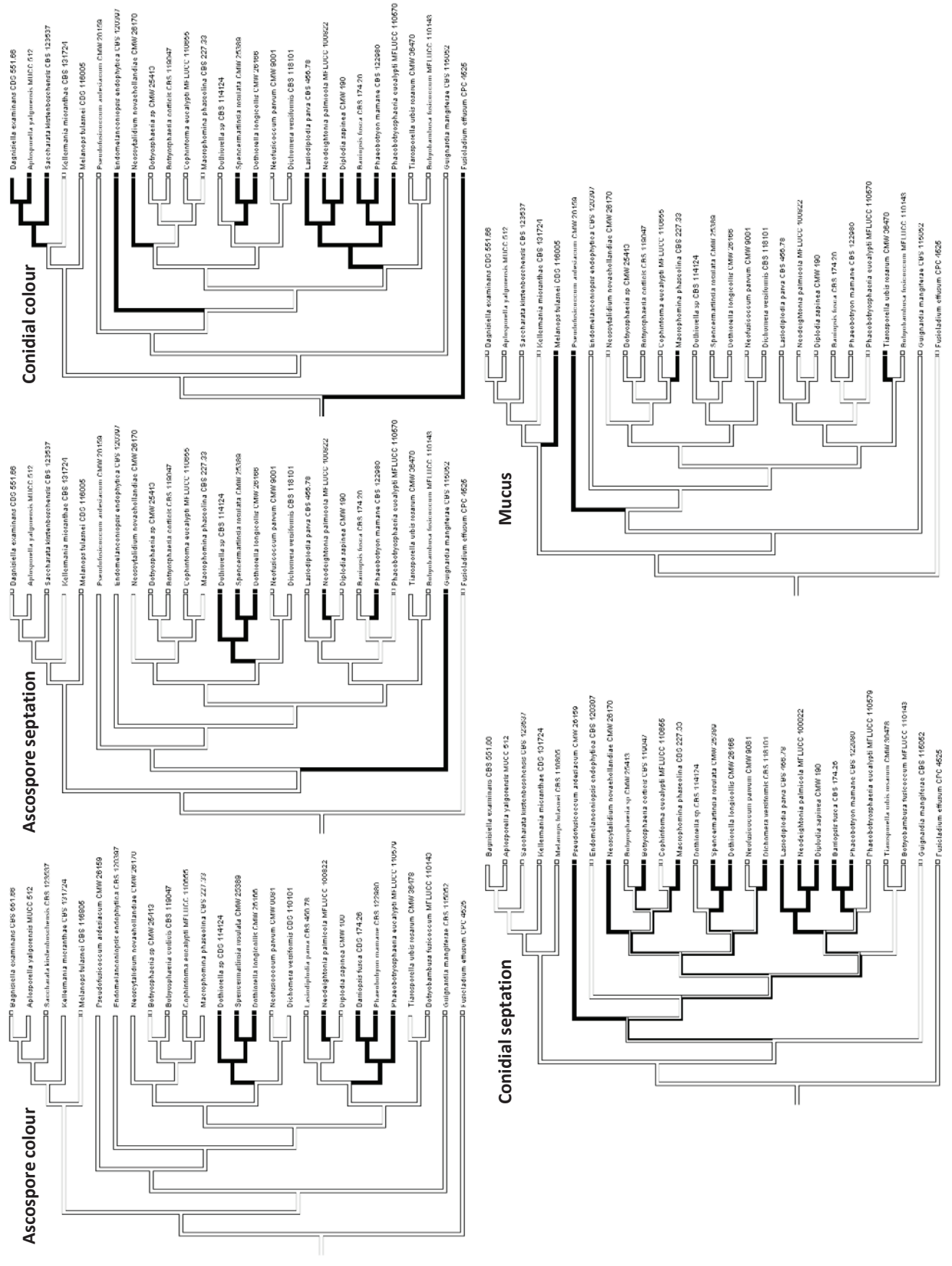

Fig. 2. Ancestral state reconstruction using parsimony and mapping the five traits onto the phylogenetic tree. Traits are coded as presence (in black) or absence (in white).

