Taxonomy, phylogeny, and epitypification of *Melanops tulasnei*, the type species of *Melanops*

Phillips, A.J.L.[1*] and Alves, A.[2]

[1]Centro de Recursos Microbiológicos, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal
[2]CESAM and Departamento de Biologia, Universidade de Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

Phillips, A.J.L. and Alves, A. (2009). Taxonomy, phylogeny, and epitypification of *Melanops tulasnei*, the type species of *Melanops*. Fungal Diversity 38: 155-166.

Melanops tulasnei was collected from dead twigs of *Quercus robur* in Germany and its identity was confirmed by comparing morphological features with the original description and with the neotype. A multi-gene phylogeny based on a portion of the 18S nuclear ribosomal gene, the nuclear rRNA cluster comprising the ITS region plus the D1/D2 variable domains of the LSU gene, together with the translation elongation factor 1-α gene and part of the β-tubulin gene was constructed. In this phylogeny, *M. tulasnei* clustered with an isolate of "*Botryosphaeria*" *quercuum* near the root of the *Botryosphaeriaceae*. On account of the morphological and phylogenetic distinctions from other genera in the *Botryosphaeriaceae*, it is recommended that the genus *Melanops* should be reinstated. An epitype specimen of *M. tulasnei* was selected and ex-epitype cultures have been deposited in the public collection of CBS.

Key words: *Botryosphaeriales*, *Botryosphaeriaceae*, *Botryosphaeria*, taxonomy, systematics, phylogeny

Article Information
Received 5 April 2009
Accepted 18 May 2009
Published online 1 October 2009
*Corresponding author: Alan Phillips; e-mail: alp@fct.unl.pt

Introduction

The genus *Melanops* (Fuckel, 1870) was introduced to accommodate *Melanops tulasnei*, the present type species of the genus, and *M. mirabilis*. The taxonomy of *M. tulasnei* has been explained by Phillips and Pennycook (2004). Briefly, *Dothidia melanops* was described by Tulasne (1856), and later transferred to *Melanops* as *M. tulasnei* (Fuckel, 1870). Winter (1887) considered that *D. melanops* would be better accommodated in *Botryosphaeria* and made the new combination *B. melanops* (Tul.) G. Winter. Subsequently, Arx and Müller (1954) included *B. melanops* under their broad concept of *B. quercuum*. Phillips and Pennycook (2004) accepted that this species belongs in *Botryosphaeria* but suggested that the correct name is *B. melanops*. Since the holotype could not be traced, Phillips and Pennycook (2004) designated a specimen in PAD as neotype. However, in the absence of cultures, the phylogenetic position of this species could not be established.

The taxonomy of the genus *Botryosphaeria* has recenly undergone a major revision. In a study of partial sequences of the 28S rDNA gene, Crous *et al*. (2006) showed that *Botryosphaeria sensu lato* is composed of ten phylogenetic lineages that correspond to individual genera, and that *Botryosphaeria* is restricted to *B. dothidea* and *B. corticis*. In their phylogeny a culture of "*B.*" *quercuum* (CBS 118.39) formed a lineage near the root of the *Botryosphaeriaceae*.

We isolated a *Botryosphaeria*-like species with characteristics of "*B.*" *melanops*, from dead branches of *Quercus robur* collected in Munich, Germany. The purpose of the work presented here was to characterise the fungus, confirm its identity as *B. melanops*, determine its correct taxonomy and phylogeny within the *Botryosphaeriaceae*, and to select a suitable epitype.

Materials and methods

Isolates

Dead twigs of *Quercus robur* bearing ascomata and conidiomata were collected from the English Garden, Munich, Germany in July 2004. To isolate the fungus, conidia and ascospores were spread over the surface of plates of Difco potato dextrose agar (PDA) and incubated at 25°C. Individual, germinating spores were transferred to fresh plates of PDA and checked microscopically to ensure that a single spore had been transferred. Cultures were maintained on PDA slopes at 6°C. Representative isolates were deposited in the culture collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.

DNA extraction and sequencing

Genomic DNA was extracted from mycelium following the method of Alves *et al*. (2004). PCR reactions were carried out with *Taq* polymerase, nucleotides and buffers supplied by MBI Fermentas (Vilnius, Lithuania) and PCR reaction mixtures were prepared according to Alves *et al*. (2004), with the addition of 5 % DMSO to improve the amplification of some difficult DNA templates. All primers used were synthesised by MWG Biotech AG (Ebersberg, Germany).

A portion of the nuclear ribosomal SSU gene was amplified with primers NS1 and NS4 (White *et al*., 1990). The amplification conditions were as follows: initial denaturation of 5 min at 95 ºC, followed by 35 cycles of 45 s at 94 ºC, 45 s at 48 ºC, and 90 s at 72 ºC, and a final extension period of 10 min at 72 ºC. The nucleotide sequence of the SSU region was determined using the above primers along with the internal sequencing primers NS2 and NS3 (White *et al*., 1990).

Part of the nuclear rRNA cluster comprising the ITS region plus the D1/D2 variable domains of the ribosomal LSU gene was amplified using the primers ITS1 (White *et al*., 1990) and NL4 (O'Donnell, 1993) as described by Alves *et al*. (2005). Nucleotide sequences of the ITS and D1/D2 regions were determined as described previously (Alves *et al*., 2004; 2005) using the primers ITS4 (White *et al*., 1990) and NL1 (O'Donnell, 1993) as internal sequencing primers.

The primers EF1-688F (Alves *et al*., 2008) and EF1-986R (Carbone and Kohn, 1999) and Bt2a and Bt2b (Glass and Donaldson, 1995) were used to amplify and sequence part of the translation elongation factor 1-alpha (EF1-α) gene and part of the β-tubulin gene, respectively. Amplification and nucleotide sequencing of the EF1-α and β-tubulin genes was performed as described previously (Alves *et al*., 2006; 2008).

The amplified PCR fragments were purified with the JETQUICK PCR Purification Spin Kit (GENOMED, Löhne, Germany). Both strands of the PCR products were sequenced by STAB Vida Lda (Portugal). Sequences were read and edited with FinchTV 1.4.0 (Geospiza Inc. http://www.geospiza.com/finchtv). All sequences were checked manually and nucleotide arrangements at ambiguous positions were clarified using both primer direction sequences.

