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Treena I. Burgess, Paul A. Barber, Sari Mohali, Geoff S. Pegg ...+2 more authors

Institutions: Murdoch University, University of Los Andes, University of Pretoria

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Three new *Lasiodiplodia* spp. from the tropics, recognized based on DNA sequence comparisons and morphology

Treena I. Burgess[1]
Paul A. Barber
School of Biological Sciences and Biotechnology, Murdoch University, Perth 6150, Australia

Sari Mohali
Universidad de Los Andes, Facultad de Ciencias Forestales y Ambientales, Laboratorio de Patologia Forestal, Merida, Venezuela

Geoff Pegg
Department of Primary Industries and Fisheries, Horticulture and Forestry Science, Indooroopilly, Brisbane 4068, Australia

Wilhelm de Beer
Michael J. Wingfield
Forestry and Agriculture Biotechnology Institute, University of Pretoria, Pretoria, 0002, Republic of South Africa

Abstract: *Botryosphaeria rhodina* (anamorph *Lasiodiplodia theobromae*) is a common endophyte and opportunistic pathogen on more than 500 tree species in the tropics and subtropics. During routine disease surveys of plantations in Australia and Venezuela several isolates differing from *L. theobromae* were identified and subsequently characterized based upon morphology and ITS and EF1-α nucleotide sequences. These isolates grouped into three strongly supported clades related to but different from the known taxa, *B. rhodina* and *L. gonubiensis*, These have been described here as three new species *L. venezuelensis* sp. nov., *L. crassispora* sp. nov. and *L. rubropurpurea* sp. nov. The three could be distinguished easily from each other and the two described species of *Lasiodiplodia*, thus confirming phylogenetic separations. Furthermore all five *Lasiodiplodia* spp. now recognized separated from *Diplodia* spp. and *Dothiorella* spp. with 100% bootstrap support.

Key words: *Botryosphaeria*, *Diplodia*, *Dothiorella*, *Fusicoccum*, ITS, molecular phylogenetics, translation elongation factor EF1-α

INTRODUCTION

Botryosphaeria Ces. & de Not. includes pathogenic fungi that cause cankers and dieback on a wide range of woody hosts (von Arx 1987). The majority of *Botryosphaeria* spp. have a cosmopolitan distribution and have been found on most continents and on numerous hosts. They are rarely primary pathogens, instead causing stress-related diseases or perennial cankers. In temperate climates *B. ribis, B. parva* and *B. dothidea* are the most common species isolated from cankers, while in the tropics *B. rhodina* predominates (Punithalingam 1980).

Numerous anamorphs have been assigned to *Botryosphaeria* spp., but recent studies based on DNA sequence comparisons have indicated there is a clear phylogenetic boundary between species with thin-walled, hyaline conidia and those with thick-walled, pigmented spores (Denman et al 2000, Zhou and Stanosz 2001). Those with hyaline conidia have been assigned a *Fusicoccum* anamorph and those with pigmented conidia a *Diplodia* anamorph (Denman et al 2000). However, among the species with pigmented conidia, *B. rhodina* always groups separately from the other species (Denman et al 2000, Zhou and Stanosz 2001, Pavlic et al 2004, Slippers et al 2004). The anamorph of *B. rhodina, Lasiodiplodia theobromae*, has conidia much larger than other *Diplodia* species and currently retains the name *Lasiodiplodia* (Punithalingam 1976, Pavlic et al 2004, Phillips et al 2005).

The taxonomic history of *B. rhodina/L. theobromae* is confused. During the past 150 y this fungus has had many names and has been treated as many different species. This trend ended with the monograph of Punithalingam (1976) which reduced most species to synonymy with *L. theobromae*. Recently Pavlic et al (2004) conducted an extensive review of *Lasiodiplodia* literature and searched for herbarium specimens associated with original descriptions of the genus (Clendinin 1896) and its species (Patouillard and de Lagerheim 1892). These could not be found and cultures from the original host and location, *Theobroma cacao* in Ecuador, also were not located. It thus was necessary to rely on descriptions from the literature and Pavlic et al (2004) found that isolates from USA, South America, South Africa and Asia typically have conidia that are 18–30 × 10–15 µm. This led to the description of the new species *L. gonubiensis*, which could be distinguished by both DNA-based phylogenies and morphological characteristics.