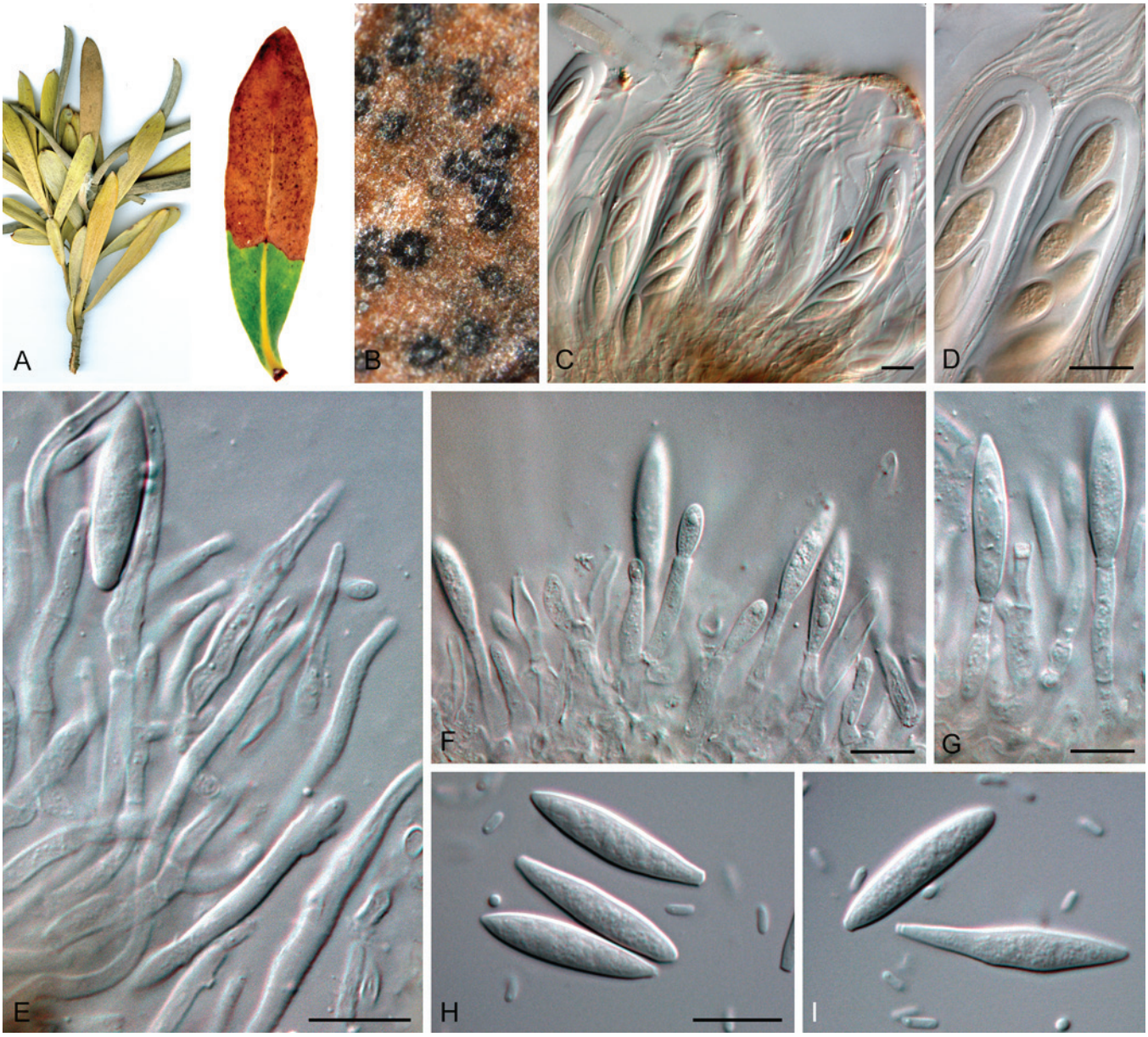

Fig. 3. *Saccharataceae* (*Saccharata proteae,* CBS 121406). A. Symptomatic leaves with tip die-back. B. Superficial view of immersed ascomata, showing clypeus-like structure. C, D. Asci and ascospores. E–G. Conidiogenous cells and paraphyses. H, I. Conidia and spermatia. Scale bars = 10 µm.

Saccharataceae Slippers, Boissin & Crous, **fam. nov.** MycoBank MB805794. Fig. 3.

Type genus: *Saccharata* Denman & Crous, In: Crous *et al.*, CBS Biodiversity Ser. (Utrecht) 2: 104. 2004.

Type species: *S. proteae* (Wakef.) Denman & Crous, In: Crous *et al.*, CBS Biodiversity Ser. (Utrecht) 2: 104. 2004.

Genus supported by DNA sequence data: *Saccharata.*

Ascomata pseudothecial, unilocular, solitary or in clusters, with multilayered dark brown walls, infrequently embedded in stromatic tissue, with upper ascomatal layer darkened and thickened. *Asci* bitunicate, fissitunicate, 8-spored, with a thick endotunica, stalked or sessile, clavate, with a well-developed apical chamber. *Pseudoparaphyses* intermixed with asci, hyaline, septate, hyphae-like, branched or not. *Ascospores* hyaline to pigmented, granular, septate or not, ellipsoid to ovoid, without mucoid appendages or sheath. *Asexual morph* has unilocular pycnidial conidiomata, infrequently embedded in stromatic tissue with thickened, darkened upper layer. *Conidiophores* sparingly branched, hyaline, subcylindrical, or reduced to conidiogenous cells. *Conidiogenous cells* hyaline, smooth, phialidic, proliferating via periclinal thickening or percurrent proliferation, with or without collarettes. *Conidia* hyaline, thin-walled, granular, fusoid, aseptate. *Synasexual morph* formed in separate conidiomata, or in same conidiomata with asexual morph. *Synasexual conidia* pigmented, thick-walled, finely verruculose, ellipsoid or oval, aseptate. *Spermatogonia* similar to conidiomata in anatomy. *Spermatogenous cells* ampulliform to lageniform or subcylindrical, hyaline smooth, phialidic. *Spermatia* developing in conidiomata or spermatogonia, hyaline, smooth, granular, subcylindrical or dumbbell-shaped, with rounded ends.

Aplosporellaceae Slippers, Boissin & Crous, **fam. nov.** MycoBank MB805795. Fig. 4.

Type genus: *Aplosporella* Speg., Anal. Soc. cient. argent. 10(5–6): 158. 1880.

Type species: *A. chlorostroma* Speg., Anal. Soc. cient. argent. 10(5–6): 158. 1880.

