Mycologia, 100(5), 2008, pp. 760–775. DOI: 10.3852/07-207
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Endomelanconiopsis, a new anamorph genus in the Botryosphaeriaceae

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Abstract: A new lineage is discovered within the Botryosphaeriaceae (Ascomycetes, Dothideomycetes, *incertae sedis*). Consistent with current practice of providing generic names for independent lineages, this lineage is described as *Endomelanconiopsis* gen. nov., with the anamorphic species *E. endophytica* sp. nov. and *E. microspora* comb. nov. (= *Endomelanconium microsporum*). *Endomelanconiopsis* is characterized by eustromatic conidiomata and holoblastically produced, brown, nonapiculate, unicellular conidia, each with a longitudinal germ slit. Phylogenetic analysis of partial sequences of LSU, ITS and translation elongation factor 1 alpha (*tef1*) indicate that *E. endophytica* is sister of *E. microspora* and that they are nested within the Botryosphaeriaceae. However because there is no support for the "backbone" of the Botryosphaeriacae we are not able to see the interrelationships among the many genera in the family. Neither species is known to have a teleomorph. *Endomelanconiopsis* differs from *Endomelanconium* because conidia of the type species of *Endomelanconium*, *E. pini*, are papillate at the base, conidiogenous cells proliferate sympodially and the pycnidial wall is thinner; we postulate that the teleomorph of *E. pini* as yet unknown is an inoperculate discomycete. *Endomelanconiopsis endophytica* was isolated as an endophyte from healthy leaves of *Theobroma cacao* (cacao, Malvaceae) and *Heisteria concinna* (Erythroplaceae) in Panama. *Endomelanconiopsis microspora* was isolated from soil in Europe.

Key words: *Austrocenangium*, *Endomelanconium*, endophytic fungi, *Heisteria concinna*, systematics, *Theobroma cacao*

INTRODUCTION

Fungal endophytes live inside plant tissues for all or part of their lives without causing apparent disease symptoms (Wilson 1995). Endophytic fungi have been found within host tissues at high density and diversity in all tropical and temperate ecosystems where endophytes have been sought (e.g. Arnold and Lutzoni 2007, Clay and Holah 1999, Hawksworth 2001). Foliar fungal endophytes have been found to be abundant and diverse in intact tropical forests with high plant diversity and low density of individual species (Dreyfuss and Petrini 1984, Lodge et al 1996, Rodrigues and Petrini 1997, Arnold et al 2000, van Bael et al 2005), and in mangroves and *Theobroma cacao* agroforestry systems (cacao, Malvaceae, Malvales) with low plant diversity and high density of individual plant species (Gilbert et al 2002, Suryanarayanan et al 1998, Arnold et al 2003).

In most tropical tree species leaves are flushed in a largely endophyte-free (E−) condition (Arnold et al 2003, Herre et al 2007). A taxonomically diverse assemblage of airborne spores land on leaf surfaces, some of which initiate endophytic associations. After the wetting of spore-laden leaf surfaces some spores germinate and penetrate directly through the cuticle into the leaf tissue, where hyphae grow between cells (Herre et al 2005, 2007). With time the initially endophyte-free leaf tissues become saturated with endophytes, with ca. 100% of sampled leaf fragments (2 × 2 mm) containing cultivable fungi (Arnold et al 2003). Data from cohorts of leaves suggest that over time the species composition of fungal endophytes within a leaf changes and that endophyte diversity (number of species per isolate recovered) usually declines as leaves pass from maturity toward senescence. Within the leaf the distribution of fungal species resembles a quilt-like patchwork: different

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species usually abutting, producing an extremely heterogeneous mix of fungal species and genotypes at fine scales (Brayford 1990a, b, Lodge et al 1996, Herre et al 2007). Colonization by endophytic fungi has been shown to reduce pathogen susceptibility in the host plant and affect host photosynthetic and hydraulic physiology in various ways, but general rules for tropical plant-endophyte interactions are not yet established (Arnold et al 2003, Herre et al 2005, 2007, Arnold and Engelbrecht 2007, Mejía et al 2003, 2008).

Arnold et al (2003) reported 344 endophytic morphospecies (determined by colony appearance in agar culture) from 126 naturally infected leaves of *T. cacao* collected during 2 y from five sites in Panama. Common among these fungal morphospecies was one that produced a dark gray or green, to nearly black, colony on potato-dextrose agar. Large eustromatic pycnidial structures developed in agar cultures; these contained dark brown, holoblastically produced conidia with a longitudinal germ slit. Several representatives of this morphospecies could be identified as an *Endomelanconium* (Corda) Petrak as characterized by Sutton (1980). What appeared to be the same species was isolated also as an endophyte from leaves of *Heisteria concinna* (Erythroplaceae, Santalales), a tree that often grows in association with cacao in lowland moist forests in Panama. The published description of *E. microsporum* Verkley & van der Aa (Verkley and van der Aa 1997), a species isolated from soil in Europe, indicated that it was the same or very closely related to the endophyte. The cultures and anamorph morphology of these species are strongly suggestive of *Botryosphaeria* and its relatives (Luque et al 2005, Crous et al 2006, Phillips 2008). At least some described *Endomelanconium* species are anamorphs of inoperculate discomycetes, although the teleomorph of the type species, *E. pini* (Corda) Petrak (Petrak 1940), is not known. Possible teleomorph relationships and subtle morphological differences among these various described *Endomelanconium* species lead us to suspect that the genus is paraphyletic.

In the present study we examine the phylogenetic position of the endomelanconium-like endophytes by means of molecular sequence data from three loci. We compare them to described species of *Endomelanconium*.

MATERIALS AND METHODS

Collection and cultures.—The endophytic cultures reported here were obtained from two surveys of endophytic fungi in the Republic of Panama. The first was carried out in 1999 and 2000 in five lowland sites with mixed forest cover across the Isthmus of Panama, including Barro Colorado Island (BCI, Canal Zone, 09°9′35″N, 79°50′33″W) and Nombre de Dios (ND, Colón, 09°34′N, 79°28′W). The mean annual rainfall in ND is 4041 mm, and the average annual temperature is approximately 26 C (Tosi 1971); at BCI average annual temperature is 27 C and rainfall is approximately 2600 mm (Leigh et al 1996). In the first survey leaves of three different ages were sampled from adult *Theobroma cacao* trees during two seasons (dry and rainy) in 1999 and 2000. In the second year of the first survey samples also were taken from two co-occurring species, *Heisteria concinna* and *Ouratea lucens* (Ochnaceae, Malpighiales) (Arnold et al 2003).

The second survey was carried during the rainy season (Jul 2000) at ND and samples were taken only from a single tree of *Theobroma cacao*. In this survey leaves of five age classes (young, 1 wk old leaves; young-mature, 2 wk old leaves; mature, 4 wk old leaves; mature–old, 8 wk old leaves; and old, > 12 wk old leaves) were sampled to address questions regarding colonization patterns. The leaves were collected at the same time and were processed the day that they were collected.

The endomelanconium-like isolates were found during the rainy season in asymptomatic foliage of *T. cacao* and *H. concinna* at BCI in our first survey; it was the most common fungus in healthy leaves of cacao for the second survey. A total of 15 isolates of endomelanconium-like endophytes were selected for DNA sequence analyses.

Characterization of colonies and morphology.—After initial isolation on 2% malt extract agar (MEA, Difco, Detroit, Michigan), isolates were cultured on potato-dextrose agar (PDA; BBL, Sparks, Maryland) and simple nutrient agar (SNA, Nirenberg 1976) at 25 C with a 12 h cool white fluorescent light/12 h dark photoperiod. In addition a piece of sterile filter paper was put on the surface of the solidified SNA to induce sporulation. Pycnidia typically formed within 3 wk on both media.

Growth rates were determined in darkness (with fluorescent light briefly introduced when the colonies were measured) on PDA and SNA (without filter paper) at five temperatures (15, 20, 25, 30 and 37 C). Inoculum for growth rate studies was taken from young, actively growing colonies on cornmeal-dextrose agar (Difco cornmeal agar + 2% w/v dextrose). A 5 mm diam plug was cut from the edge of the colony and placed in the middle of the test medium (PDA or SNA). Measurements of colony radius from the edge of the inoculum plug to the edge of the colony were taken at 24 h intervals. The growth trial was repeated twice on succeeding weeks and the two measurements were averaged.