Phylogenetic analysis

Sequences were aligned with ClustalX v. 1.83 (Thompson *et al*,. 1997), using the following parameters: pairwise alignment parameters (gap opening = 10, gap extension = 0.1) and multiple alignment parameters (gap opening = 10, gap extension = 0.2, transition weight = 0.5, delay divergent sequences = 25 %). Alignments were checked and manual adjustments were made where necessary. Phylogenetic information contained in indels (gaps) was incorporated into the phylogenetic analyses using simple indel coding as implemented by GapCoder (Young and Healy, 2003).

Phylogenetic analyses of sequence data were done using PAUP v. 4.0b10 (Swofford, 2003) for maximum-parsimony (MP) analyses and Mr Bayes v. 3.0b4 (Ronquist and Huelsenbeck, 2003) for Bayesian analyses. Trees were visualised with TreeView (Page, 1996).

Maximum-parsimony analyses were performed using the heuristic search option with 1000 random taxa addition and tree bisection and reconnection (TBR) as the branch-swapping algorithm. All characters

Table 1. Isolates studied in this paper.

Species	Accession number	Host	Locality	GenBank				
				SSU	LSU	ITS	EF1-α	β-tubulin
'Botryosphaeria' tsugae	CBS 418.64	*Tsuga heterophylla*	Canada	EU673208	DQ377867	DQ458888	DQ458873	DQ458855
Barriopsis fusca	CBS 174.26	*Citrus* sp.	Cuba	EU673182	DQ377857	EU673330	EU673296	EU673109
Botryosphaeria corticis	CBS119047	*Vaccinium corymbosum*	USA	EU673175	EU673244	DQ299245	EU017539	EU673107
Botryosphaeria corticis	ATCC 22927	*Vaccinium* sp.	USA	EU673176	EU673245	DQ299247	EU673291	EU673108
Botryosphaeria dothidea	CBS 115476	*Prunus* sp.	Switzerland	EU673173	AY928047	AY236949	AY236898	AY236927
Botryosphaeria dothidea	CBS 110302	*Vitis vinifera*	Portugal	EU673174	EU673243	AY259092	AY573218	EU673106
Diplodia corticola	CBS 112549	*Quercus suber*	Portugal	EU673206	AY928051	AY259100	AY573227	DQ458853
Diplodia corticola	CBS 112546	*Quercus ilex*	Spain	EU673207	EU673262	AY259090	EU673310	EU673117
Diplodia cupressi	CBS 168.87	*Cupressus sempervirens*	Israel	EU673209	EU673263	DQ458893	DQ458878	DQ458861
Diplodia cupressi	CBS 261.85	*Cupressus sempervirens*	Israel	EU673210	EU673264	DQ458894	DQ458879	DQ458862
Diplodia mutila	CBS 112553	*Vitis vinifera*	Portugal	EU673213	AY928049	AY259093	AY573219	DQ458850
Diplodia mutila	CBS 230.30	*Phoenix dactylifera*	USA	EU673214	EU673265	DQ458886	DQ458869	DQ458849
Diplodia pinea A	CBS 393.84	*Pinus nigra*	Netherlands	EU673219	DQ377893	DQ458895	DQ458880	DQ458863
Diplodia pinea A	CBS 109727	*Pinus radiata*	South Africa	EU673220	EU673269	DQ458897	DQ458882	DQ458865
Diplodia pinea C	CBS 109725	*Pinus patula*	South Africa	EU673222	EU673270	DQ458896	DQ458881	DQ458864
Diplodia pinea C	CBS 109943	*Pinus patula*	Indonesia	EU673221	EU673271	DQ458898	DQ458883	DQ458866
Diplodia rosulata	CBS 116470	*Prunus africana*	Ethiopia	EU673211	DQ377896	EU430265	EU430267	EU673132
Diplodia rosulata	CBS 116472	*Prunus africana*	Ethiopia	EU673212	DQ377897	EU430266	EU430268	EU673131
Diplodia scrobiculata	CBS 113423	*Pinus greggii*	Mexico	EU673217	EU673267	DQ458900	DQ458885	DQ458868
Diplodia scrobiculata	CBS 109944	*Pinus greggii*	Mexico	EU673218	EU673268	DQ458899	DQ458884	DQ458867
Diplodia seriata	CBS 112555	*Vitis vinifera*	Portugal	EU673215	AY928050	AY259094	AY573220	DQ458856
Diplodia seriata	CBS 119049	*Vitis* sp.	Italy	EU673216	EU673266	DQ458889	DQ458874	DQ458857
Dothiorella iberica	CBS 115041	*Quercus ilex*	Spain	EU673155	AY928053	AY573202	AY573222	EU673096
Dothiorella iberica	CBS 113188	*Quercus suber*	Spain	EU673156	EU673230	AY573198	EU673278	EU673097
Dothiorella sarmentorum	IMI 63581b	*Ulmus* sp.	United Kingdom	EU673158	AY928052	AY573212	AY573235	EU673102
Dothiorella sarmentorum	CBS 115038	*Malus pumila*	Netherlands	EU673159	DQ377860	AY573206	AY573223	EU673101
Guignardia bidwellii	CBS 111645	*Parthenocissus quinquefolia*	USA	EU673223	DQ377876	*FJ824766*	*FJ824772*	*FJ824777*
Guignardia citricarpa	CBS 102374	*Citrus aurantium*	Brasil	*FJ824759*	DQ377877	*FJ824767*	FJ538371	*FJ824778*
Guignardia philoprina	CBS 447.68	*Taxus baccata*	Netherlands	*FJ824760*	DQ377878	*FJ824768*	*FJ824773*	*FJ824779*
Lasiodiplodia crassispora	CBS 110492	Unknown	Unknown	EU673189	EU673251	EF622086	EF622066	EU673134
Lasiodiplodia crassispora	CBS 118741	*Santalum album*	Australia	EU673190	DQ377901	DQ103550	EU673303	EU673133
Lasiodiplodia gonubiensis	CBS 115812	*Syzygium cordatum*	South Africa	EU673193	DQ377902	DQ458892	DQ458877	DQ458860
Lasiodiplodia gonubiensis	CBS 116355	*Syzygium cordatum*	South Africa	EU673194	EU673252	AY639594	DQ103567	EU673126
Lasiodiplodia parva	CBS 356.59	*Theobroma cacao*	Sri Lanka	EU673200	EU673257	EF622082	EF622062	EU673113
Lasiodiplodia parva	CBS 494.78	Cassava-field soil	Colombia	EU673201	EU673258	EF622084	EF622064	EU673114

Table 1 (continued). Isolates studied in this paper.