In a study using SSR markers to examine host relationships and geographic isolation among isolates

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[1] Corresponding author. E-mail: tburgess@murdoch.edu.au

of *L. theobromae*, Mohali et al (2005) identified several isolates that shared no common alleles with 177 other isolates at any of the eight SSR loci. This is considered to be indicative of the presence of related but different species. In addition disease surveys in tropical Australia have led to the discovery of several *Lasiodiplodia* isolates with conidia morphologically different from those of *L. theobromae*. These observations prompted the current study aimed at characterizing isolates of *Lasiodiplodia* based on morphology and multiple gene genealogies.

MATERIALS AND METHODS

Isolates.—During routine sampling in 2002/2003 and subsequent studies using SSR markers, a number of fungi resembling *Lasiodiplodia theobromae* were collected. Closer examination of some showed cultural characteristics that distinguished them from *L. theobromae sensu stricto*. These collections included two isolates from cankered sandalwood (*Santalum album*) in Kununnara, Western Australia, one isolate from *Eucalyptus urophylla* in Venezuela, three isolates from *Acacia mangium* in Venezuela and four isolates collected from cankered *E. grandis* near Tully, North Queensland (TABLE I).

DNA sequence comparisons.—For each isolate approximately 50 mg of fungal mycelium was scraped from the surface of 7 d old cultures, ground with a glass rod, suspended in 200 µL of DNA extraction buffer (200 mM Tris-HCL pH 8.0, 150 mM NaCl, 25 mM EDTA, 0.5% SDS) and incubated 1 h at 70 C. DNA was purified with the Ultrabind® DNA purification kit following the manufacturer's instructions (MO BIO Laboratories, Solana Beach, California).

Two gene regions were used for phylogenetic comparisons. A part of the internal transcribed spacer (ITS) region of the ribosomal DNA operon was amplified with the primers ITS-1F (5′ CTT GGT CAT TTA GAG GAA GTA A) (Gardes and Bruns 1993) and ITS4 (5′ TCC TCC GCT TAT TGA TAT GC) (White et al 1990). In addition a part of the elongation factor 1-α was amplified with primers EF1-728F (5′ CAT CGA GAA GTT CGA GAA GG) and EF1-986R (5′ TAC TTG AAG GAA CCC TTA CC) (Carbone and Kohn 1999). The PCR reaction mixture (25 µL), PCR conditions and visualization of products were as described by Pavlic et al (2004) except that 0.5 U of Taq polymerase (Biotech International, Needville, Texas) were used in each reaction. PCR products were cleaned with Ultrabind® DNA purification kit (MO BIO Laboratories). Products were sequenced with the BigDye terminator cycle sequencing kit (PE Applied Biosystems) with the same primers used in the initial amplification. The products were separated with an ABI 3730 48 capillary sequencer (Applied Biosystems, Foster City, California) and a BioRad Biofocus 2000 capillary gel electrophoresis system. Data were collected with ABI data collection software.

To compare the *Botryosphaeria* isolates used in this study with other *Botryosphaeria* spp., 30 ITS rDNA and 15 EF-1α sequences obtained from GenBank were included in the phylogenetic analysis (TABLE I). Sequence data were analyzed with Sequence Navigator version 1.0.1™ (Perkin Elmer Corp., Foster City, California) and manually aligned by inserting gaps. PCR products of approximately 500 bp and 300 bp were amplified for the ITS and EF-1α regions respectively. Ambiguous sequences at the 5′ and 3′ ends were deleted in the aligned dataset. *Lasiodiplodia* spp. have a large deletion (35–38 bp), compared with other *Botryosphaeria* spp. in this study, in the ITS1 region, which was excluded and coded. Two regions in EF-1α sequences were also excluded and liberally coded. The first region was a 9 bp insertion found only in *B. rhodina* and the second a 13 bp insertion found only in *B. ribis*. Gaps were treated as a fifth character, all ambiguous characters and parsimony uninformative characters were excluded before analysis. The initial analysis was performed on an ITS dataset that included 33 isolates of *B. rhodina* (TABLE I). Subsequent analyses, including 11 isolates of *B. rhodina*, were performed on individual datasets as well as combined datasets after partition homogeneity tests (PHT) were performed in PAUP version 4.0b10 (Swofford 2000) to determine statistical congruence (Farris et al 1995, Huelsenbeck et al 1996).