Morphological descriptions were made from isolates sporulating on PDA or SNA (with filter paper) at 25 C for ca. 10 d with photoperiod 12 h darkness/12 h cool white fluorescent light. Thirty conidia and up to 30 conidiogenous cells were measured for each isolate. Sections of pycnidia for microscopic examination were prepared with a freezing microtome (IEC-CTF Microtome-Cryostat, International Equipment Co., Needham Heights, Massachusetts).

Images were captured with a Nikon DXM1200 digital camera and Nikon ACT 1 software or with a Nikon Ds-Fi1 camera and NIS Elements Basic Research 2.30, SP4 (Nikon). Some composite images were made with Helicon Focus version 4.21.5 Pro (Helicon Soft, www.heliconfocus.com). Measurements were made with Scion Image (release Beta 4.0.2; Scioncorp, Frederick, Maryland). Data were analyzed statistically with Systat 10.0 (SPSS, Chicago, Illinois). Representative cultures of the endophytic endomelanconium-like species were deposited in the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands.

DNA extraction, PCR amplification and sequence assembly.—Mycelium for DNA extraction was prepared in potato-dextrose broth (Difco). DNA was extracted following the manufacturer's protocol for fresh plant tissue with the PureGene genomic DNA isolation kit (Gentra Systems, Minneapolis, Minnesota).

For each isolate 25 μL PCR reactions were employed to amplify sections of the nuclear ribosomal large subunit (LSU), internal transcribed spacers and 5.8 s gene (ITS), and translation elongation factor 1 alpha (*tef1*). PCR mixtures contained 2.5 μL 10× PCR buffer (New England Biolabs, Ipswich, Massachusetts), 200 μM dNTPs, 25 pmole each primer, 1.25 units Taq DNA Polymerase and 10–50 ng template DNA. Primers LROR and LR7 were used to amplify ca. 1100 base pairs of LSU, followed by sequencing of ca. 600 bp with LROR and LR3 (Rehner and Samuels 1994). Primers ITS5 and ITS4 (White et al 1990) were used to amplify the approximately 540 bp ITS, followed by sequencing with the same primers. Primers EF1-728F (Carbone and Kohn 1999) and tef1 rev (Samuels et al 2002) were used to amplify and sequence ca. 600 bp of *tef1*. PCR was carried out on a PT-200 PCR thermal cycler (MJ Research, Waltham, Massachusetts) according to these conditions: 95 C for 5 min; 35 cycles denaturation at 95 C for 30 s, annealing at 51 C for 30 s, extension at 72 C for 1 min; and a final extension at 72 C for 10 min. The concentration of PCR products in ng/μL was determined on 1% agarose gel via electrophoresis with Lambda Hind III DNA as a marker. PCR products were purified with ExoSAP-IT® reagent following the manufacturer's instructions (USB Corp., Cleveland, Ohio). Bidirectional sequences were obtained with BigDye Terminator cycle sequencing kit (Applied Biosystems, Foster City, California). Products were analyzed on a 3100 DNA sequencer (Applied Biosystems). Eight representative cultures were sequenced for LSU and 15 cultures were sequenced for both ITS and *tef1*.

Phylogenetic analyses.—Sequences were edited and assembled with Sequencher 4.1 (Gene Codes, Wisconsin) and Clustal X version 1.8 (Thompson et al 1997) was used to align sequences, followed by manual adjustment in MacClade 4 (Maddison and Maddison 2000). New DNA sequences were deposited in GenBank (TABLE I).

Two datasets were prepared. One alignment included LSU sequences from representatives of the major lineages within the Botryosphaeriaceae (Crous et al 2006), two species representing the endomelanconium-like group, and species of *Guignardia* as outgroup, for a total of 56 terminals. The second dataset included a concatenated alignment of ITS and *tef1* sequences for 15 endomelanconium-like isolates from *T. cacao* and *H. concinna*, *Endomelanconium microsporum* (ex type culture, CBS 353.97), representative species of the Botryosphaeriaceae, and two species of *Guignardia*, identified as closely related to *Botryosphaeria* (Schoch et al 2006) as outgroup taxa (total of 50 terminals).

The LSU dataset was analyzed with PAUP* v. 4.0b10 (Swofford 2002) using the neighbor joining (NJ) method with Kimura 2-parameter distance calculation and maximum parsimony (MP) using the heuristic search option with simple taxa additions and tree bisection and reconnection (TBR) as the branch-swapping algorithm. Robustness of the resulting topology was assessed with 1000 bootstrap replicates in both neighbor joining and parsimony analyses.

The ITS and *tef1* datasets were analyzed separately and in the absence of phylogenetic conflict combined for analysis with NJ and MP as described above and with Bayesian methods (Rannala and Yang 2005) as implemented with the program MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001). MrModeltest 2.2 (Nylander 2004) was used to select the best model, taking into account both the hierarchical likelihood ratio test (LRT) and Akaike information criteria (AIC). Preference was given to the latter (AIC). Bayesian analysis was performed with models specific to each partition of the data (GTR + I + G for ITS, and GTR + G for *tef*). The Bayesian MCMC (Markov chain Monte Carlo) analysis was run with three hot chains and one cold chain for 1 500 000 generations, sampling every 500 generations. The first 400 trees were discarded as burn-in on the basis of likelihood scores, and PAUP* was used to construct a 50% majority rule consensus tree from the remaining 5600 trees.

RESULTS

The endomelanconium-like endophyte was found only during the rainy season. At Nombre de Dios it was not found in the first survey, but it was isolated from *Theobroma cacao* and *Heisteria concinna* at BCI. It was the most abundant species isolated from the single tree (*Theobroma cacao*) that was the subject of the second survey. Of 16 cultures identified as being the morphospecies that included the endomelanconium-like fungus, nine were confirmed by microscopy to be endomelanconium-like. The remaining seven cultures either had died before they could be fully identified or were a different fungus. The endomelanconium-like fungus was isolated from mature and old leaves; it never was found in leaves younger than 1 wk.

Phylogenetic analysis.—BLAST analyses of the NCBI GenBank database with LSU sequences of the endomelanconium-like cultures showed that the closest matches (maximum identity 97% and sequence coverage of 100%) were with members of

the Botryosphaeriaceae. We aligned our sequences of the endomelanconium-like endophyte with the ex-type culture of *E. microsporum* CBS 353.97 and with one representative taxon selected from each clade in the Botryosphaeriaceae tree of Crous et al (2006, TreeBase M2707); we added to this tree *Aplosporella prunicola* (Damm et al 2007). Because no variation was observed among the eight endophytic isolates sequenced for the LSU locus, only two were selected for the analysis. The two sequences of the endophyte in the tree (FIG. 1) are from cultures that represent the phenotype-groups described below. The LSU dataset had 933 characters, 102 of which were parsimony informative, 47 were parsimony uninformative and 784 were constant when gaps were treated as missing characters. A heuristic search found 100 equally parsimonious trees (TL = 339 steps; CI = 0.522; RI = 0.787; RC = 0.411). Neighbor joining analysis with Kimura 2-parameter distance calculation on the sequence data yielded trees with similar topology (see FIG. 1). The two isolates of the endomelanconium-like endophyte clustered with *E. microsporum* in a highly supported clade (FIG. 1), indicating that they are congeneric. Although there was high BS support (FIG. 1) for *Botryosphaeria s. lat.*, (including *Pseudofusicoccum*), only internal clades received significant support, and there was no support for a backbone. Thus it was impossible to determine relationships among the clades.

The manually adjusted alignment of 15 isolates of the endomelanconium-like species with species of the Botryosphaeriaceae for the concatenated ITS and *tef1* datasets contained 34 ambiguously aligned characters in the ITS region (25–42 bp, 54–69 bp insertion found only in *G. mangiferae* and *G. bidwellii*) that were excluded, leaving 892 characters. Of these 385 were parsimony informative, 46 were parsimony uninformative and 461 were constant when gaps were treated as missing characters. A heuristic search found six equally parsimonious trees (TL = 967 steps; CI = 0.698; RI = 0.908; RC = 0.634) and same topology was found with Kimura 2-parameters distance calculations in neighbor joining and Bayesian analyses.

Together these analyses placed the endomelanconium-like endophyte isolates and *E. microsporum* in the Botryosphaeriaceae (FIG. 2). The 15 endophytic cultures form a highly supported (100% bootstrap, 1.0 posterior probability) clade. There was no distinction between endophytic isolates from *T. cacao* and those from *H. concinna*, indicating that the endomelanconium-like endophytes collected from both hosts likely are one species. The endophytes and the ex-type culture of *E. microsporum* formed a strongly supported clade but *E. microsporum* differed markedly (FIG. 2). The closest morphological comparison within the Botryosphaeriaceae is with *Sphaeropsis subglobosa*, the anamorph of *Neodeightonia subglobosa* Booth in Punithalingham (1969) and which Crous et al (2006) referred to as '*Botryosphaeria*' *subglobosa* (FIG. 1). It is not closely related to *E. microsporum* or the endomelanconium-like endophyte (FIG. 1).