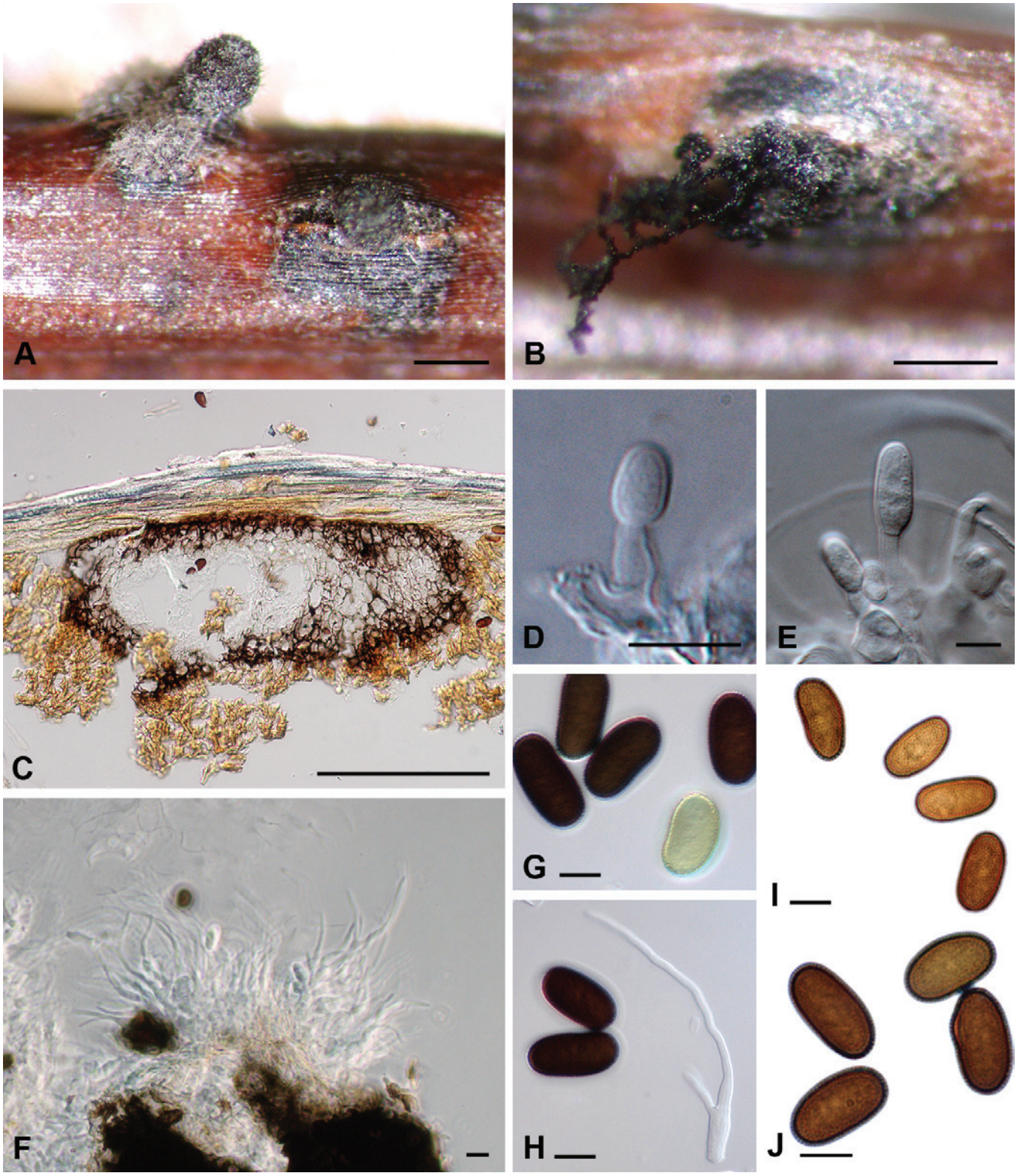

Fig. 4. *Aplosporellaceae* (*Aplosporella prunicola,* CBS 121167). A, B. Oozing spore masses from submerged conidiomata. C. Transverse section through multilocular conidioma. D, E. Conidiogenous cells. F. Paraphyses. H. Conidia and branched paraphyses. G–J. Conidia. Scale bars: A–C = 250 µm, F = 20 µm, D, E, G–J = 10 µm (adapted from Damm *et al.* 2007).

Genera supported by DNA sequence data: *Aplosporella*, *Bagnisiella.*

Ascomata pseudothecial, mostly multilocular with multilayered dark brown walls, embedded in stromatic tissue. *Asci* bitunicate, with a thick endotunica, stalked or sessile, clavate, with a well-developed apical chamber, intermixed with hyaline, septate, hyphal-like pseudoparaphyses, branched or not. *Ascospores* hyaline to pigmented, septate or not, ellipsoid to ovoid, without mucoid appendages or sheath. *Asexual morphs* with uni- to multilocular pycnidial conidiomata, embedded in stromatic tissue. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* hyaline, phialidic, proliferating percurrently or with periclinal thickening at apex. *Paraphyses* present or absent, hyaline, smooth-walled, septate, branched or not, hyphae-like. *Conidia* ellipsoid to subcylindrical, initially hyaline becoming pigmented, aseptate, thin-walled and smooth, becoming thick-walled and spinulose.

Melanopsaceae Phillips, Slippers, Boissin & Crous, **fam. nov.** MycoBank MB805796. Figs 5, 6.
Type genus: *Melanops* Nitschke ex Fuckel, In: Fuckel, Jahrb. Nassau. Ver. Naturk. 23–24: 225. 1870.
Type species: *Melanops tulasnei* Nitschke, In: Fuckel, Jahrb. Nassau. Ver. Naturk. 23–24: 225. 1870.

Genus supported by DNA sequence data: *Melanops* (see Phillips & Alves 2009).

Ascomata pseudothecial, multiloculate, immersed, partially erumpent at maturity, black, subglobose, thick-walled; wall composed of thick-walled *textura angularis*. *Asci* 8-spored, bitunicate, fissitunicate, stipitate, clavate. *Pseudoparaphyses* hyaline, thin-walled, hyphal-like, septate. *Ascospores* hyaline, aseptate, thin-walled, ellipsoid to rhomboid, with a persistent mucus sheath. *Conidiomata* indistinguishable from ascomata and often formed in the same stroma. *Paraphyses* hyaline, septate, branched or not, filiform, arising from between the conidiogenous cells. *Conidiophores* hyaline, smooth, 1–2-septate, branched or not, or reduced to conidiogenous cells. *Conidiogenous cells* subcylindrical, hyaline, branched or unbranched, discrete, formed from the inner wall of the conidioma, proliferating percurrently at apex, or with periclinal thickening. *Conidia* hyaline, aseptate, fusoid, with a persistent mucus sheath, rarely with minute marginal frill.

Phyllostictaceae Fr. (as "Phyllostictei"), Summa veg. Scand., Section Post. (Stockholm): 420. 1849.
Type genus: *Phyllosticta* Pers., Traité sur les Champignons Comestibles (Paris): 55. 147. 1818.
Type species: *P. convallariae* Pers., Traité sur les Champignons Comestibles (Paris): 148. 1818.

Genus supported by DNA sequence data: *Phyllosticta* (see Wikee *et al*. 2013b, this volume).

Planistromellaceae M.E. Barr, Mycotaxon 60: 433. 1996. Fig. 7.
Type genus: *Planistromella* A.W. Ramaley, Mycotaxon 47: 260. 1993.
Type species: *P. yuccifoliorum* A.W. Ramaley, Mycotaxon 47: 261. 1993 (= *Kellermania yuccifoliorum*)

Genus supported by DNA sequence data: *Kellermania* (= *Alpakesa, Piptarthron, Planistroma, Planistromella, Septoplaca* (possibly), see Minnis *et al*. 2012).