Species	Accession number	Host	Locality	GenBank				
				SSU	LSU	ITS	EF1-α	β-tubulin
Lasiodiplodia pseudotheobromae	CBS 116459	*Gmelina arborea*	Costa Rica	EU673199	EU673256	EF622077	EF622057	EU673111
Lasiodiplodia pseudotheobromae	CBS 447.62	*Citrus aurantium*	Suriname	EU673198	EU673255	EF622081	EF622060	EU673112
Lasiodiplodia rubropurpurea	CBS 118740	*Eucalyptus grandis*	Queensland	EU673191	DQ377903	DQ103553	EU673304	EU673136
Lasiodiplodia theobromae	CBS 124.13	Unknown	USA	EU673195	AY928054	DQ458890	DQ458875	DQ458858
Lasiodiplodia theobromae	CBS 164.96	Fruit along coral reef coast	New Guinea	EU673196	EU673253	AY640255	AY640258	EU673110
Lasiodiplodia theobromae	CAA 006	*Vitis vinifera*	USA	EU673197	EU673254	DQ458891	DQ458876	DQ458859
Lasiodiplodia venezuelensis	CBS 118739	*Acacia mangium*	Venezuela	EU673192	DQ377904	DQ103547	EU673305	EU673129
Melanops sp.	CBS 118.39	*Quercus borealis*	USA	*FJ824763*	DQ377856	*FJ824771*	*FJ824776*	*FJ824782*
Melanops tulasnei	CBS 116805	*Quercus robur*	Germany	*FJ824761*	*FJ824764*	*FJ824769*	*FJ824774*	*FJ824780*
Melanops tulasnei	CBS 116806	*Quercus robur*	Germany	*FJ824762*	*FJ824765*	*FJ824770*	*FJ824775*	*FJ824781*
Neodeightonia phoenicum	CBS 169.34	*Phoenix dactylifera*	USA	EU673203	EU673259	EU673338	EU673307	EU673138
Neodeightonia phoenicum	CBS 123168	*Phoenix canariensis*	Spain	EU673204	EU673260	EU673339	EU673308	EU673115
Neodeightonia phoenicum	CBS 122528	*Phoenix dactylifera*	Spain	EU673205	EU673261	EU673340	EU673309	EU673116
Neodeightonia subglobosa	CBS 448.91	keratomycosis in eye	United Kingdom	EU673202	DQ377866	EU673337	EU673306	EU673137
Neofusicoccum luteum	CBS 110299	*Vitis vinifera*	Portugal	EU673148	AY928043	AY259091	AY573217	DQ458848
Neofusicoccum luteum	CBS 110497	*Vitis vinifera*	Portugal	EU673149	EU673229	EU673311	EU673277	EU673092
Neofusicoccum mangiferae	CBS 118531	*Mangifera indica*	Australia	EU673153	DQ377920	AY615185	DQ093221	AY615172
Neofusicoccum mangiferae	CBS 118532	*Mangifera indica*	Australia	EU673154	DQ377921	AY615186	DQ093220	AY615173
Neofusicoccum parvum	CMW 9081	*Pinus nigra*	New Zealand	EU673151	AY928045	AY236943	AY236888	AY236917
Neofusicoccum parvum	CBS 110301	*Vitis vinifera*	Portugal	EU673150	AY928046	AY259098	AY573221	EU673095
Phaeobotryon mamane	CPC 12264	*Sophora chrysophylla*	Hawaii	EU673183	DQ377898	EU673331	EU673297	EU673125
Phaeobotryon mamane	CPC 12440	*Sophora chrysophylla*	Hawaii	EU673184	EU673248	EU673332	EU673298	EU673121
Phaeobotryon mamane	CPC 12442	*Sophora chrysophylla*	Hawaii	EU673185	DQ377899	EU673333	EU673299	EU673124
Phaeobotryon mamane	CPC 12443	*Sophora chrysophylla*	Hawaii	EU673186	EU673249	EU673334	EU673300	EU673120
Phaeobotryon mamane	CPC 12444	*Sophora chrysophylla*	Hawaii	EU673187	DQ377900	EU673335	EU673301	EU673123
Phaeobotryon mamane	CPC 12445	*Sophora chrysophylla*	Hawaii	EU673188	EU673250	EU673336	EU673302	EU673122
Phaeobotryosphaeria citrigena	ICMP 16812	*Citrus sinensis*	New Zealand	EU673180	EU673246	EU673328	EU673294	EU673140
Phaeobotryosphaeria citrigena	ICMP 16818	*Citrus sinensis*	New Zealand	EU673181	EU673247	EU673329	EU673295	EU673141
Phaeobotryosphaeria porosa	CBS 110496	*Vitis vinifera*	South Africa	EU673179	DQ377894	AY343379	AY343340	EU673130
Phaeobotryosphaeria visci	CBS 100163	*Viscum album*	Luxembourg	EU673177	DQ377870	EU673324	EU673292	EU673127
Phaeobotryosphaeria visci	CBS 186.97	*Viscum album*	Germany	EU673178	DQ377868	EU673325	EU673293	EU673128
Pseudofusicoccum stromaticum	CBS 117448	*Eucalyptus* hybrid	Venezuela	EU673146	DQ377931	AY693974	AY693975	EU673094
Pseudofusicoccum stromaticum	CBS 117449	*Eucalyptus* hybrid	Venezuela	EU673147	DQ377932	DQ436935	DQ436936	EU673093
Spencermartinsia vitícola	CBS 117009	*Vitis vinifera*	Spain	EU673165	DQ377873	AY905554	AY905559	EU673104
Spencermartinsia vitícola	CBS 117006	*Vitis vinifera*	Spain	EU673166	EU673236	AY905555	AY905562	EU673103

were unordered and of equal weight and gaps were treated as missing data. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the most parsimonious trees was evaluated from 1000 bootstrap replications (Hillis and Bull, 1993). Other measures used were consistency index (CI), retention index (RI) and homoplasy index (HI).

Bayesian analyses employing a Markov Chain Monte Carlo method were performed. The general time-reversible model of evolution (Rodriguez *et al*., 1990), including estimation of invariable sites and assuming a discrete gamma distribution with six rate categories (GTR+Γ+G) was used. Four MCMC chains were run simultaneously, starting from random trees for 1 000 000 generations. Trees were sampled every 100th generation for a total of 10 000 trees. The first 1 000 trees were discarded as the burn-in phase of each analysis. Posterior probabilities (Rannala and Yang, 1996) were determined from a majority-rule consensus tree generated with the remaining 9 000 trees. This analysis was repeated three times starting from different random trees to ensure trees from the same tree space were sampled during each analysis.