Although ITS sequences for all the endophytic cultures were identical, there was slight variation in the *tef1* gene. Two groups were formed with > 0.95 Bayesian posterior probability and high NJ and MP bootstrap support, and one isolate (culture 3370 from *T. cacao*, BCI, 1999) formed a third group that differed from the other two groups by two bp in the same locus. These three groups correlated with the same three groups that were observed based on the morphological characters of their colonies (FIGS. 3–5, see below). There was no obvious relationship between observed groups and their occurrence in particular sites or host species.

Morphological analyses.—The endomelanonium-like endophyte was not observed sporulating in nature. Pycnidia (FIGS. 6–9) formed in culture were eustromatic (Sutton 1980), tending to be cylindrical, large and the wall was formed of pseudoparenchymatous cells (FIGS. 6–9). The locule was convoluted (FIGS. 8, 9) and was lined entirely with conidiogenous cells (FIGS. 8–10). The conidiogenous cells arose directly from the cells of the pycnidial wall (FIG. 11) and possessed a single, terminal, holoblastic conidiogenous locus (FIGS. 11, 12). Macroconidia (FIGS. 13–15) were opaque, brown, unicellular and bilaterally symmetrical, elliptical in face view and flattened with a narrow edge; a germ slit was positioned on the flattened edge. Often conidia were flattened at the base at the point of abscission from the conidiogenous cell. In addition to these macroconidia, microconidia (spermatia?, FIG. 18) formed either in the same pycnidium as the macroconidia or in separate pycnidia (FIG. 16) on SNA and PDA. Microconidia were variable in shape, ellipsoidal to subcylindrical, hyaline, and arose from phialidic conidiogenous cells (FIG. 17). Two size classes were observed: those with microconidia 2.0–4.5 × 1–2 μm (Mean = 2.9 × 1.3 μm) and those with microconidia 3–10 × 1.0–2.7 μm (Mean = 6.9 × 1.7 μm).

Two size classes of macroconidia were observed (FIG. 19, TABLE II), designated respectively as Groups 1 and 2. Macroconidia of Group 1 were shorter (5.7 ± 0.7 × 3.8 ± 0.3 μm; 95% confidence interval (CI) 5.6–5.9 × 3.7–3.8 μm), macroconidia of Group 2 were longer (7.2 ± 0.9 × 4.4 ± 0.45 μm; 95% CI 7.1–7.3 × 4.3–4.4 μm), the macroconidia of a single culture (3370), referred to below as Group 3 because of its

TABLE I. Cultures used in phylogenetic analysis, their origin and GenBank numbers

Species	Cultures[1]	Collector	Geography[2]	Substratum	GenBank accession number[3]		
					ITS1 + 2	*tef1*	LSU
Endomelanconiopsis microspora	**CBS 353.97**	H.A. van der Aa	Papua New Guinea	soil	EU683655	EU683636	EU683628
E. endophytica	**CBS 120397 = 7463**	E. Rojas, L. Mejía & Z. Maynard	Panama (ND)	*Theobroma cacao*, leaf	EU683656	EU683637	EU683629
E. endophytica	CBS 122550 = 7394	E. Rojas, L. Mejía & Z. Maynard	Panama (ND)	*T. cacao*, leaf	EU683664	EU683645	EU683634
E. endophytica	CBS 122549 = 3370	E. Rojas, L. Mejía & Z. Maynard	Panama (BCI)	*T. cacao*, leaf	EU683659	EU683640	—
E. endophytica	7473	E. Rojas, L. Mejía & Z. Maynard	Panama (ND)	*T. cacao*, leaf	EU683660	EU683641	EU683630
E. endophytica	CBS 122548 = 7759	E. Rojas, L. Mejía & Z. Maynard	Panama (BCI)	*T. cacao*, leaf	EU683657	EU683638	—
E. endophytica	7329	E. Rojas, L. Mejía & Z. Maynard	Panama (ND)	*T. cacao*, leaf	EU683662	EU683643	EU683632
E. endophytica	CBS 122547 = 7370	E. Rojas, L. Mejía & Z. Maynard	Panama (ND)	*T. cacao*, leaf	EU683666	EU683647	EU683635
E. endophytica	CBS 122546 = 7331	E. Rojas, L. Mejía & Z. Maynard	Panama (ND)	*T. cacao*, leaf	EU683661	EU683642	EU683631
E. endophytica	CBS 122545 = 7326	E. Rojas, L. Mejía & Z. Maynard	Panama (ND)	*T. cacao*, leaf	EU683663	EU683644	EU683633
E. endophytica	CBS 122544 = 8145	E. Rojas, L. Mejía & Z. Maynard	Panama (BCI)	*T. cacao*, leaf	EU683658	EU683639	—
E. endophytica	CBS 122551 = 7752	E. Rojas, L. Mejía & Z. Maynard	Panama (BCI)	*T. cacao*, leaf	EU683665	EU683646	—
E. endophytica	CBS 122543 = 9090	E. Rojas, L. Mejía & Z. Maynard	Panama (BCI)	*Heisteria concinna*, leaf	EU683670	EU683651	—
E. endophytica	CBS 122542 = 9185	E. Rojas, L. Mejía & Z. Maynard	Panama (BCI)	*H. concinna*, leaf	EU683669	EU683650	—
E. endophytica	9188	E. Rojas, L. Mejía & Z. Maynard	Panama (BCI)	*H. concinna*, leaf	EU683668	EU683649	—
E. endophytica	CBS 122552 = 9181	E. Rojas, L. Mejía & Z. Maynard	Panama (BCI)	*H. concinna,* leaf	EU683667	EU683648	—
Botryosphaeria ribis	CBS 122553 = PI006 (E.A. Herre)	E. Rojas, L. Mejía & Z. Maynard	Panama (BT)	*T. cacao*, leaf	EU683673	EU683654	—
B. ribis	CBS 115475	G. Hudler	USA, New York	*Ribes* sp.	AY236935	AY236877	—
B. ribis	C.M.W. 7773	G. Hudler	USA, New York	*Ribes* sp.	*AY236936*	*AY236878*	—
B. dothidea	C.M.W. 8000	B. Slippers	Switzerland, Crocifisso	*Prunus* sp.	*AY236949*	*AY236898*	—
B. dothidea	CBS 110302	A.J.L. Phillips	Portugal, Montemor-o-Novo	*Vitis vinifera*	*AY259092*	*AY573218*	—
B. lutea	C.M.W. 9076	S.R. Pennycook	New Zealand	Malus × domestica	*AY236946*	*AY236893*	—
B. lutea	**CBS 110299**	A.J.L. Phillips	Portugal, Oeiras	*V. vinifera*	*AY259091*	*AY573217*	AY928043
B. australis	CBS 112872	F. Halleen	South Africa	*V. vinifera*	*AY343388*	*AY343347*	—
B. australis	CBS 112877	F. Halleen	South Africa	*V. vinifera*	*AY343385*	*AY343346*	—
Fusicoccum viticlavatum	**CBS 112977**	F. Halleen	South Africa	*V. vinifera*	*AY343381*	*AY343341*	—
F. vitifusiforme	**CBS 110880**	J.M. van Niekerk	South Africa	*V. vinifera*	*AY343382*	*AY343344*	—
B. parva	**C.M.W. 9081**	G.J. Samuels	New Zealand, Te Puke	*Populus nigra*	*AY236943*	AY236888	—
B. parva	CBS 110301	A.J.L. Phillips	Portugal, Palmela	*V. vinifera*	*AY259098*	*AY573221*	AY928046
B. stevensii	CBS 431.82	H.A. van der Aa	Netherlands, Maarseveen	*Fraxinus excelsior*	AY236955	*AY236904*	DQ377863
B. stevensii	CBS 112553	A.J.L. Phillips	Portugal, Montemor-o-Novo	V. vinifera	*AY259093*	*AY573219*	—