The most parsimonious trees were obtained by using heuristic searches with random stepwise addition in 100 replicates, with the tree bisection-reconnection branch-swapping option on and the steepest-descent option off. MAXTREES were unlimited, branches of zero length were collapsed and all multiple equally parsimonious trees were saved. Estimated levels of homoplasy and phylogenetic signal (retention and consistency indices) also were determined with PAUP (Hillis and Huelsenbeck 1992). In the initial analysis all characters were unweighted and unordered; for the ITS analysis, characters were reweighted according to the consistency index. Branch and branch node supports were determined with 1000 bootstrap replicates (Felsenstein 1985) and characters were sampled with equal probability but weights were applied. ITS trees were rooted with *Saccharata protea* (Wakefield) Denman & Crous. This study focussed on *Botryosphaeria* spp. with *Lasiodiplodia* anamorphs, and to avoid long-branch attraction associated with phylogenetically distant outgroups trees from the combined dataset were rooted with *B. ribis*, a species with hyaline conidia and a *Fusicoccum* anamorph.

Morphological characteristics.—A total of 32 single conidium isolates representing the different cultural morphologies were used in this study (TABLE I). Sporulation was induced by transferring isolates to tap water agar overlaid with pine needles and/or eucalypt twigs as a substrate and exposing these to near UV light on a 24 h light cycle at 22 C for 2–4 wk. Cultures were maintained on one-half strength potato-dextrose agar (one-half PDA; Becton, Dickinson & Co., Sparks, Maryland) at 25 C and stored on this medium at 4 C. Cultures also were stored at room temperature in sterile water.

Colony morphology, color (Rayner 1970) and growth rates at 5–35 C of representative isolates were determined

on one-half strength PDA. Fruiting structures were mounted in lactoglycerol. Observations and measurements of conidial characteristics (30–40 per isolate) were made with a light microscope and an Axiocam digital camera (Carl Zeiss, Germany) and drawings prepared with a drawing tube. Approximately 30 conidia were measured for each isolate. All isolates in this study are maintained in the culture collection (CMW) of the Forestry and Agriculture Biotechnology Institute, University of Pretoria, South Africa, and the Department of Agriculture, Perth, Western Australia (WAC). Herbarium material is held at the Murdoch University Herbarium (MURU).

RESULTS

DNA sequence comparisons.—Initially 44 *Lasiodiplodia* isolates, including 33 isolates of *B. rhodina* from different hosts and locations, were compared based on ITS sequence alone (TABLE I). The aligned dataset consisted of 541 characters, of which a 38 bp indel was coded and excluded, resulting in 133 parsimony informative characters. The dataset contained significant phylogenetic signal compared to 1000 random trees ($P < 0.01$, g1 = −1.02). Heuristic searches of unweighted characters in PAUP resulted in 1224 most parsimonious trees of 257 steps (CI = 0.74, RI = 0.92). The large number of trees was due to the small (1–5 bp) differences among isolates of *L. theobromae*. Of the 133 informative characters, 47 characters had a weight of less than 1, indicating homoplasy. Reweighting of characters based on the consistency index resulted in only 18 trees of 190 steps (CI = 0.83, RI = 0.95). Sequence alignments are available from TreeBASE (SN2399-9015). In the analysis *Lasiodiplodia* isolates grouped in five strongly supported clades (FIG. 1). One large clade contained isolates of *L. theobromae* from a wide range of hosts and locations, another clade of two isolates represented *L. gonubiensis* and three further clades were thought to represent undescribed taxa.