The possibility of combining the individual data sets was assessed by comparing highly supported clades among trees generated from the different data sets to detect conflict. High support typically refers to bootstrap support values ≥ 70 % and Bayesian posterior probabilities ≥ 95 % (Alfaro *et al*., 2003). If no conflict exists between the highly supported clades in trees generated from these different data sets, it is likely that the genes share similar phylogenetic histories and phylogenetic resolution and support could ultimately be increased by combining the data sets (Miller and Huhndorf, 2004).

Morphology

To induce sporulation, cultures were grown on plates of 2% water agar bearing a piece of autoclaved *Quercus ilex* twig. Cultures for sporulation were incubated at room temperature (*ca*. 22°C) exposed to indirect sunlight. Asci and ascospores were dissected from ascomata on the host and mounted in 100% lactic acid. Conidiomata on the host, or from culture were cut through horizontally, the conidiogenous layer excised and mounted in 100% lactic acid. Sections were cut by hand and mounted in 100% lactic acid.

Digital images were recorded with a Leica DFC320 camera. At least 50 ascospores or conidia were measured with the Leica IM500 measurement module from images recorded with the ×100 objective lens.

Growth rates in relation to temperature were determined on PDA. Plugs of agar cut from the margin of actively growing cultures on PDA were placed about 1 cm from the edge of freshly prepared PDA plates. Colony radii, measured from the edge of the agar plug, were recorded daily. Growth rates are expressed as the average increase in radius in mm per day of three replicate plates.

Results

Phylogenetic analyses

Partial nucleotide sequences of the SSU ribosomal DNA (1134 bp), the ITS region (500–600 bp), the D1/D2 variable domains of the LSU ribosomal DNA (614 bp), β-tubulin (approx. 400 bp) and EF1-α genes (approx. 300 bp) were determined. The other sequences used in the analyses were retrieved from GenBank (Table 1). Sequences of the five genes were aligned and analysed separately by MP and Bayesian analyses, and the resulting trees were compared. No conflicts were detected between single gene phylogenies indicating that the datasets could be combined. New sequences were deposited in GenBank (Table 1) and the alignments in TreeBase (SN4365). Sequences of *Guignardia bidwellii*, *G. citricarpa* and *G. philoprina* were used as outgroup taxa

The combined dataset contained 3642 characters, of which 165 were variable and parsimony-uninformative, and 2468 were constant. Maximum parsimony analysis of the remaining 1009 parsimony-informative characters resulted in two equal, most parsimonious trees (TL = 2689 steps, CI =0.6073, RI = 0.8692, RC = 0.5278, HI = 0.3927). The 50 % majority-rule consensus tree of 10 000 trees sampled during the Bayesian analysis had an overall topology similar to the MP trees. One of the MP trees is shown in Fig. 1 with

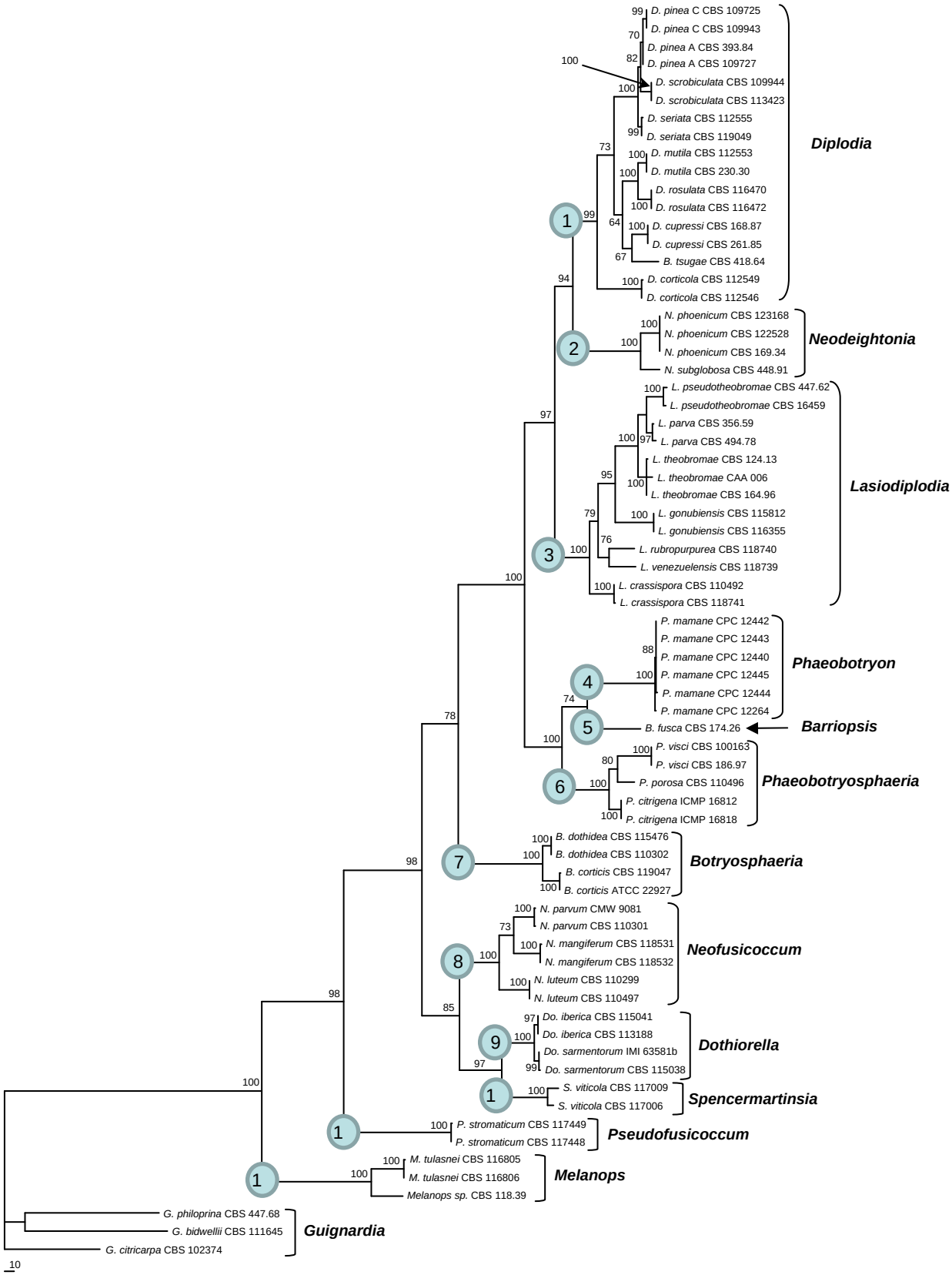


Fig. 1. One of two most parsimonious trees from a heuristic search with 1000 random taxon additions of the combined SSU+ITS+LSU+EF1-α+β-tubulin sequences. The bar indicates 10 changes. The numbers at the nodes represent bootstrap support from 1000 replicates. The tree was rooted to *Guignardia bidwellii*, *G. citricarpa* and *G. philoprina*.

bootstrap support at the branches. The Bayesian tree is available in TreeBase (SN4365). In both analyses 12 clades were identified within the ingroup. For convenience these clades are numbered 1–12 in Fig. 1. All of the clades received high bootstrap (99–100 %) and posterior probabilities (1.00) support.