TABLE I. Continued

Species	Cultures[1]	Collector	Geography[2]	Substratum	GenBank accession number[3]		
					ITS1 + 2	*tef1*	LSU
B. obtusa	CBS 112555	A.J.L. Phillips	Portugal, Montemor-o-Novo	*V. vinifera*	*AY259094*	*AY573220*	AY928050
B. obtusa	C.M.W. 7775	B. Slippers & G. Hudler	USA, New York	*Ribes* sp.	*AY236954*	*AY236903*	—
B. corticola	**CBS 112549**	A. Alves	Portugal, Aveiro	Quercus suber	*AY259100*	*AY573227*	—
B. corticola	CBS 112545	M.E. Sánchez & A. Trapero	Spain, Cádiz	*Q. suber*	*AY259089*	*AY573226*	—
B. rhodina	C.M.W. 10130	J. Roux	Uganda	*Vitex donniana*	*AY236951*	*AY236900*	—
B. rhodina	C.M.W. 9074	T. Burgess	Mexico	*Pinus* sp.	*AY236952*	*AY236901*	—
Diplodia porosum	**CBS 110496**	J.M. van Niekerk	South Africa, Stellenbosch	V. vinifera	*AY343379*	*AY343340*	DQ377894
D. porosum	CBS 110574	J.M. van Niekerk	South Africa	V. vinifera	*AY343378*	*AY343339*	—
B. viticola	**CBS 117009**	J. Luque & S. Martos	Spain, Vimbodí	*V. vinifera* cv. Garnatxa Negra	*AY905554*	*AY905559*	DQ377873
B. viticola	CBS 117008	J. Luque & J. Reyes	Spain, Sant Sadurní d'Anoia	*V. vinifera* cv. Xarel-lo	*AY905557*	*AY905560*	—
B. viticola	CBS 117006	J. Luque & R. Mateu	Spain, Vimbodí	*V. vinifera* cv. Garnatxa Negra	*AY905555*	*AY905562*	—
Diplodia sp.	CBS 112870	J.M. van Niekerk	South Africa, Western Cape	V. vinifera	*AY343376*	*AY343337*	—
Diplodia sp.	CBS 112869	J.M. van Niekerk	South Africa, Western Cape	V. vinifera	*AY343373*	*AY343336*	—
B. sarmentorum	**IMI 63581b**	E.A. Ellis	England, Warwickshire	*Ulmus* sp.	*AY573212*	*AY573235*	AY928052
B. sarmentorum	CBS 115038	A.J.L. Phillips	Netherlands, Delft	*Malus pumila*	*AY573206*	*AY573223*	—
B. iberica	**CBS 115041**	J. Luque	Spain, Aragon	*Q. ilex*	*AY573202*	*AY573222*	DQ377853
B. iberica	CBS 115040	J. Luque	Spain, Catalonia	*Q. ilex*	*AY573214*	*AY573232*	—
Guignardia mangiferae	1095	E. Rojas, L. Mejía & Z. Maynard	Panama (DI)	*T. cacao*, leaf	EU683671	EU683652	—
G.bidwellii	CBS 111645	G. Carroll	USA, Missouri	*Parthenocissus quinquefolia*	EU683672	EU683653	DQ377876

[1] Abbreviations of culture collections: CBS = Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; IMI = CABI Genetic Resource Collection, Egham, UK; C.M.W. = C.M. Wingfield. Accession numbers in boldface signify ex-type cultures.
[2] Geographic regions in Panama abbreviated as ND = Nombre de Dios, BCI = Barro Colorado Island, DI = Divisa, BT = Bocas del Toro.
[3] Sequence numbers in italic were retrieved from GenBank. All others were obtained in this study.

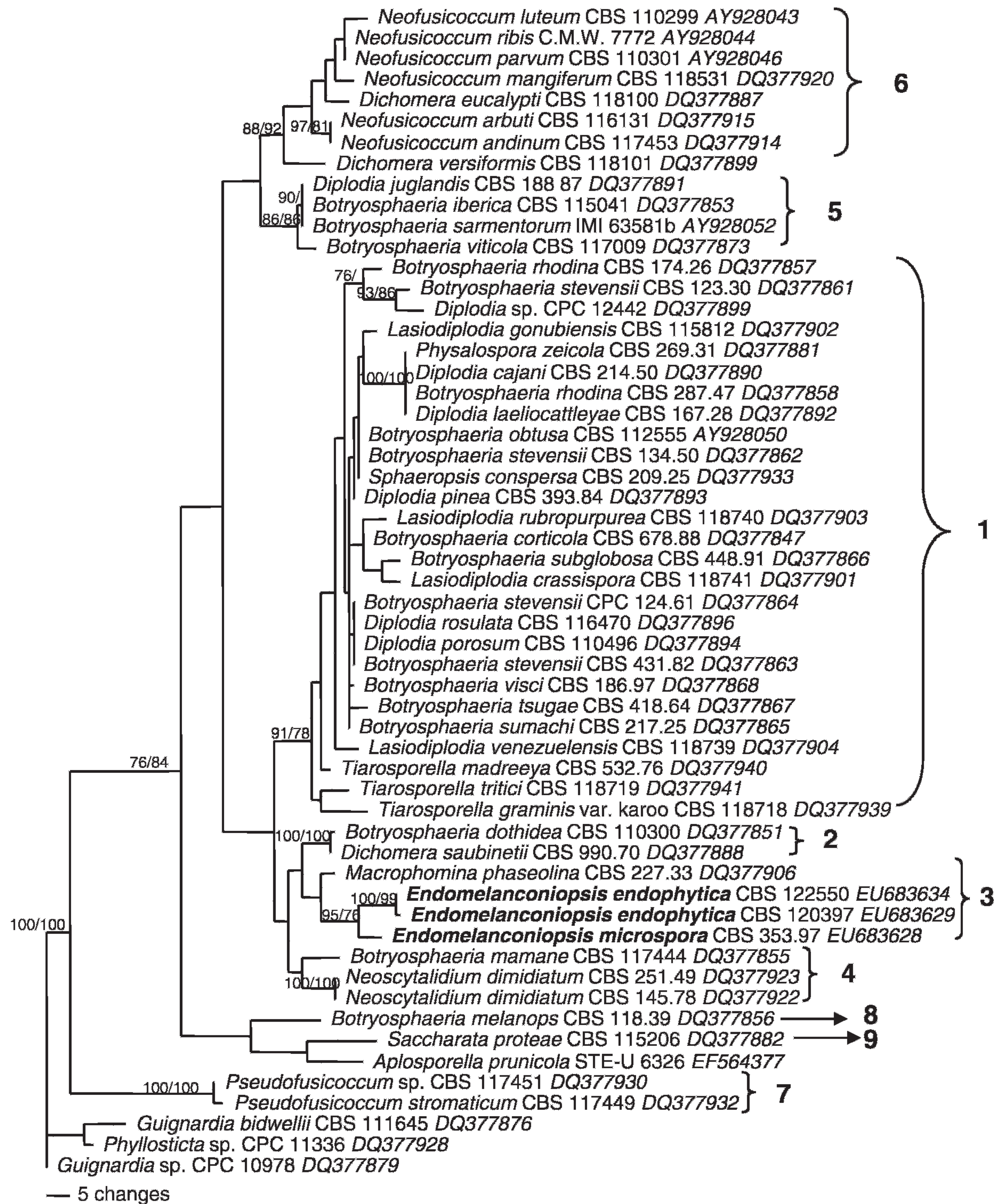


FIG. 1. Parsimony tree based on LSU sequence. The tree was rooted to *Guignardia bidwellii*, *Guignardia* sp, and *Phyllosticta* sp. This tree is modified from Crous et al (2006), to which the reader should refer for full discussion of the clades; numbers on the right side refer to clades in Crous et al (2006). Numbers in the nodes refer to bootstrap support values from 1000 replicates of NJ and MP respectively.

differing growth rate, were not significantly different in size from others (6.7 ± 0.3 × 4.1 ± 0.2 μm; 95% CI 6.6–6.8 × 4.1–4.2). Macroconidia of *E. microsporum* were of intermediate length but wider (FIG. 19; 6.1 ± 0.4 × 4.6 ± 0.4 μm; 95% CI 6.0–6.3 × 4.4–4.7 μm) than those of either of the endophyte size groups (FIG. 19). No differences were observed in the size of the conidiogenous cells of the endophyte groups.