Ascomata pseudothecial, multi- or uniloculate, immersed to erumpent, solitary to gregarious, with papillate, periphysate ostiole; walls of several layers of dark brown *textura angularis*. Hamathecium mostly lacking pseudoparaphyses at maturity. *Asci* 8-spored, bitunicate, fissitunicate, thick-walled, oblong to clavate or subcylindrical, stipitate, with well-developed ocular chamber. *Ascospores* 1–3-seriate, hyaline or pale brown, guttulate, ellipsoid to broadly obovoid, aseptate or with 1–2 transverse septa, thin-walled, with or without a gelatinous sheath. *Conidiomata* pycnidial to acervular, subepidermal, dark brown, immersed to semi-erumpent, solitary to gregarious; wall comprising several layers with cells of dark brown *textura angularis*, becoming hyaline towards the inner region. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* ampulliform to sub-cylindrical, hyaline, smooth, phialidic, proliferating via percurrent proliferation or periclinal thickening. *Conidia* obclavate to ellipsoid-cylindrical, aseptate or transversely multiseptate, hyaline to brown, smooth to verruculose, with or without one or more apical appendages, a persistent mucoid sheath, and a basal marginal frill. *Spermatogonia* similar to conidiomata in anatomy. *Spermatogenous cells* ampulliform to lageniform or subcylindrical, hyaline smooth, phialidic. *Spermatia* developing in conidiomata or spermatogonia, hyaline, smooth, granular, sub-cylindrical or dumbbell-shaped, with rounded ends.

DISCUSSION

Using the DNA sequence data for the six loci analysed in this study, together with unique morphological and ecological characteristics (as discussed below), we have distinguished six families in the *Botryosphaeriales*. The *Planistromellaceae* that has recently been defined based on DNA sequence data (Minnis *et al*. 2012) has been retained. Furthermore, the *Aplosporellaceae*, *Melanopsaceae*, and *Saccharataceae* are distinguished from the *Botryosphaeriaceae* and introduced as novel families. These families are also phylogenetically distinct from the *Phyllostictaceae*, which has been defined in a separate study (Wikee *et al*. 2013b, this volume).

Botryosphaeriaceae

The *Botryosphaeriaceae* as it has been defined in this study includes the type genus *Botryosphaeria* (asexual morph *Fusicoccum*), as well as 16 other genera. This group corresponds to a group traditionally referred to as botryosphaeria-like. This term is, however, now understood to be much more restricted, including *B. dothidea* and a few closely related species (as defined in Slippers *et al*. 2004, Crous *et al*. 2006, Phillips *et al*. 2013, this volume). Using *Botryosphaeria* to refer to the assemblage of genera including *Diplodia*, *Lasiodiplodia*, *Neofusicoccum* and others, of which the sexual morphs were formerly described in *Botryosphaeria*, is thus taxonomically incorrect. These groups are now referred to using a single genus name, which is typically the asexual morph, irrespective of whether a sexual morph is known or not. This convention has been applied subsequent to the taxonomic changes introduced by Crous *et al*. (2006), and is also consistent with the recent decisions to abolish the dual nomenclatural system for fungal taxonomy (Hawksworth *et al*. 2011, Wingfield *et al*. 2012).

The data presented in this study, together with those emerging from more focused earlier studies, as well as the recent changes resulting in the abandonment of a dual nomenclature, necessitates reducing a number of genera in the *Botryosphaeriaceae* to synonymy with others. Most importantly, there is no longer just cause to maintain *Botryosphaeria* and *Fusicoccum* as distinct genera. In the interests of maintaining taxonomic stability, and the fact that *B. dothidea* is the type species of the order and family, it is recommended that *Botryosphaeria* be retained (Slippers *et al*. 2004, Schoch *et al*. 2006, Phillips *et al*. 2013, this volume). *Botryosphaeria* must thus be redefined to include species where only the asexual (*Fusicoccum* and dichomera-like) morphs are known. Species such as *F. ramosum* and *Dichomera saubinetti* must then be redefined in *Botryosphaeria*. For *F. ramosum,* an ex-type isolate was available and it has thus been redescribed in *Botryosphaeria* in a companion paper (Phillips *et al*. 2013, this volume).