In both analyses (MP and Bayesian) the cultures identified as *M. tulasnei* formed a distinct and highly supported clade at the base of the *Botryosphaeriaceae*. This clade included a culture CBS 118.39 identified as "*Botryosphaeria*" *quercuum*, which formed a sister clade to *M. tulasnei* cultures.

Morphology

Morphological characters of the teleomorph and anamorph on the host, and anamorph in culture corresponded in all ways with the original description (Tulasne, 1856) and with the detailed descriptions and illustrations made by Tulasne and Tulasne (1863). The collections also corresponded entirely with the morphological characters of the neotype in PAD. For these reasons we considered that the specimen collected from *Q. robur* is a typical example of *M. tulasnei*. This specimen was selected as epitype.

Taxonomy

Melanops Nitschke, in Fuckel, *Jahrb. Nassauischen Vereins Naturk*. 23–24: 225 ('1869-70').

Type species. *Melanops tulasnei* Nitschke

Anamorph: *Fusicoccum*-like but the conidia have a persistent mucus sheath.

Ascomata pseudothecial, multiloculate, immersed, partially erumpent at maturity, black, subglobose, thick-walled, wall composed of thick-walled *textura angularis*. *Pseudoparaphyses* hyaline, thin-walled, septate. *Asci* bitunicate, stipitate, clavate, eight-spored. *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* filiform, arising from between the conidiogenous cells. *Conidiogenous cells* cylindrical, hyaline, branched or unbranched, discrete, formed from the inner wall of the conidioma, forming a single conidium at the tip and proliferating percurrently to form one or two indistinct annellations, or proliferating at the same level giving rise to periclinal thickenings. *Conidia* hyaline, aseptate, fusiform, with a persistent mucus sheath.

Melanops tulasnei Nitschke, in Fuckel, *Jahrb. Nassauischen Vereins Naturk*. 23–24: 225, 1870 ('1869–70'), **nom. nov.** (Figs 3-20)

Basionym. *Dothidea melanops* Tul., *Ann. Sci. Nat. Bot*., 4^{e} Sér., **5**: 116, 1856.

≡ *Botryosphaeria melanops* (Tul.) G. Winter, *Rabenh. Krypt.-Fl*. Ed. 2, **1**(2): 800. 1886 ('1887').

Misapplied name:

Botryosphaeria advena sensu Sacc., *Michelia* **1**(1): 42, 1877, non (Ces.) Ces. & De Not., 1863 [fide Winter, 1886; Traverso, 1907].

Neotype. Labelled as *Botryosphaeria advena* [misapplied name], on *Quercus*, Altichiero, Italy, June 1876, Herbarium Mycologicum PA Saccardo, Padova.

Anamorph: *Fusicoccum*-like

Basionym. *Dothiorella advena* Sacc., *Michelia* **2**(8): 620, 1882.

≡ *Fusicoccum advenum* (Sacc.) Died., *Krypt. Fl. Brandenburg* **9**: 314. 1912 ('1915').

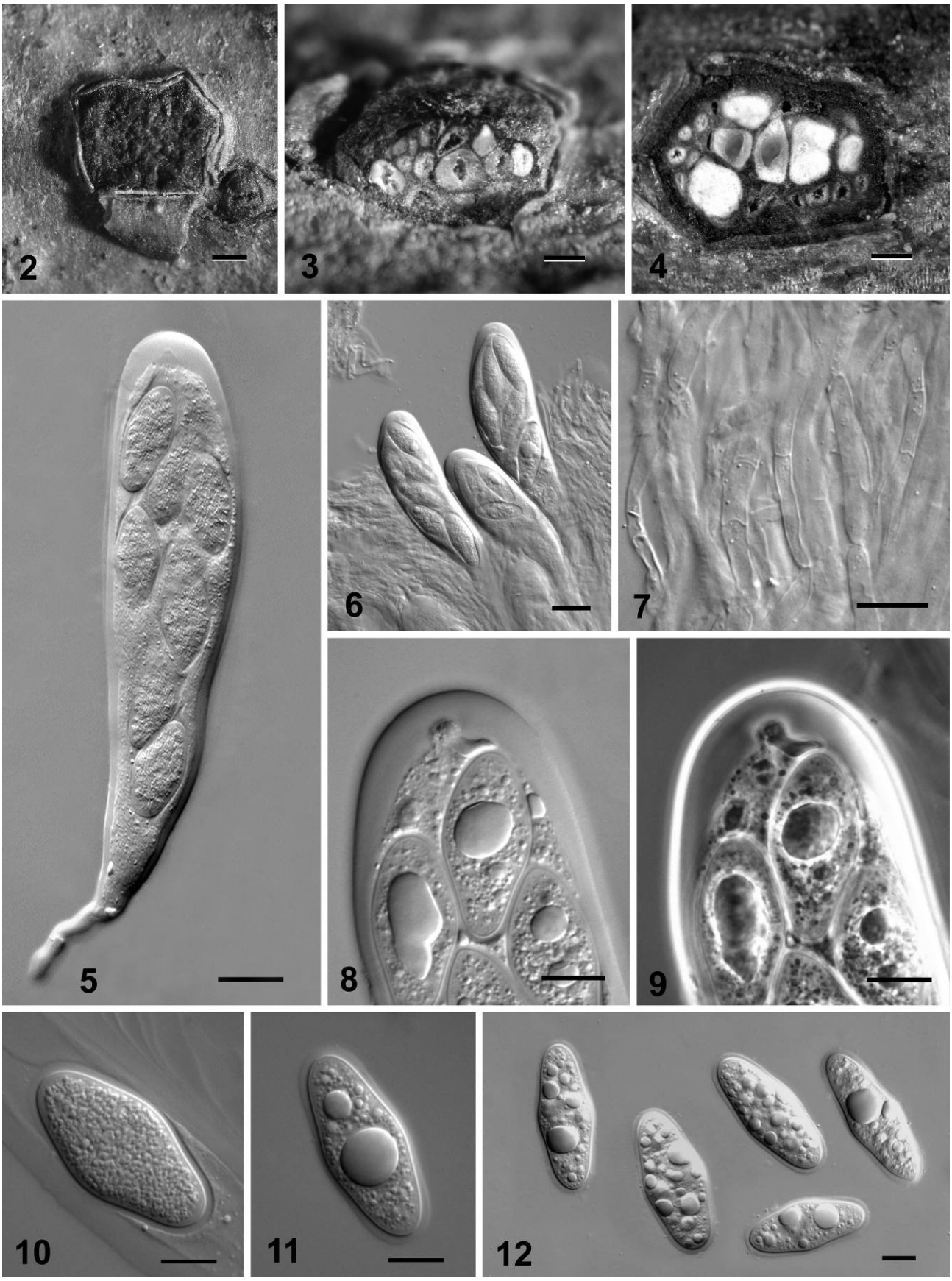
= *Fusicoccum testudo* Höhn., *Ann. Mycol*. **1**(5): 399, 1903 [fide Diedicke, 1915; Shear & Davidson, 1936].