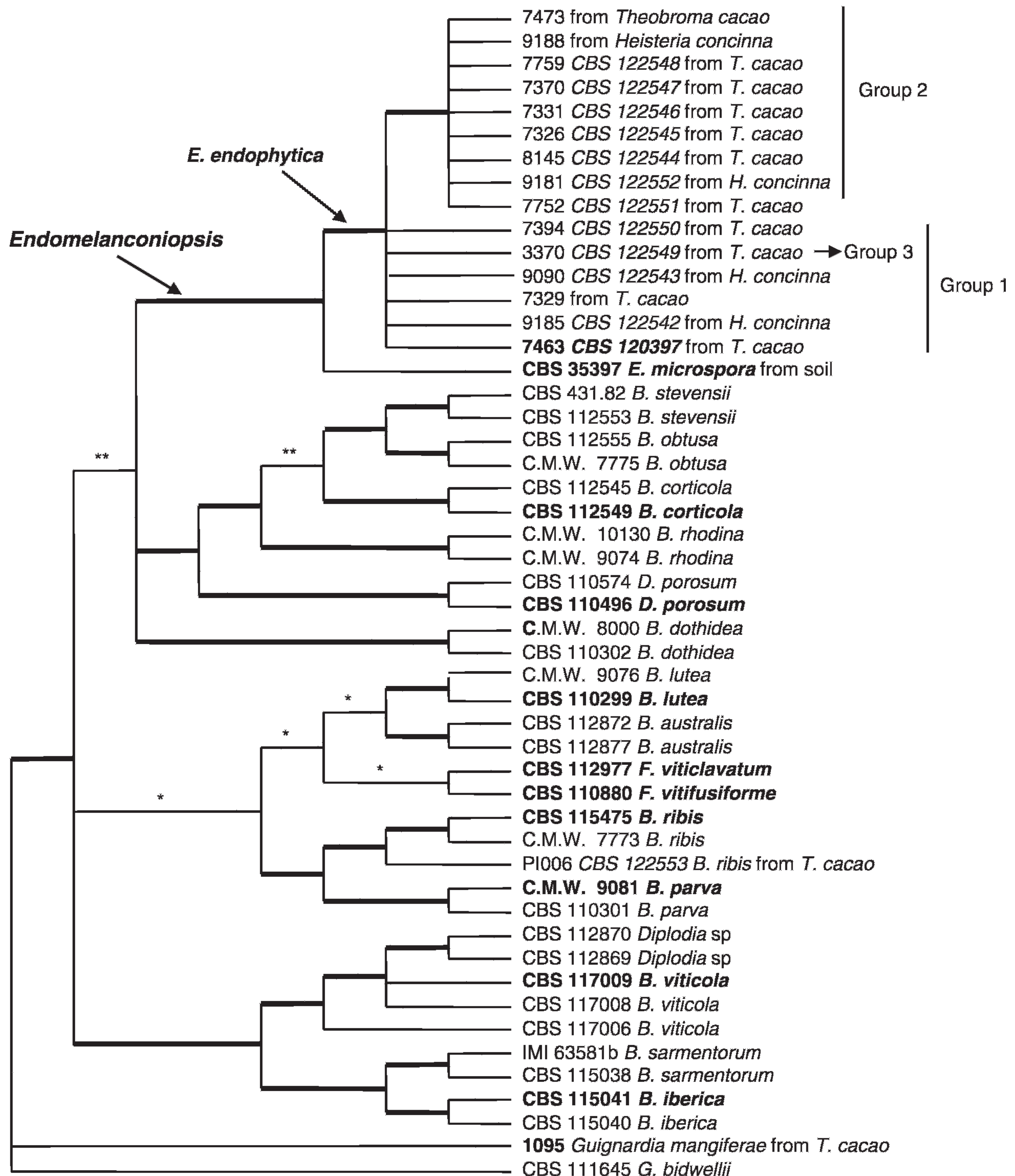
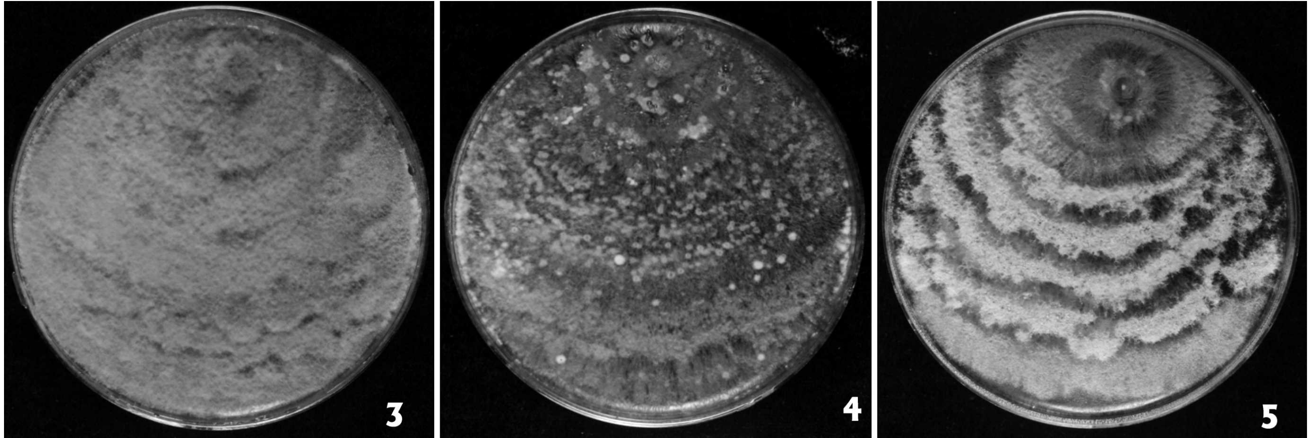


FIG. 2. Bayesian inference tree (majority rule consensus of 5600 trees) based on the combined ITS and *tef1* sequences. Fifteen *Endomelanconiopsis* isolates from *T. cacao* and *H. concinna* were included. Tree was rooted with *Guignardia mangiferae* and *G. bidwellii.* Black lines are $\geq$ 0.95 posterior probability coefficients and $\geq$ 70% NJ and MP bootstrap support; (*) represents < 0.95 posterior probability coefficients and $\geq$ 70% NJ and MP bootstrap support; (**) represents < 0.95 posterior probability coefficients and < 70% NJ and MP bootstrap support. Group numbers refer to phenotype-defined groups in *Endomelanconiopsis endophytica.* Accession numbers in boldface signify ex-type cultures.



FIGS. 3–5. *Endomelanconiopsis endophytica*, three groups on PDA after10, 25 C, 12 h cool white fluorescent light/12 h dark. 3 Group 1 (7394). 4. Group 2 (7463). 5. Group 3 (3370).

Colonies on PDA become dark gray to black and the quantity of aerial mycelium was variable. Optimum temperature for growth on PDA and SNA was 30–35 C. Three growth curves were observed after 5 d on PDA (FIG. 20). Two of these curves corresponded to the macroconidium-size groups noted above: Group 1 (n = 5) colony radius at 30 C the same as the colony radius at 37 C (ca. 50 mm); Group 2 (n = 9) colony radius at 30 C (ca. 50 mm) that was less than the radius at 37 C (55–60 C); Group 3 (n = 1) colony radius at 30 C (ca. 45 mm) greater than the radius at 37 C (ca. 35 mm). All endophytic isolates grew well at 37 C, although isolate 3370 (Group 3) was significantly slower at 30 and 37 C than were the other cultures. Pycnidia usually formed within 1 wk at 25 C under alternating light and dark.

In the phylogram (FIG. 2) Group 2 forms a well supported clade that is sister of Group1/Group 3. Thus Group 1 isolates had shorter conidia and relatively slower growth at 37 C while Group 2 isolates had longer conidia and faster growth at 37 C. Group 3, which comprised a single culture, had conidia of intermediate size and a significantly slower rate of growth than Groups 1 or 2. Despite the phenotypic differences, the phylogenetic distance between these sister groups is small (only five bp difference in *tef*). We consider the phenotypic differences to represent intraspecific variation, but additional collections might support taxonomic subdivision.

Conidia of the endophytic endomelanconium-like species are most similar in size to those of *E. microsporum* and smaller than the reported measurements of all other *Endomelanconium* species (TABLE II). As noted above conidia of *E. microsporum* are wider (4.4–4.7 μm) than the endophytes. A further difference between the endophytes and *E. microsporum* is the absence of chlamydospores in the former (see Verkley and van der Aa 1997). Microconidia have not been reported for any other *Endomelanconium* species, but the describing authors could have overlooked them. We did not notice them in the ex-type culture of *E. microsporum*.