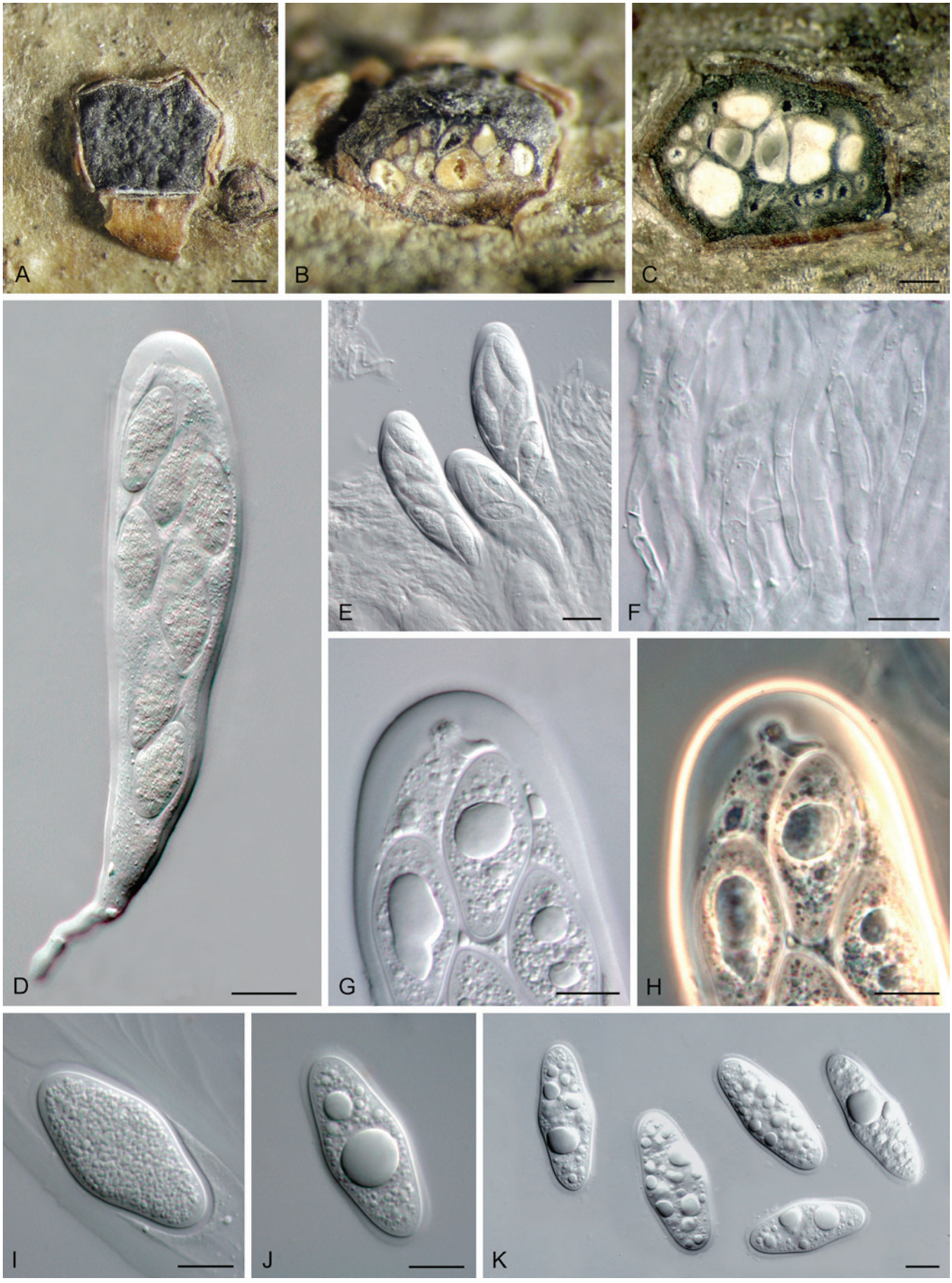

Fig. 5. *Melanopsaceae* (*Melanops tulasnei*, LISE 95179). A. Stroma erumpent through bark. B, C. Sections through stromata revealing ascomata and conidiomata. D. Ascus. E. Asci and pseudoparaphyses. F. Pseudoparaphyses. G, H. Ascus tips viewed under differential interference contrast (G) and phase contrast (H). I–K. Ascospores. Scale bars: A–C = 250 µm, D, E = 20 µm, F–K = 10 µm (adapted from Phillips & Alves 2009).

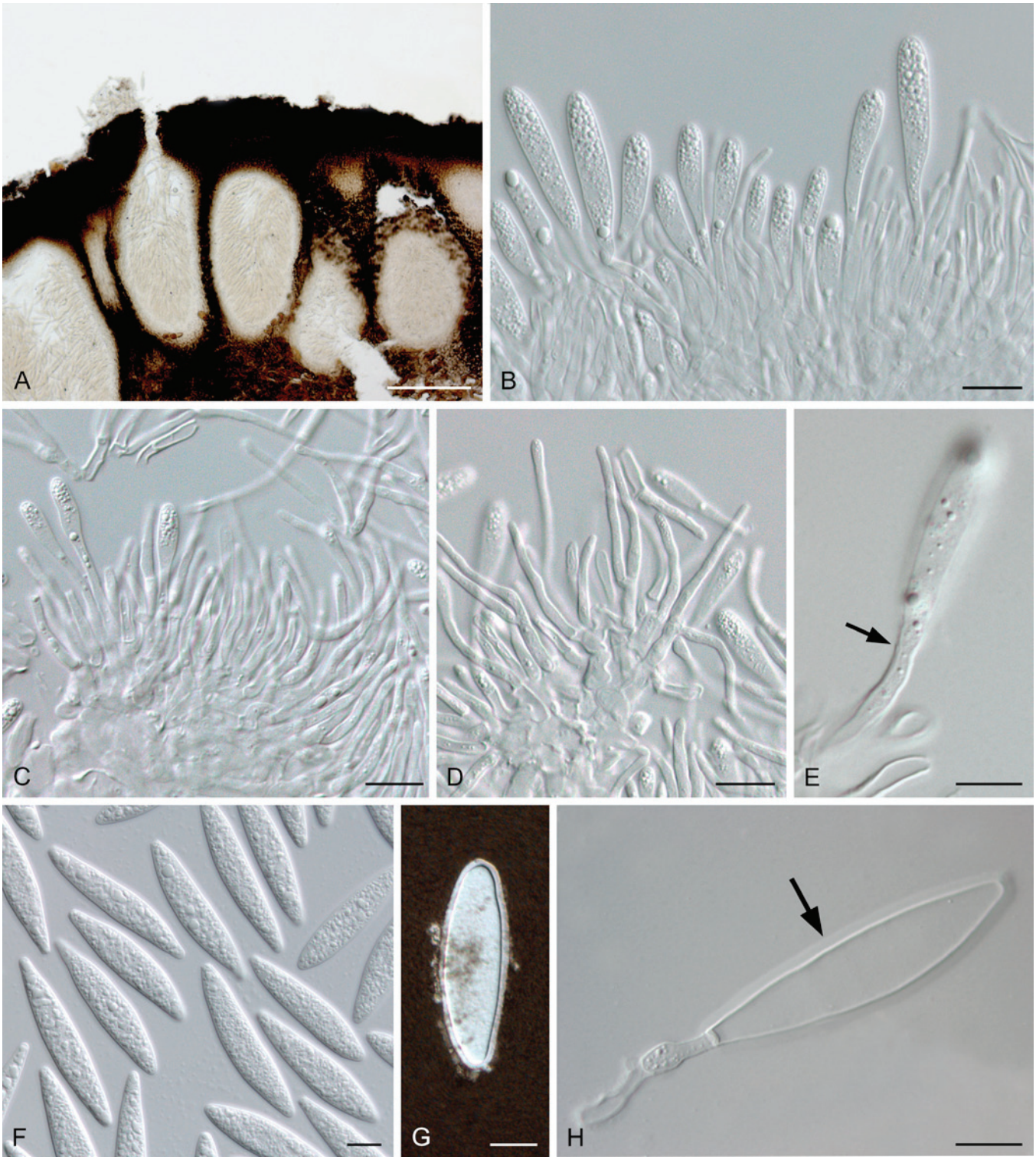

Fig. 6. *Melanopsaceae* (*Melanops tulasnei*, LISE 95179). A. Section through conidiomata. B. Conidiogenous layers with developing conidia among paraphyses. C. Immature conidiogenous cells. D. Paraphyses. E. Conidiogenous cell with percurrent proliferations (arrowed). F. Conidia. G. Conidium in indian ink, revealing sheath. H. Conidium attached to conidiogenous cell with mucus sheath (arrowed). Scale bars: A = 200 µm, B–D, F, G = 10 µm, E, H = 5 µm (adapted from Phillips & Alves 2009).