= *Dothiorella melanops* Traverso, *Fl. Ital. Crypt., Fungi* **2**(2): 409, 1907 [microconidial state].

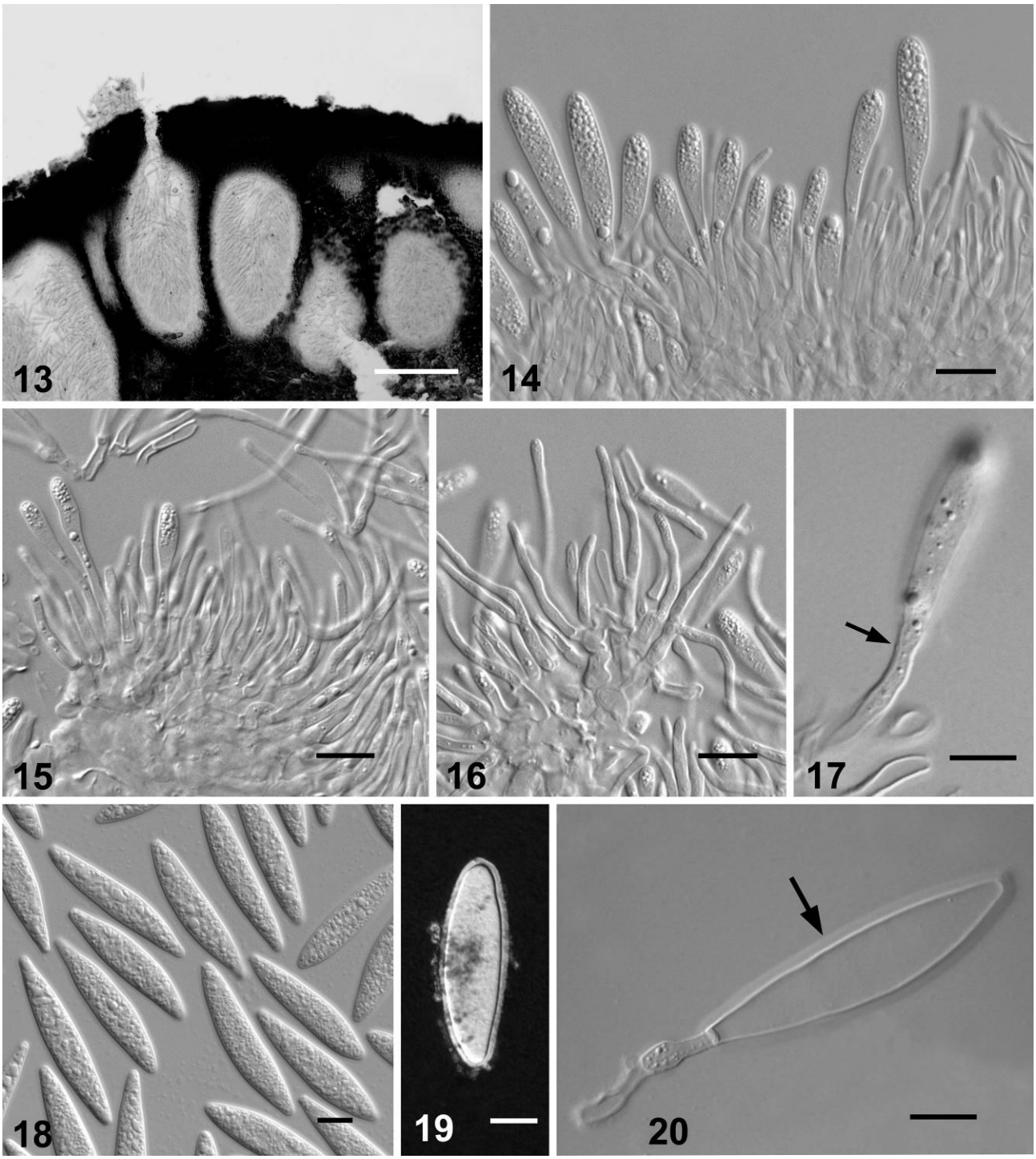
Misapplied name:

Sphaeria quercina sensu Fr., Scler. Suec. Exs. Nº 143, 1821, non Pers. : Fr., 1794 [fide Tulasne & Tulasne, 1865; Shear & Davidson, 1936].

Ascomata up to 2 mm diameter, pseudothecial, initially immersed, partially erumpent at maturity, black, multilocular, thick-walled, outer layers composed of thick-walled, brown *textura angularis*, becoming progressively thinner-walled and paler towards the loculi, individual locules 150–300 µm diameter. *Ostioles* circular and central on each locule, non-papillate. *Pseudoparaphyses* 3–4 µm diameter, thin-walled, hyaline, septate, constricted at the septum. *Asci* 150–240 × 30–48 µm, clavate, stipitate, bitunicate with a well developed apical chamber, eight-spored, spores irregularly biseriate in the ascus. *Ascospores* (30–)37.5–40.9(–47) × (13.0–)15.1–16.3(–19.0) µm, with a persistent mucus sheath, rhomboid, widest in the middle, thin-walled, smooth, hyaline, contents granular with small or large guttules, aseptate.