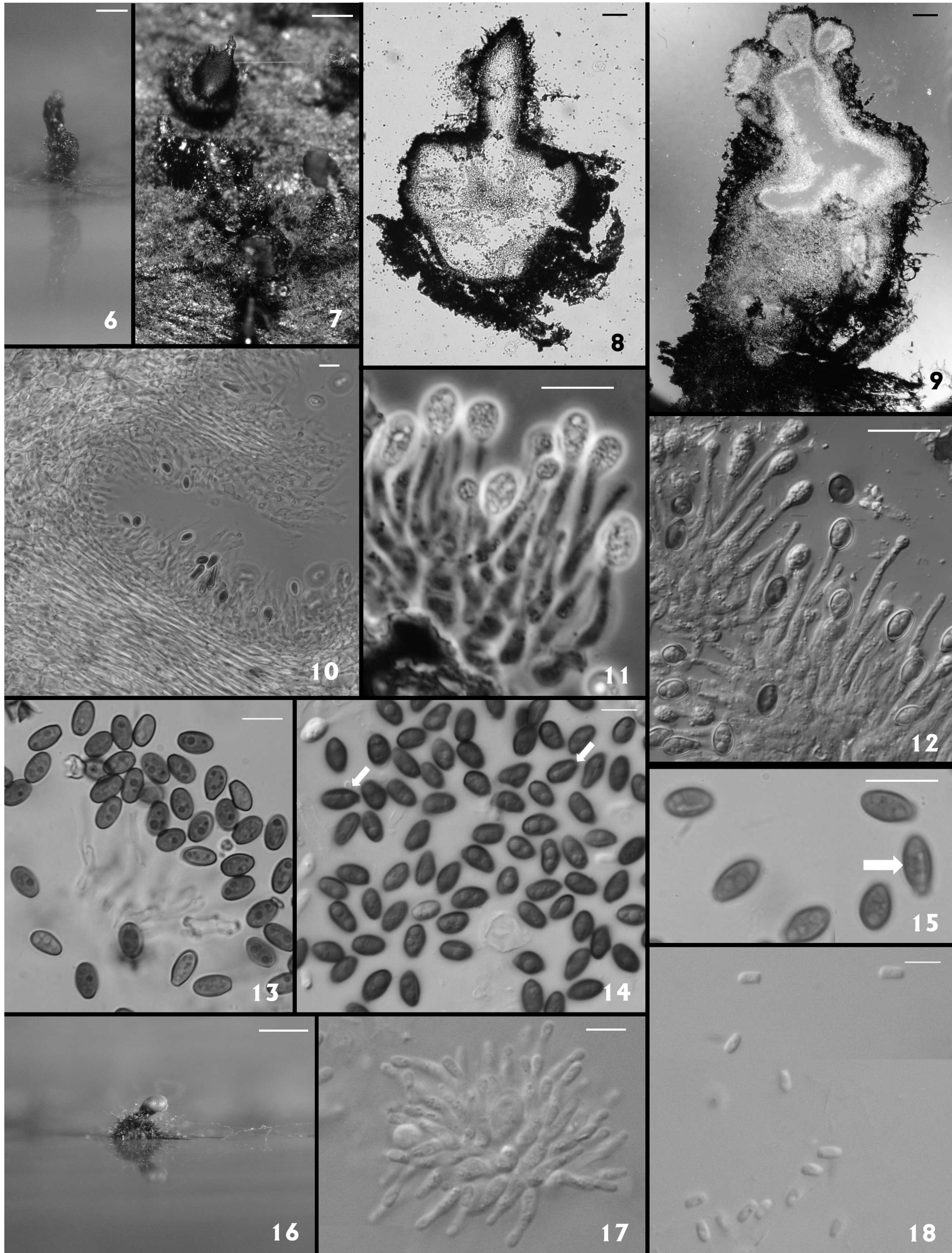
These differences, combined with the respective differences in habitat (leaf endophyte vs. soil) and geography (Central America vs. southwestern Pacific) and our phylogenetic results, distinguish the endophytes from *E. microsporum* at the species level.

Comparison with Endomelanconium *species*.—Only five species have been included in *Endomelanconium*, including *E. microsporum* discussed above and the unnamed anamorph of *Austrocenangium australe* (Speg.) Gamundí (TABLE II). The type species of *Endomelanconium*, *E. pini* (Corda) Petrak, has been collected from dead trunks and branches of *Abies* from Switzerland, Austria, Germany, the Czech Republic and Slovakia but apparently has not been grown in culture. As a result we did not include it in our phylogenetic analysis. No teleomorph is known for it.

There are small but significant differences between *E. pini* and the two endomelanconium-like species noted above. We studied one specimen of *E. pini*

→

FIGS. 6–18. *Endomelanconiopsis endophytica/Botryosphaeria* anamorph. 6–9. Pycnidia. 6. From SNA. 7. From PDA. 8, 9. Pycnidia showing convoluted hymenium in the locule. 10–12. Conidiogenous cells. 10, 11. Conidiogenous cell arising from pycnidial wall cells. 11, 12. Holoblastic conidiogenous locus. 13–15. Macroconidia, opaque brown, unicellular, often flattened



at the base. 14, 15. Germ slit on the flattened edge marked with arrow. 16. Pycnidium producing microconidia on SNA. 17. Phialidic microconidial conidiogenous cells. 18. Microconidia ellipsoidal to subcylindrical, hyaline. FIG. 6 from 9090; 7, 8, 10, 14 from 7463; 9 from 9181; 11, 12 from 9188; 13 from 7331; 15 from 3370; 16–18 from 8145. Bars: 6, 7 = 0.5 mm; 8, 9 = 200 μm; 10 = 20 μm; 11–15 = 10 μm; 16 = 0.5 mm; 17, 18 = 10 μm.

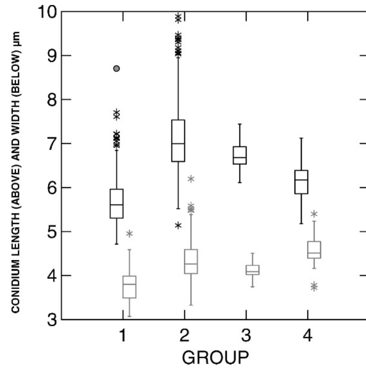


FIG. 19. *Endomelanconiopsis endophytica*. Box plot showing conidium length (above, black line) and width (below, gray line) of three phenotype groups. Group 4 is *E. microsporum*.

(Krieger, Fungi Saxonici 1450; BPI 402518) and made the following observations. Conidiogenous cells of *E. pini* tend to proliferate by pushing aside the first-formed conidiogenous cell, which remains as a spur, to form up to two more conidiogenous cells (FIGS. 21–24). Conidiogenesis is holoblastic, but the point of attachment of the conidium to the conidiogenous cell is narrow, which results in the conidia having a papillate base when they are discharged (FIGS. 25–28). Papillate conidia also were noted by Petrak (1940) when he proposed *Endomelanconium*. Conidiogenous cells of *E. nanum* Gamundí & Arambari (Gamundí and Arambari 1983, Suarez et al 2000) and the unnamed *Endomelanconium* anamorph of *Austrocenangium australe* (Gamundí 1997) are illustrated as being narrowed at the point of dehiscence of conidia and conidia are papillate. Conidia of *E. phoenicicola* Yanna et al (Yanna et al 1999) have a flat, somewhat protuberant base but the conidiogenous cell is not illustrated as being constricted at the point of dehiscence. Pycnidia of *E. pini* are subcortical and have a convoluted hymenium; the walls are made of small-celled pseudoparenchyma (Petrak 1940, Sutton 1980) but there is minimal stromatic development (see also Sutton 1980), unlike typical botyosphaeriaceous anamorphs. Even though our endomelanconium-like fungi are known only from cultures their pycnidial walls are formed of thick-walled pseudoparenchymatous cells (see Verkley and van der Aa 1997, FIG. 2). Conidia of *E. pini* are extruded in a slimy, black mass that may extend considerably beyond the erumpent pycnidia (also described by Petrak 1940).

The intimate juxtaposition of *E. nanum* (Cash) Gamundí and the unnamed *Endomelanconium* anamorph with their respective *Austrocenangium* Gamundí teleomorphs (Helotiales) leave no doubt of their respective conspecficity (Gamundí et al 1983, Gamundí 1997). Based on the observations noted above, the endomelanconium-like fungi that we discussed are unlikely to be phylogenetically related to *E. pini*, which appears to be more similar to the helotiaceous anamorphs of *Austrocenangium*.