The aligned dataset of the combined ITS and EF-1α sequences consisted of 877 characters of which three indels of 57 bp were coded and excluded leaving 276 parsimony informative characters that were included in the analysis. A partition homogeneity test showed no significant difference ($P = 0.77$) between the data from the different gene regions (sum of lengths of original partition was 549, range for 1000 randomizations was 543–552) and these therefore could be combined. The combined dataset contained significant phylogenetic signal compared to 1000 random trees ($P < 0.01$, g1 = −0.75). Initial heuristic searches of unweighted characters in PAUP resulted in eight most parsimonious trees of 552 steps (CI = 0.75, RI = 0.93). Sequence alignments are available from TreeBASE (SN2399-9016). The resulting tree (FIG. 2) clearly separated isolates (with 100% bootstrap support) with *Lasiodiplodia* anamorphs from those with *Diplodia*, *Dothiorella* and *Fusicoccum* anamorphs. The *Lasiodiplodia* isolates were grouped further into five clades corresponding to *L. theobromae*, *L. gonubiensis* and three undescribed taxa labeled Clade III, Clade IV and Clade V (FIG. 2). All clades were supported strongly by high bootstrap value and no further subdivision was seen for clades II–V. Within the *B. rhodina* clade (I), there was some substructure but this had low bootstrap support.

Morphology.—Anamorph structures were produced by isolates representing the three unique phylogenetic groups, on both pine needles and eucalypt twigs after 3 wk. No sexual structures were observed, either on the original wood from which isolations were made or on the needles or twigs in culture. The conidia produced by the cultures were similar to those described for *L. theobromae*, although in all cases they were slightly larger, although not as large, as those observed for *L. gonubiensis* (TABLE II). Isolates in clades III and IV had septate paraphyses, whereas those observed for *L. theobromae* and *L. gonubiensis* were aseptate. Pycnidia of isolates in Clade V were reddish/purple and covered with mycelium. Pycnidia of *L. theobromae*, and those for isolates in clades III and IV are smooth.

TAXONOMY

DNA sequence comparisons have shown that isolates collected from Western Australia and Venezuela, forming the basis of this study, represent three distinct and strongly supported phylogenetic groups. These groups separated clearly from *L. theobromae* and *L. gonubiensis*. The isolates in these three clades also could be separated from *L. theobromae* and *L. gonubiensis* as well as from each other, based on morphological characteristics. They consequently are described here as three new species:

Lasiodiplodia crassispora MB500235 Burgess, Barber, sp. nov. FIGS. 3A–C, 4

Pycnidia superficialia, pro parte maxima solitaria, conica laevia, chalybea, 0.5–1 mm diam. Paraphyses cylindricae, septatae, hyalinae. Cellulae conidiogenae holoblasticae, subcylindricae, cylindricae vel ampulliformes, percurrenter proliferantes. Conidia in cultura facta (Statura media 28.8 × 16.0 µm, longitudo/latitudo 1.8), primo hyalina, unicellularia, ellipsoidea vel obovoidea, parietibus crassis cum contento granulari, apice rotundata, interdum basin truncata, cum