Figs 2-12. *Melanops tulasnei*. **2**. Stroma erumpent through the bark. **3**. Stroma cut through vertically revealing ascomata and conidiomata embedded at various levels. **4**. Stroma cut through horizontally revealing ascomata and conidiomata. **5**. Ascus. **6**. Asci and pseudoparaphyses. **7**. Pseudoparaphyses. **8**. Ascus tip under differential interference contrast illumination. **9**. Ascus tip under phase contrast illumination. **10-12**. Ascospores. Bars: 2–4 = 250 μm; 5, 6 = 20 μm; 7–12 = 10 μm.



Figs 13-20. *Melanops tulasnei*. **13**. Sectioned conidioma. **14**. Conidiogenous layer with conidia developing on filiform conidiogenous cells arising amongst paraphyses. **15**. Young conidiogenous cells. **16**. Paraphyses. **17**. Conidiogenous cell with percurrent proliferations (arrow). **18**. Conidia. **19**. Conidium mounted in indian ink revealing the mucous sheath. **20**. Conidium attached to a conidiogenous cell with mucus sheath around the conidium but absent from the conidiogenous cell. Bars: 13 = 200 µm; 14–16, 18, 9 = 10 µm; 17, 20 = 5 µm.

Conidiomata indistinguishable from ascomata and often formed in the same stroma. *Paraphyses* filiform, branched, arising between the conidiogenous cells, 1.5–2 µm wide, up to 40 µm long, tip rounded or slightly swollen. *Conidiogenous cells* cylindrical, hyaline, sometimes branched at the base, discrete, formed from the inner wall of the conidioma, 2–3 × 12–18 µm, forming a single conidium at the tip and proliferating percurrently to form one or two annellations, rarely proliferating at the same level giving rise to periclinal thickenings. *Conidia* (37.2–)45–46.8(–53) × (7.2–)9.1–9.7 (–12.1) µm, hyaline, aseptate, fusiform, widest in the middle, apex acute, base truncate with a minute marginal frill, surrounded by a persistent mucus sheath, contents granular. *Cardinal temperatures for growth*: min 5ºC, opt 20–25ºC, max below 35ºC.

Habitat: *Quercus* spp.

Known distribution: USA, Canada, Germany, Italy

Material examined: ITALY. Altichiero, on *Quercus* twig, June 1876, P.A. Saccardo, Herbarium Mycologicum P.A. Saccardo, Padova, labelled as *Botryosphaeria advena* (misapplied name), (**neotype** designated by Phillips and Pennycook, 2004). GERMANY. BAVARIA. Munich, English Garden, on dead twigs of *Quercus robur*, 8 July 2004, A.J.L. Phillips, LISE 95179 (herein designated **epitype**), cultures ex-epitype CBS 116805, CBS 116806, CBS 116807, CBS 116808.

Discussion

In this paper *Melanops* was shown to be a distinct genus in the *Botryosphaeriaceae*. This conclusion was based on a specimen found on dead twigs of *Quercus robur* collected in Munich, Germany. Identification was based on a morphological comparison with the protologue (Tulasne, 1856), the detailed description and illustrations by Tulasne and Tulasne (1863) and a comparison with the neotype specimen in PAD. The specimen considered here correlated in all aspects with the neotype and the original descriptions. In terms of the 95% confidence limits for ascospore dimensions, ascospores of the collection studied here were somewhat larger than was reported for the neotype and other specimens examined by Phillips and Pennycook (2004). However, the range of ascospore dimensions overlapped considerably and they could not be separated from one another. Therefore, any differences in ascospore dimensions were thought to reflect normal variation within the species. Similarly, the 95% confidence limits of conidial dimensions for the collection studied here were larger than was reported for the type of *Dothiorella advena* (≡ *Fusicoccum advenum*) (Phillips and Pennycook, 2004). However, the ranges of conidial dimensions overlapped considerably and again this was regarded as intra-species variation. This specimen was considered to be a typical example of *M. tulasnei* and was selected as epitype.

In the phylogenetic analyses *M. tulasnei* formed a clade at the base of the *Botryosphaeriaceae*. This corresponds to the clade identified by Crous *et al*. (2006) as "*Botryosphaeria*" *quercuum* (anamorph *Diplodia*-like). In fact, in our phylogenetic analyses "*Botryosphaeria quercuum*" CBS 118.39 used by Crous *et al*. (2006) formed a highly supported clade with *M. tulasnei*. Unfortunately, CBS 118.39 could not be induced to sporulate, no doubt because it has been in culture for many years, and thus we were unable to study its morphology. However, given the nucleotide differences in the sequences of all the genes analysed this isolate clearly represents a species distinct from *M. tulasnei*.

Characteristic features of *Melanops* are the large ascomata and conidiomata that occur within the same stroma. Unlike other genera in the *Botryosphaeriales* locules in *Melanops* are distributed at different levels within the stroma. Although ascomata and conidiomata of *Botryosphaeria* and *Neofusicoccum* can often be found in the same stroma (Slippers *et al*., 2004), they are placed at similar levels. Another feature that defines *Melanops* is the mucus sheath surrounding the ascospores and conidia. The only other genus in the *Botryosphaeriales* reported to have conidia with a mucus sheath is *Pseudofusicoccum* (Crous *et al*., 2006; Mohali *et al*., 2006) and this feature is present in all of the known species (Pavlic *et al*., 2008). Although mucus sheaths are uncommon in the *Botryosphaeriales*, mucus appendages are found on *Guignardia* ascospores and are occasionally seen in *B. dothidea* (Sivanesan, 1984;

Wulandari *et al*., 2009). Conidia of *M. tulasnei* are very large and the ascospores have a characteristic shape. Shoemaker (1964) reported that the large conidia with a length:width ratio of between 4:1 and 5:1 clearly distinguish this fungus from *B. quercuum*, the conidia of which are sub-globose with a length:width ratio of 1.5:1. Another characteristic feature of this species is the slow growth rate in culture (< 1 mm per day at 25°C) that was first pointed out by Shear and Davidson (1936). It can be speculated that this slow growth rate is the reason for the relatively few reports of *M. tulasnei* since cultures in isolation plates would be rapidly overgrown by faster growing fungi.

Although Tulasne (1856), reported that this fungus was common around Paris and Versailles on the bark of *Quercus* (Tulasne and Tulasne, 1863), since then it has been reported rarely. Saccardo (1877) described a specimen on *Quercus* collected from Altichiero (Padova) while Shear and Davidson (1936) examined a specimen collected on *Quercus borealis* in Connecticut, USA in 1935. On the basis of cultures derived from single ascospores, Shear and Davidson (1936) established the connection between the teleomorph and the anamorph. This connection was confirmed in the present study.