Dichomera is polyphyletic and most likely also includes synasexual morphs of other genera. Two of the species included in this analysis, *D. eucalypti* and *D. versiformis*, clearly group in *Neofusicoccum* and we consider them as synonyms of species in this genus. *Dichomera saubinetti* grouped with *Botryosphaeria* and should be redescribed in this genus. Unfortunately no ex-type isolates of these species are presently available.

A number of genera grouped in *Lasiodiplodia* s. lat. in our analyses and are possibly synonyms of this genus. *Macrovalsaria* (see Sivanesan 1975) clearly grouped amongst species of *Lasiodiplodia*. This was also pointed out by Liu *et al.* (2012), but they did not find the available LSU and SSU data sufficiently convincing to make taxonomic changes. Isolates of *Macrovalsaria* however, grouped extremely closely with *L. theobromae* and we view them as representing a synonym of *Lasiodiplodia*, rather than *Lasiodiplodia* being polyphyletic. *Lasiodiplodia* and *Macrovalsaria* are both tropical fungi. Our analyses differ from those of Liu *et al.* (2012), indicating that *Auerswaldia* is a synonym of *Lasiodiplodia*. This was also confirmed in Phillips *et al*. (2013, this volume), who redescribed *A. lignicola* as *L. lignicola*. These findings suggest that

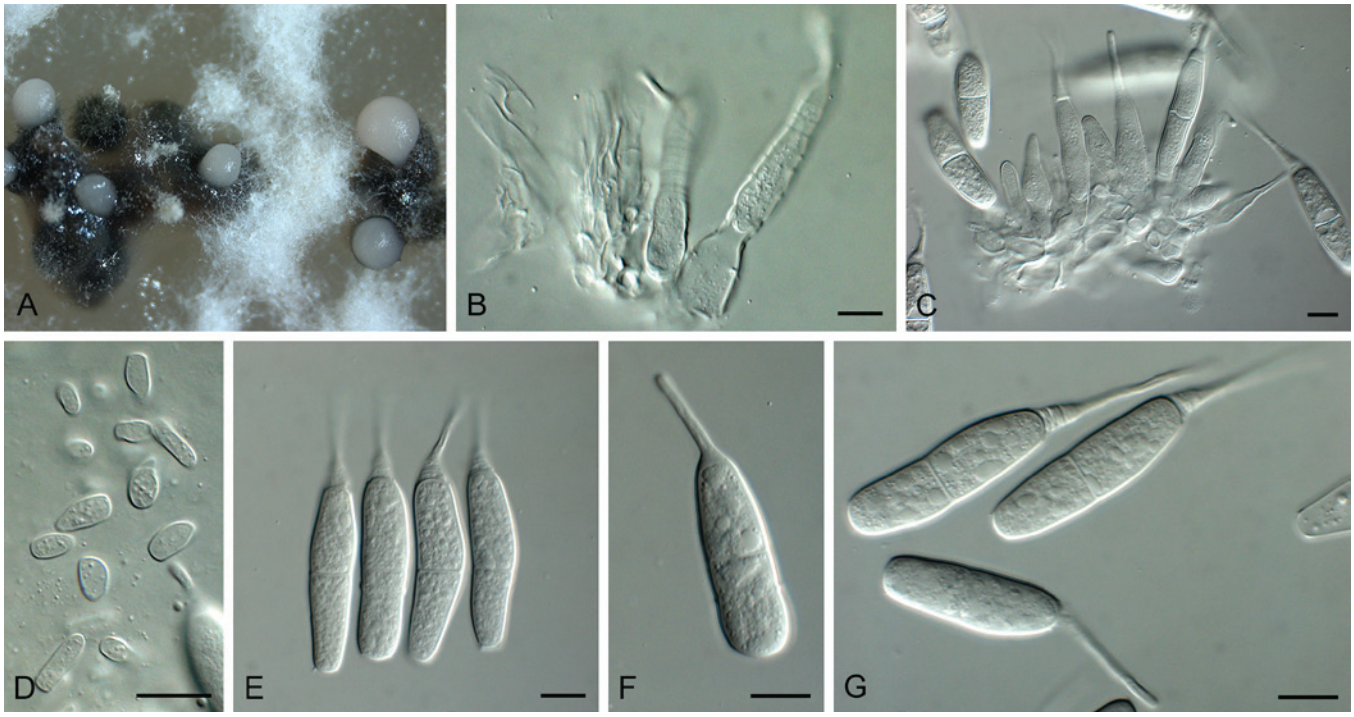

Fig. 7. *Planistromellaceae* (*Kellermania yuccigena*, CPC 20627). A. Conidiomata sporulating on OA. B, C. Conidiogenous cells showing percurrent proliferation. D. Spermatia. E–G. One-septate macroconidia with apical appendages. Scale bars = 10 µm.

the taxonomy of *Lasiodiplodia* needs to be re-evaluated, but for the present we do not recognize *Auerswaldia* as a genus in the Botryosphaeriaceae.

Diplodia juglandis and *D. corylii* both grouped in *Dothiorella*, which is consistent with the results of a previous study (Phillips *et al*. 2008). Type specimens were not available for these species and they were, therefore, not re-described. These species require epitypification. It is likely that a number of other *Diplodia* species will similarly reside in *Dothiorella* or *Spencermartinsia*, or *vice versa*, given the confusion of these names in the past (Phillips *et al*. 2005, 2008). The conidia of these genera remain difficult to distinguish, which can create problems when interpreting older descriptions or poorly preserved herbarium specimens.