The anamorph genus *Endomelanconium* Petrak is paraphyletic. There are significant differences in pycnidial and conidial morphology and in proliferation of conidiogenous cells among the known '*Endomelanconium*' species (TABLE II). *Endomelanconium nanum* and the unnamed *Endomelanconium* anamorph of *A. australe* (Gamundí and Arambarri Gamundí 1997), and most likely *E. pini*, are helotiaceous anamorphs. Whether the *Austrocenangium* anamorphs are congeneric with *E. pini* remains to be proven, and proliferating conidiogenous cells are not reported for either anamorph. Our endomelanconium-like endophyte and *E. microsporum* are obviously Dothideomycetes and not likely to be congeneric with *E. pini*. However, apart from a broadly circumscribed *Endomelanconium*, we do not know of any genus that can accommodate them. Consequently we propose a new genus, *Endomelanconiopsis*. The illustrations of *E. phoenicicola* (Hyde et al 1999) suggest that it is also botryosphaeriaceous, possibly a species of *Endomelanconiopsis*; the describing authors did not report the deposit of cultures of this species.

TAXONOMY

Endomelanconiopsis Rojas et Samuels, gen. nov.

Genus *Endomelanconii* Petrak simile sed ad *Botryosphaeriaceas* pertinens et conidia non papillata.

Species typica: *Endomelanconiopsis endophytica* Rojas et Samuels

Endomelanconiopsis endophytica Rojas et Samuels, sp. nov. FIGS. 3–18

Endomelanconio microsporo Verkley & van der Aa simile, sed conidia (4.7–)5.5–7.5(–10.0) × (3.0–)3.5–4.5(–6.2) µm producens et chlamydosporae absunt; species endophytica.

Holotypus: Ex culto agaro sicco BPI 878370.

Mycobank 11838

Optimum temperature at 30–37 C, colony radius 43–55 mm after 5 d (n = 15) on PDA. *Colonies* at first colorless with hyaline immersed hyphae, after 4 d colonies olivaceous in center and concentric rings with irregular shape, after 10 d aerial mycelium dense dark olivaceous or gray (Groups 1, 3) or shiny black with little aerial mycelium (Group 2). *Conidiomata* eustromatic, scattered throughout colony, varying from globose to cylindrical, 1–3 cylindrical necks, superficial or immersed in the agar; often cylindrical papillae protruding from the agar in groups of a few.

TABLE II. Comparison of *Endomelanconiopsis* and *Endomelanconium* species

Character	*Endomelanconiopsis endophytica*[1]	*Endomelanconiopsis microspora*[1]	*Endomelanconium nanum* Gamundí and Arambarri 1983	*E. phoenicicola* Yanna et al 1999	*E. pini* Sutton 1980	*Endomelanconium* anamorph, *Austrocenangium australe* Gamundí 1997
Teleomorph	Unknown, Botryosphaeriaceae	Unknown, Botryosphaeriaceae	*Austrocenangium*, Helotiales	Unknown	Unknown	*Austrocenangium*, Helotiales
Conidium, length (µm)	4.7–8.7 (Group 1) 6.1–7.4 (Group 2) 5.1–9.9 (Group 3)	5.4–6.4	7.2–10	10–12 (av = 11.23)	11.5–13.5	12–13
Conidium, width (µm)	3.1–4.9 (Group 1) 3.7–4.5 (Group 2) 3.3–6.2 (Group 3)	4–4.6	3.8–5.8	9–11 (av = 10.33)	6.5–7.5	9–10
Conidium base	flat to rounded	flat to rounded	papillate	flat	papillate	papillate
Conidiogenous cell, length (µm)	7.8–23.4	6–10	7.9–10.5	14–25 (av = 19)	7–16	9–12
Conidiogenous cell, apex width (µm)	0.7–3.5	—	—	2–4 (av = 2.88)	—	
Conidiogenous cell, base width (µm)	1.3–4.1	5–7	4.1–4.7	2–5 (av = 3.25)	3–7	2.5–4.5
Microconidia, length (µm)	1.8–4.4 (Group 1) 2.9–10 (Group 3)	—	—	—	—	—
Microconidia, width (µm)	0.9–1.9 (Group 1) 0.9–2.8 (Group 3)	—	—	—	—	—
Chlamydospores (µm)	—	9–17 × 6–10	—	—	—	—
Host	*Theobroma cacao* and *Heisteria concinna*, Leaf endophytes	Soil	Plant debris	*Phoenix hanceana*, dead petiole	*Abies pectinata*, *A. alba*, *A. excelsa*; dead trunks and branches	*Nothofagus betuloides*, fallen branches

[1] Upper and lower 95% confidence intervals.

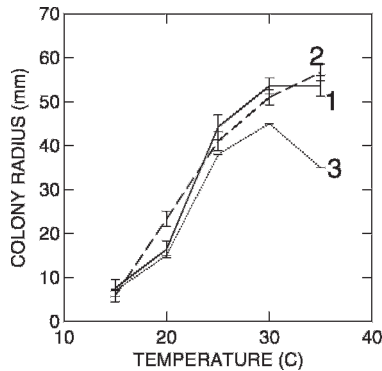


FIG. 20. *Endomenanconiopsis endophytica*. Growth curves of three groups on PDA after 72 h in intermittent light. Lines = standard error. A total of 5, 9 and 1 isolates were measured respectively in Groups 1, 2 and 3.

Wall comprising pale brown and black angular cells, becoming hyaline and more hyphal toward the *conidiogenous cells*. *Pycnidial* locule convoluted, completely lined with conidiogenous cells. *Conidiogenous cells* formed from the inner cells all over the conidiomata wall, discrete, determinate, cylindrical, tapered toward the apex, hyaline, holoblastic, rarely with a single percurrent proliferation, (7.8–23.4 × 0.8–3.5 [apex] 1.3–4.1 [base] µm [Mean = 14.2 µm × 1.6 µm apex × 2.2 µm base]). *Conidia* ellipsoidal to limoniform, apex rounded, base flat to rounded, aseptate, hyaline when immature, dark brown with a single longitudinal slit three-quarters of the length of the conidia when mature, (4.7–)5.5–7.5(–10.0) × (3.0–)3.5–4.5(–6.2) µm (n = 399). Microconidia forming in the same locules as macroconidia from densely arranged, enteroblastic, phialidic conidiogenous cells, appearing to arise from the inner cells of the pycnidial wall. *Microconidia* ellipsoidal to allantoid, 2.0–7.0(–10.0) × (1.0–2.0(–3.0) µm (n = 80), formed on PDA and SNA. *Chlamydospores* not observed.

Etymology. *endophytica*, referring to the occurrence of this fungus as an endophyte.

HOLOTYPE: PANAMA. Nombre de Dios, isolated from leaves of *Theobroma cacao*, 2000, *E. Rojas, L. Mejía & Z. Maynard 7463* (BPI 878370, ex-type culture CBS 120379).

Commentary. Additional collections are cited (TABLE I). The description given above is based on several of these cultures grown on PDA, and it amalgamates the diverse measurements of the groups discussed in RESULTS. On SNA there was little mycelial production and colonies were transparent. Pycnidia formed often at the edge of the colony at the interface between the agar and plastic of the Petri plate; they were much smaller than on PDA, nearly flask-shaped with one or more papillae.

Endomelanconiopsis microspora (Verkley & van der Aa) Rojas et Samuels, comb. nov.

≡ *Endomelanconium microsporum* Verkley & van der Aa, Mycologia 89:967. 1997.