MycoBank (http://www.mycobank.org/) lists 102 species that have been described under *Melanops*. As far as we could establish, no cultures are available for any of these species. Therefore the types of all these species will have to be re-examined and epitypes collected and studied in order to determine their correct taxonomic and phylogenetic position

Acknowledgements

This work was financed by the European Regional Development Fund (FEDER) and Fundação para a Ciência e a Tecnologia (FCT) under project number POCI/AGR/56140/2004. A. Alves was supported by grant No. SFRH/BPD/24509/2005, and A. Phillips by grant No. SFRH/BCC/15810/2006 both from FCT. This research received support from the SYNTHESYS Project http://www.synthesys.info/ which is financed by European Community Research Infrastructure Action under the FP6 "Structuring the European Research Area" Programme.

References

Alfaro, M.E., Zoller, S. and Lutzoni, F. (2003). Bayes or bootstrap? A simulation study comparing the performance of Bayesian Markov chain Monte Carlo sampling and bootstrapping in assessing phylogenetic confidence. Molecular Biology and Evolution 20: 255-266.

Alves, A., Correia, A., Luque, J. and Phillips, A.J.L. (2004). *Botryosphaeria corticola* sp. nov. on *Quercus* species, with notes and description of *Botryosphaeria stevensii* and its anamorph *Diplodia mutila*. Mycologia 96: 598-613.

Alves, A., Correia, A. and Phillips, A.J.L. (2006). Multigene geneologies and morphological data support *Diplodia cupressi* sp. nov., previously recognized as *D. pinea* f. sp. *cupressi*, as a distinct species. Fungal Diversity 23: 1-15.

Alves, A., Crous, P.W., Correia, A. and Phillips, A.J.L. (2008). Morphological and molecular data reveal cryptic speciation in *Lasiodiplodia theobromae*. Fungal Diversity 28: 1-13.

Alves, A., Phillips, A.J.L., Henriques, I. and Correia, A. (2005). Evaluation of amplified ribosomal DNA restriction analysis as a method for the identification of *Botryosphaeria* species. FEMS Micribiology Letters 245: 221-229.

Arx, J.A. von and Müller, E. (1954). Die Gattungen der Amerosporen Pyrenomyceten. Beiträge zur Kryptogemenflora der Schweiz 11: 1-434.

Carbone, I. and Kohn, L.M. (1999). A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia 91: 553-556.

Crous, P.W., Slippers, B., Wingfield, M.J., Rheeder, J., Marasas, W.F.O., Phillips, A.J.L., Alves, A., Burgess, T., Barber, P. and Groenewald, J.Z. (2006). Phylogenetic lineages in the *Botryosphaeriaceae*. Studies in Mycology 55: 235-253.

Fuckel, L. (1870). Symbolae mycologicae. Jahrb. Nassauischen Vereins Naturk. 23-24: 1-459.

Hillis, D.M. and Bull, J.J. (1993). An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. Systematic Biology 42: 182-192.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16: 111-120.

Miller, A.N. and Huhndorf, S.M. (2004). Using phylogenetic species recognition to delimit species boundaries within *Lasiosphaeria*. Mycologia 96: 1106-1127.

Mohali, S., Slippers, B. and Wingfield, M.J. (2006). Two new *Fusicoccum* species from *Acacia* and *Eucalyptus* in Venezuela, recognised based on morphology and DNA sequence data. Mycological Research 110: 405-413.

Page, R.D. (1996). TreeView: an application to display phylogenetic trees on personal computers. Computer Applications in the Biosciences 12: 357-358.

Pavlic, D., Wingfield, M.J., Barber, P., Slippers B., Hardy, G.E. StJ. and Burgess, T. (2008). Seven new species of the Botryosphaeriaceae from Baobab and other native trees in Western Australia. Mycologia 100: 851-866.

Phillips, A.J.L. and Pennycook, S.R. (2004). Taxonomy of *Botryosphaeria melanops* and its anamorph *Fusicoccum advenum*. Sydowia 56: 68-75.

Rannala, B. and Yang, Z. (1996). Probability distribution of molecular evolutionary trees: a new method of phylogenetic inference. Journal of Molecular Evolution 43: 304-311.

Rodriguez, F., Oliver, J.F., Marin, A. and Medina, J.R. (1990). The general stochastic model of nucleotide substitutions. Journal of Theoretical Biology 142: 485-501.

Ronquist, F.R. and Huelsenbeck, J.P (2003). MrBayes3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572-1574.

Saccardo, P.A. (1877). Fungi veneti novi vel critici vel Mycologiae Venetae addendi. Michaelia 1: 1-72.

Shear, C.L. and Davidson, R.W. (1936). The life histories of *Botryosphaeria melanops* and *Massaria platani*. Mycologia 28 : 476-482.

Shoemaker, R.A. (1964). Conidial states of some *Botryosphaeria* species on *Vitis* and *Quercus*. Canadian Journal of Botany 42: 1297-1301.

Sivanesan, A. (1984). *The bitunicate ascomycetes and their anamorphs*. J. Cramer, Vaduz.

Slippers, B., Crous, P.W., Denman, S., Coutinho, T.A., Wingfield, B.D. and Wingfield, M.J. (2004). Combined multiple gene genealogies and phenotypic characters differentiate several species previously identified as *Botryosphaeria dothidea*. Mycologia 96: 83-101.

Swofford, D.L. (2003). *PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4.0*. Sunderland, Massachusetts: Sinauer Associates.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. (1997). The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 25: 4876-4882.

Tulasne, L.-R. (1856). Note sur l'appareil reproducteur multiple des Hypoxylées (DC) ou Pyrénomycetes (Fr.). Annales des Sciences Naturelles, Botanique, 4^{e} Série, 5: 107-118.

Tulasne, L.-R. and Tulasne, C. (1863). Selecta Fungorum Carpologia 2: 1-319.

White, T.J., Bruns, T., Lee, S. and Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: A Guide to Methods and Applications* (eds. M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White). San Diego, California: Academic Press: 315-322.

Winter, G. (1887). Ascomyceten: Gymnoasceen und Pyrenomyceten. Rabenhort's Kryptogamen-Flora 2rd edn. 1: 1-928.

Wulandari, N.F., To-anun, C., Hyde, K.D., Duong, L.M., de Gruyter, J., Meffert, J.P., Groenewald, J.Z. and Crous, P.W. (2009). *Phyllosticta citriasiana* sp. nov., the cause of Citrus tan spot of *Citrus maxima* in Asia. Fungal Diversity 34: 239.

Young, N.D. and Healy, J. (2003). GapCoder automates the use of indel characters in phylogenetic analysis. BMC Bioinformatics 4: 6.