There was no statistically significant pattern that could be discerned in the *Botryosphaeriaceae* with respect to the evolution of hyaline or pigmented conidia or ascospores. These characters appear to be more or less randomly spread amongst the clades of this section of the phylogenetic tree for the family. This would suggest that these characters predate the divergence of the genera in this family, and that they have been independently lost or suppressed (character not expressed under all conditions) in different groups. This would also explain the "appearance" of darker or even dark muriform conidia in genera such as *Botryosphaeria* and *Neofusicoccum* that were traditionally considered not to have such synasexual morphs (Barber *et al.* 2005, Phillips *et al*. 2005b, Crous *et al*. 2006). Clearly these characters, which have traditionally been commonly used for phylogenetic and taxonomic purposes, have very little phylogenetic and taxonomic value above the genus level.

The distinction and more narrow definition of the *Botryosphaeriaceae* is important when considering the economic and ecological importance of this group. Many of the *Botryosphaeriaceae* species share a common ecology in being endophytic and latent pathogens in virtually all parts of woody plants (Slippers & Wingfield 2007). While not all species have been isolated as endophytes, or from all plant parts, most of those that have been carefully studied have conformed to this pattern, and it is thus expected for the group as a whole. Many of the genera in the family are also very widespread, with wide host ranges and broad levels of environmental tolerance (e.g. *N. parvum*, *N. australe*, *B. dothidea*, *L. theobromae*, *L. pseudotheobromae*). Sakalidis *et al*. (2013) for example reported *N. parvum* from 90 hosts in 29 countries on six continents. This broad ecological range, together with their cryptic nature as endophytes, makes these fungi important to consider as a group prone to being spread with living plant material. Ample evidence exists that these fungi can infect both native and non-native trees, once they have been introduced into a region. The observed (Dakin *et al*. 2010, Piškur *et al*. 2011) and expected (Desprez-Loustau *et al*. 2006) increase of the importance of this group due to pressure on plant communities as a result of climate change provides another reason to focus future efforts on characterising the diversity, distribution and pathogenicity of this group of fungi.

Aplosporellaceae

An unexpected outcome of this study was the consistent connection between *Aplosporella* and *Bagnisiella* and their distinction from other members of the *Botryosphaeriales*. While it has been suggested that some *Aplosporella* spp. might be asexual morphs of *Bagnisiella*, this connection has never been proven. Neither of these genera were treated in molecular phylogenetic re-evaluations of the *Botryosphaeriaceae* until very recently. The first analyses to include DNA sequence data for *Aplosporella* (Damm *et al*. 2007, Liu *et al*. 2012) and *Bagnisiella* (Schoch *et al*. 2009) hinted at a distant relationship with other *Botryosphaeriales*. However, none of these studies included both genera. The phylogenetic relationship between these genera revealed in this study is further supported by their remarkably similar multiloculate sporocarps, expressed in both the asexual morphs and sexual morphs, which is thus

not the product of parallel evolution in two distinct groups. There are, however, undoubtedly many species of *Aplosporella* and *Bagnisiella* that would not be connected to the phylogenetic clade identified here, because both genera are heterogeneous and likely contain unrelated species.

The data presented in this study suggest that *Aplosporella* and *Bagnisiella* are not only related, but that they should be synonymized. Both genera were described in 1880, and historical precedence can thus not be used to choose an appropriate genus. *Aplosporella* includes many more species (352) than *Bagnisiella* (65) (www.MycoBank.org, accessed August 2013). More species have also recently been described in the former genus, possibly because the asexual structures are more common than the sexual structures (as is found in other *Botryosphaeriales*). An argument based on taxonomic stability, relevance and frequency of occurrence is thus favoured and has led us to decide that *Bagnisiella* should be reduced to synonymy with *Aplosporella*.

Examination of the distribution of species of *Aplosporella* and *Bagnisiella* is complicated by the fact that the literature is old (which means the taxonomic accuracy is difficult to judge) and commonly lacking relevant information. However, most of the well-known and recently characterised species, and all species included here, are from the Southern Hemisphere (Damn *et al*. 2007, Taylor *et al*. 2009). This result suggests the possibility of a Southern Hemisphere and Gondwanan origin and divergence pattern, which should be considered in future studies based on more robust sampling.

Melanopsaceae

Melanops tulasnei and an undescribed *Melanops* sp. grouped most basal in the *Botryosphaeriales*, together with the *Aplosporellaceae*, *Planistromellaceae* and *Saccharataceae*. This group is unique amongst these families in having persistent mucous sheath around its ascospores and conidia (Phillips & Alves 2009). Conidia in *M. tulasnei* are typically hyaline and fusoid and it resembles the *Aplosporellaceae* in having multiloculate ascomata and conidiomata, often with locules at different levels. Little is known regarding the ecology and distribution of this group, given the paucity of recent reports that could be verified using DNA sequence data. It appears similar, however, to other *Botryosphaeriales* that infect woody tissue of plants, and sporulates on the dead tissue. Whether it is pathogenic or endophytic is not known.

Saccharataceae

Saccharata (the only genus in the *Saccharataceae*) grouped separately from all other families that were basal in the phylogenetic tree, suggesting a long, separate evolutionary history. The genus was first described by Crous *et al.* (2004) from *Proteaceae* in the South Western Cape region of South Africa. Subsequently, three additional species were added to the genus, two also from *Proteaceae* and one from *Encephalartos* (Marincowitz *et al*. 2008, Crous *et al*. 2008, 2009). All known species are thus from the same region on indigenous flora. These species are typically associated with leaf spots and stem cankers and they appear to be pathogens. Separate studies have also shown that they are endophytes (Swart *et al*. 2000, Taylor *et al.* 2001), similar to members of the *Botryosphaeriaceae*.

Apart from its restricted distribution and host range, *Saccharata* is also unique in its asexual morphology, which includes a hyaline, fusicoccum-like and a pigmented diplodia-like asexual morph. These characters are shared with its related families; fusicoccum-like conidia in *Melanopsaceae* and pigmented diplodia-like conidia in *Aplosporellaceae*. It is clear that these variations in conidial morphology are very old (tens of millions of years) ancestral characters that must have existed prior to the divergence of this group from other *Botryosphaeriales*. It is thus remarkable how similar, especially in the fusicoccum-like conidia and ascospore morphology, the spores of these fungi have remained over time. The diplodia-like state is somewhat different from other *Botryosphaeriales* in that the conidia are typically almost half the size of other *Diplodia* conidia. We do not currently have enough data to address the selective pressure that could have played a role in the development these interesting morphological changes.