TABLE I. Isolates considered in this phylogenetic study

[1]Culture no.	[1]Other no.	Identity	Host	Location	Collector	Reference (sequence)	GenBank Accession no. ITS	GenBank Accession no. EF-1α
CMW 7772		*Botryosphaeria ribis*	*Ribes* sp.	New York, USA	B. Slippers/ G. Hudler	Slippers et al 2004	AY236935	AY236877
CMW 7773		*B. ribis*	*Ribes* sp.	New York, USA	B. Slippers/ G. Hudler	Slippers et al 2004	AY236936	AY236878
CMW 7054	CBS121	*B. ribis*	*Ribes* sp.	New York, USA	N.E. Stevens	Smith and Stanosz 2001	AF241177	AY236879
CMW 7780		*B. dothidea*	*Fraxinus excelsior*	Switzerland	B. Slippers	Slippers et al 2004	AY236947	AY236896
CMW 8000		*B. dothidea*	*Prunus* sp.	Switzerland	B. Slippers	Slippers et al 2004	AY236949	AY236898
CMW 991	ATCC58188	*B. dothidea*	*Pinus nigra*	New Zealand	G.J. Samuels	Smith and Stanosz 2001	AF241175	AY236895
CMW 7060	CBS431	*B. stevensii*	*F. excelsior*	Netherlands	H.A. van der Aa	Slippers et al 2004	AY236955	AY236904
CMW 7774		*B. obtusa*	*Ribes* sp.	New York, USA	B. Slippers/ G. Hudler	Slippers et al 2004	AY236953	AY236902
	KJ94.07	*Diplodia pinea*	*P. resinosa*	Wisconsin, USA	D.R. Smith	Jacobs and Rehner 1998	AF027758	
CMW189		*D. scrobiculata*	*P. radiata*	California, USA	M.J. Wingfield	de Wet et al 2003	AY623704	
	CBS120.41	*B. sarmentorum*	*Vitex*	South Africa	J.M. van Niekerk	van Niekerk et al 2004	AY343377	
	CBS115038	*B. sarmentorum*	*Malus pumila*	Netherlands	A.J.L. Phillips	Phillips et al 2005	AY573206	AY573223
	IMI63581b	*B. sarmentorum*	*Ulmus* sp.	England	E.A. Ellis	Phillips et al 2005	AY573212	AY573235
	CBS115041	*B. iberica*	*Quercus ilex*	Spain	J. Luque	Phillips et al 2005	AY573202	AY573222
	CBS115035	*B. iberica*	*Q. ilex*	Spain	N. Ibarra	Phillips et al 2005	AY573213	AY573228
	CBS418.64	*B. tsugae*	*Tsuga heterophylla*	Canada	A. Funk	Zhou and Stanosz 2001	AF243405	
	CBS112551	*B. corticola*	*Q. suber*	Portugal	A. Alves	Alves et al 2004	AY259101	
	31-M-Mexico	*B. rhodina*	*Musa acuminata*	Mexico	M. Espinoza-Ortega	Yanez-Morales unpub	AY568635	
		B. rhodina	*P. nigra*	Kentucky, USA	J.B. Magnin	Flowers et al 2003	AY160214	
		B. rhodina	*P. nigra*	Kentucky, USA	J. Flowers	Flowers et al 2003	AY160201	
	96-172	*B. rhodina*	*Theobroma cacao*	Sri Lanka	E. Müller	Zhou and Stanosz 2001	AF243400	
	KJ93.27	*B. rhodina*	*Quercus* sp.	California, USA	E. Hecht-Poinar	Jacobs and Rehner 1998	AF027761	
	KJ93.40	*B. rhodina*	*Pistacia* sp.	California, USA	T.J. Michailides	Jacobs and Rehner 1998	AF027760	
	KJ94.41	*B. rhodina*	*Pistacia* sp.	California, USA	T.J. Michailides	Jacobs and Rehner 1998	AF027762	
CMW13510		*B. rhodina*	*Acacia mangium*	Venezuela	S. Mohali	This study	[2]DQ103526	
CMW13496		*B. rhodina*	*A. mangium*	Venezuela	S. Mohali	This study	[2]DQ103529	
CMW13489		*B. rhodina*	*Eucalyptus urophylla*	Venezuela	S. Mohali	This study	[2]DQ103525	
CMW13479		*B. rhodina*	*E. urophylla*	Venezuela	S. Mohali	This study	[2]DQ103531	
CMW13464		*B. rhodina*	*E. urophylla*	Venezuela	S. Mohali	This study	[2]DQ103530	
CMW18421	BOT1279	*B. rhodina*	*E. urophylla*	Mexico	M.J. Wingfield	This study	[2]DQ103542	[2]DQ103560
CMW14701		*B. rhodina*	*E. pellita*	Kuranda, Australia	T. Burgess	This study	[2]DQ103536	
CMW14702		*B. rhodina*	*E. pellita*	Kuranda, Australia	T. Burgess	This study	[2]DQ103535	
CMW18420	BOT979	*B. rhodina*	*Casuarina cunninghamii*	Uganda	J. Roux	This study	[2]DQ103534	[2]DQ103564
CMW13520		*B. rhodina*	*P. carribeae*	Venezuela	S. Mohali	This study	[2]DQ103527	