Mycobank 5111839

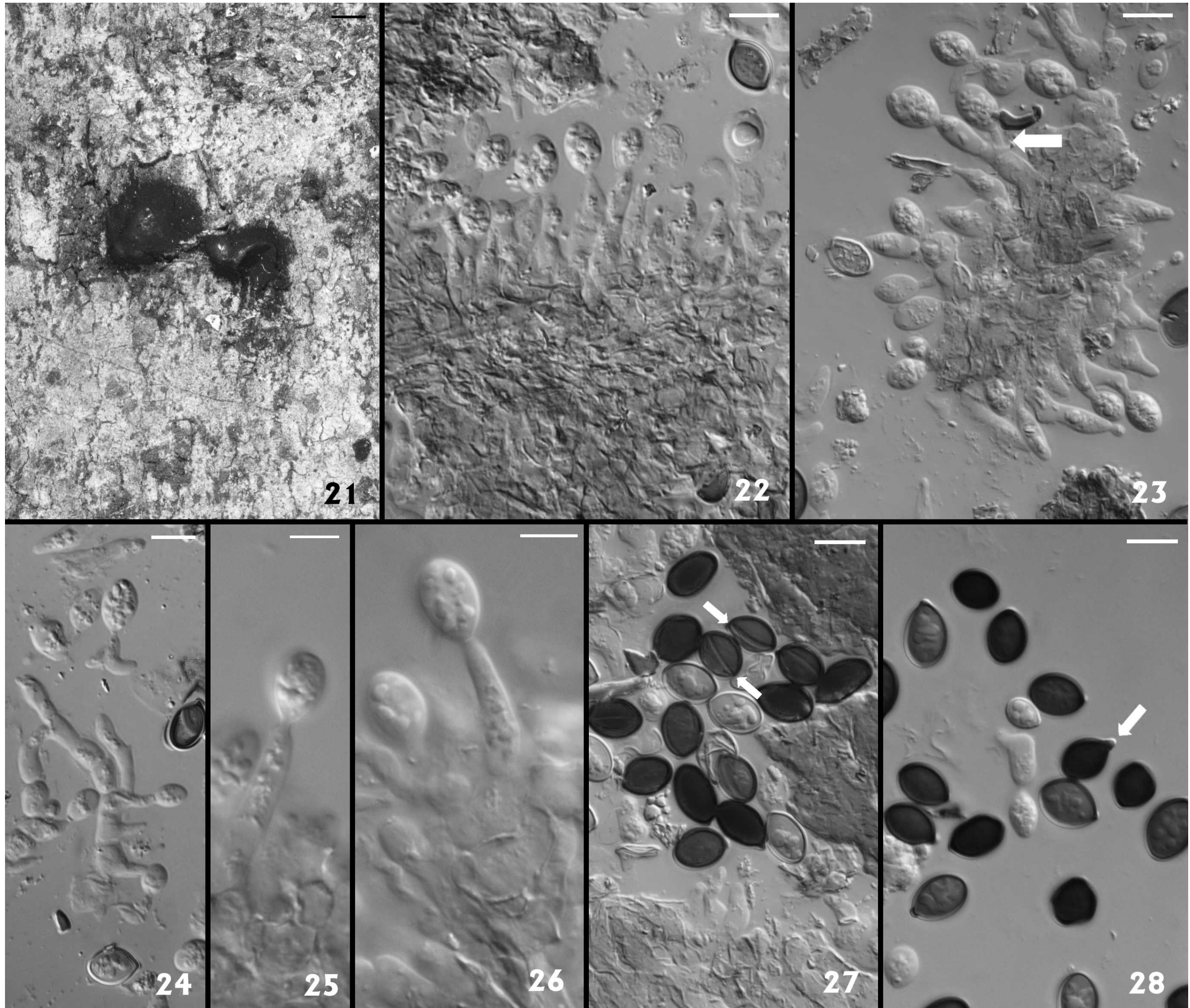
This species is described and illustrated in Verkley and Van der Aa (1997).

DISCUSSION

It comes as no surprise that *Endomelanconiopsis* is derived from within the Botryosphaeriaceae. Rapidly spreading dark colonies on PDA that characterize *E. endophytica* and *E. microspora* (Verkley and van der Aa 1997) are typical of *Botryosphaeria s.l.* (see Phillips 2008 and colony descriptions in Punithalingham 1978, Samuels and Singh 1986, Alves et al 2004, Luque et al 2005). The eustromatic pycnidia with often convoluted locules (appearing multiloculate) and holoblastic conidiogenous cells arising directly from cells of the pycnidial wall are further characteristics as are the rather large, unicellular conidia that are often truncate at the base. Microconidia (spermatia) usually are not reported for '*Botryosphaeria*' anamorphs (e.g. Pennycook and Samuels 1985, Samuels and Singh 1986, Phillips et al 2005), but they probably are produced by all species.

Endomelanconiopsis is unusual in having a germ slit in the conidia. In the Botryosphaeriaceae we are aware only of *Neodeightonia subglobosa* C. Booth (teleomorph *Botryosphaeria subglobosa* [C. Booth] Arx. & E. Müller) as having similar conidia (see Punithalingham 1969). Crous et al (2006) observed germ slits in conidia of this species and compared it to *Lasiodiplodia theobromae*, the striations of which were postulated to be germ slits. Despite the similarity of conidia of *E. endophytica* and *N. subglobosa*, the two species are not closely related (FIG. 1).

Botryosphaeria and the Botryosphaeriaceae have attracted considerable attention in recent years, with the result that the number of identified species is increasing rapidly along with the number of associated anamorph generic names (see Crous et al 2006, Damm et al 2007). A process of dismembering *Botryosphaeria* based in part on the anamorph phenotype and in part on phylogenetic analysis is under way (Crous et al 2006, Shenoy et al 2007). There are challenges to defining genera on these bases. First, as can be seen in (FIG. 1) there is no support for the backbone of the Botryosphaeriaceae when species of the closely related *Guignardia* are used as outgroup. The strongly supported lineages (FIG. 1), which is derived from Crous (2006) but with a different outgroup, could be interpreted equally as genera or as species of *Botryosphaeria*. That being the



FIGS. 21–28. *Endomelanconium pini*. 21. Two immersed pycnidia marked by glistening black masses of extruded conidia. 22–26. Holoblastic conidiogenous cells. 23. A spur-like sympodial proliferation (arrow). 24. A chain of conidiogenous cells. 23, 25, 26. Constriction at the point of attachment of the conidium to the conidiogenous cell. 27–28. 27. Conidia, germ slits at arrow. 28. A papillate conidial base at arrow. From Krieger, Fungi Saxonici 1450; BPI 04024518. Bars: 21 = 0.5 mm, 22–28 = 10 μm.

case the argument for recognizing the lineages must depend on phenotype and/or biology. There is a uniform biology in the Botryosphaeriaceae (see Phillips 2008); they cause cankers, root diseases and postharvest fruit rot. Teleomorphs are very similar. Most generic differences are attributed to conidial morphology, but while there is broad agreement of anamorph phenotype with clades (see Crous et al 2006) this is not consistent. Two primary examples include the distantly related yet apparently morphologically indistinguishable *Endomelanconiopsis*, described here, and *Neodeightonia subglobosa*. Similarly the highly distinctive *Lasiodiplodia* morphology occurs in two or more clades (Crous et al 2006). Thus whether these lineages represent genera or species can be questioned reasonably. We have adopted the nomenclature summarized in Crous et al (2006) and in the *Botryosphaeria* Website (Phillips 2008) but not without misgivings.

Endomelanconiopsis endophytica was the dominant endophytic fungus isolated from mature to old cacao leaves taken from the one tree that was the subject of the second survey at Nombre de Dios in Jul 2000. However it was not encountered in any other tree at Nombre de Dios during the first survey, 1999 and 2000. Thus it appears to vary greatly in its occurrence over time, across sites and possibly among hosts. In the present work we discuss only endophytic isolates

from *Theobroma* and *Heisteria*. However isolates with identical ITS sequences and consistent colony morphology have been recovered from additional trees and shrubs representing the Flacourtiaceae, Meliaceae and Ochnaceae at Barro Colorado Island in Panama (Arnold unpubl). It is likely that *E. endophytica* has a much wider host and geographic range than we record here. It also is strongly possible that reports of endophytic isolates of species such as *Diplodia juglandis*, *Botryosphaeria sarmentorum*, *B. viticola* or *B. iberica*, all of which give 97% maximum identity and 100% sequence coverage (LSU locus), actually could refer to *E. endophytica*. The wide host range is reminiscent of *Guignardia mangiferae* (anam: *Phyllosticta capitalensis*), a ubiquitous, cosmopolitan endophyte of many genera of woody plants (Baayen et al 2002), and a species that we also have recovered as an endophyte from leaves of cacao. These fungi are known only as endophytes in a wide range of hosts.

ACKNOWLEDGMENTS

The authors thank Amy Rossman for comments on this manuscript; Adnan Ismaiel (BPI) for valuable advice during the sequencing phase of this project; Zuleyka Maynard, Luis Ramirez and Terri Shirshac for help with the culture collection; and USDA-ARS-SMML and the Smithsonian Tropical Research Institute for research infrastructure and logistical support. Walter Gams and Orlando Petrini corrected the Latin diagnoses. Financial support was provided by World Cocoa Foundation (WCF), the John Clapperton Fellowship of Mars Inc., the Andrew W. Mellon Foundation and the Smithsonian Tropical Research Institute. ER was the recipient of a Norman E. Borlaug Fellowship in support of this work.

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