Phyllostictaceae and Planistromellaceae

Phyllosticta clearly warrants a separate family to accommodate this morphologically and ecologically unique, widespread and economically important genus in the *Phyllostictaceae*. These fungi typically infect leaves and fruit, rather than woody tissue, and they can cause serious damage (Glienke *et al.* 2011, Wong *et al*. 2012). Species of *Phyllosticta* are also known to have an endophytic phase (Wikee *et al.* 2013a), as is true for most other *Botryosphaeriales*. The *Phyllostictaceae* is also morphologically unique in terms of the ascospores and conidia (Van der Aa & Vanev 2002). The species included in this study are only representative of a small extent of the diversity in this group and a more complete analysis of the *Phyllostictaceae* is presented in Wikee *et al*. (2013b, this volume).

The *Planistromellaceae* has previously been recognised as distinct within the *Botryosphaeriales* (Minnis *et al*. 2012) and this is supported by the analyses in the present study. The family is currently considered to include only species of *Kellermania*. Species in the *Planistromellaceae* have unique conidia with fairly long appendages that are quite distinct from other genera in the *Botryosphaeriales*. *Kellermania* spp. are mostly leaf infecting, and one species has been associated with Yucca Leaf Blight in California and Florida in the USA (Horst 2008). Species are commonly collected sporulating on dead leaves of *Agavaceae*, and appear to be endophytic, as they have been isolated from healthy leaves in many countries where *Yucca* spp. are grown as ornamentals (P.W. Crous, unpubl data). Minnis *et al*. (2012) have also shown apparent patterns of host specificity and co-evolution with major plant lineages. As is true for many other families in the *Botryosphaeriales*, additional sampling is needed for a better understanding of species diversity, host range and geographic distribution.

Molecular dating

The molecular dating on the radiations within the *Botryosphaeriales* in this study, based on general estimated mutation rates of the rDNA SSU locus by Taylor & Berbee (2010), must be viewed as preliminary. From the dating that was conducted, the group appears to have originated in the Cretaceous period around 103 (45–188) mya, with most of the subsequent diversification within the families occurring in the Tertiary period. This date is well within the estimated date of the emergence of the *Dothideomycetes* (Berbee & Taylor 2010, Gueidan *et al*. 2011). This coincides with important periods of Angiosperm radiation and spread, which is the main group of plants on which these fungi are found and that might be expected to have influenced the diversification of these fungi. Of

particular relevance is the rapid diversification of the Eurosid and other dominant woody Angiosperm groups and their prominence in Angiosperm dominated forests from around 110 mya onwards (Soltis *et al.* 2008, Fawcett *et al.* 2009, Wang *et al*. 2009, Bell *et al*. 2010). De Wet *et al.* (2008) pointed out that the members of the *Botryosphaeriaceae* are most diverse on Angiosperms, and showed that ancestral state reconstruction suggests that this is the main group of plants on which the *Botryosphaeriaceae* co-evolved. A much smaller number of species, especially those in *Diplodia*, occur commonly on coniferous hosts and appear to have emerged and diversified more recently. They also tend to be more host-specific than some of the other genera discussed above that are more common on Angiosperms (De Wet *et al*. 2008, Sakalidis *et al*. 2013), suggesting a specific acquired trait that allowed them to infect coniferous hosts.

The major changes in dominant plant hosts in forests globally during the Cretaceous period would be expected to have influenced fungal evolution beyond the Botryosphaeriales, and this indeed appears to be the case. For example, studies on another *Dothideomycetes* order, the sooty molds in the *Capnodiales*, also appear to have been influenced by the rise of Angiosperm forests in the Cretaceous period (Schmidt *et al*. 2013). Furthermore, the divergence of a prominent fungal complex, *Fusarium*, was estimated to be later at around 93 mya, and is also thought to have been influence by this Angiosperm divergence (O'Donnell *et al*. 2013).

Molecular dating, together with the geographic distribution (and restriction) of different groups in the *Botryosphaeriales* should provide rich information to explore in future to understand the patterns that have shaped the diversity of this important group of plant-associated fungi. For example, the *Saccharataceae* has previously been known only from southern Africa, and is most diverse on the *Proteaceae*. Recent research has shown, however, that it has also been introduced as endophyte into other countries where South African *Proteaceae* are now being cultivated (Marincowitz *et al*. 2008). It is known that this plant family, which has a high endemic richness in southern Africa, has evolved in the region for more than 100 million years (Barker *et al.* 2007). This date allows for the estimated 57 (28–100) mya (based on rDNA SSU) of separation of the *Saccharataceae* to have evolved with these endemic plant hosts in the region.

Diversification within the most diverse family, the *Botryosphaeriaceae*, occurred between 52–65 (27–112) mya for the two earliest diverging (and least diverse) genera, *Pseudofusicoccum* and *Endomelanconiopsis*. These dates correspond to the diversification between some other families in the order [e.g. the *Aplosporellaceae*, *Melanopsaceae*, *Planistromellaceae* and *Saccharataceae* that split between 57–75 (28–136) mya]. At present, however, there does not appear sufficiently robust morphological or ecological validation for a further split of the *Botryosphaeriaceae* to accommodate *Pseudofusicoccum* and *Endomelanconiopsis* in distinct families. If these genera were to be considered as residing in distinct families, the question would arise as to whether further family level distinction in the *Botryosphaeriaceae* is necessary. The other major lineages within the *Botryosphaeriaceae* (clades 1–4) appeared around 11–35 (3–58) mya. The most recent diversification for which there was support was within *Neofusicoccum* clade, dated at around 11 (3–23) mya.

This study has provided a systematic framework for future taxonomic and ecological studies of the *Botryosphaeriales*. It has also highlighted a number of interesting host association and geographic patterns amongst the genera that are worthy of further investigation. It is hoped that this framework, in conjunction with the growing body of DNA–based sequence data reflecting the species diversity and their distribution will ultimately lead to a model supporting an improved understanding of the co-evolution of woody plants and their fungal endophytes/latent pathogens.

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