TABLE I. Continued

[1]Culture no.	[1]Other no.	Identity	Host	Location	Collector	Reference (sequence)	GenBank Accession no. ITS	GenBank Accession no. EF-1α
CMW13530		*B. rhodina*	*P. carribeae*	Venezuela	S. Mohali	This study	[2]DQ103528	
CMW13519		*B. rhodina*	*P. carribeae*	Venezuela	S. Mohali	This study	[2]DQ103532	
CMW18419	BOT554	*B. rhodina*	*P. ellioti*	Richards Bay, S. Africa	W. de Beer	This study	[2]DQ103541	[2]DQ103525
CMW18426	BOT1728	*B. rhodina*	*P. ellioti*	Richards Bay, S. Africa	W. de Beer	This study	[2]DQ103543	[2]DQ103525
CMW18422	BOT1479	*B. rhodina*	*P. patula*	Mpumalanga, S. Africa	W. de Beer	This study	[2]DQ103544	[2]DQ103562
CMW18423	BOT1493	*B. rhodina*	*P. patula*	Mpumalanga, S. Africa	W. de Beer	This study	[2]DQ103545	[2]DQ103563
CMW18425	BOT1519	*B. rhodina*	*P. patula*	Mpumalanga, S. Africa	W. de Beer	This study	[2]DQ103546	[2]DQ103561
	96-112	*B. rhodina*	*P. radiata*	South Africa	W. Swart	Zhou and Stanosz 2001	AF243401	
CMW 9074		*B. rhodina*	*Pinus sp.*	Mexico	T.I. Burgess	This study	[2]DQ103533	[2]DQ103565
CMW14695	SW9A	*B. rhodina*	*Santalum album*	Kununurra, Australia	T.I. Burgess/B. Dell	This study	[2]DQ103537	
CMW14693	SW6B	*B. rhodina*	*S. album*	Kununurra, Australia	T.I. Burgess/B. Dell	This study	[2]DQ103538	
CMW14690	SW5D	*B. rhodina*	*S. album*	Kununurra, Australia	T.I. Burgess/B. Dell	This study	[2]DQ103539	
CMW14689	SW3B	*B. rhodina*	*S. album*	Kununurra, Australia	T.I. Burgess/B. Dell	This study	[2]DQ103540	
STE-U 4419		*B. rhodina*	*Vitis vinifera*	South Africa	J.M. van Niekerk	van Niekerk et al 2004	AY343478	AY343368
STE-U 5051		*B. rhodina*	*V. vinifera*	South Africa	J.M. van Niekerk	van Niekerk et al 2004	AY343483	AY343369
CMW 10130		*B. rhodina*	*Vitex donniana*	Uganda	J. Roux	Slippers et al 2004	AY236951	AY236900
CMW 14077	CBS 115812	*Lasiodiplodia gonubiensis*	*Syzygium cordatum*	Eastern Cape, S. Africa	D. Pavlic	Pavlic et al 2004	AY637595	[2]DQ103566
CMW 14078	CBS 116355	*L. gonubiensis*	*Sy. cordatum*	Eastern Cape, S. Africa	D. Pavlic	Pavlic et al 2004	AY639594	[2]DQ103567
CMW 13488		*L. crassispora*	*E. urophylla*	Acarigua, Venezuela	S. Mohali	This study	[2]DQ103552	[2]DQ103559
CMW 14691	WAC12533	*L. crassispora*	*S. album*	Kununurra, Australia	T.I. Burgess/B. Dell	This study	[2]DQ103550	[2]DQ103557
CMW 14688	WAC12534	*L. crassispora*	*S. album*	Kununurra, Australia	T.I. Burgess/B. Dell	This study	[2]DQ103551	[2]DQ103558
CMW 14700	WAC12535	*L. rubropurpurea*	*E. grandis*	Tully, Queensland	T.I. Burgess/G. Pegg	This study	[2]DQ103553	[2]DQ103571
CMW 15207	WAC12536	*L. rubropurpurea*	*E. grandis*	Tully, Queensland	T.I. Burgess/G. Pegg	This study	[2]DQ103554	[2]DQ103572
	WAC12537	*L. rubropurpurea*	*E. grandis*	Tully, Queensland	T.I. Burgess/G. Pegg	This study	[2]DQ103555	[2]DQ103573
	WAC12538	*L. rubropurpurea*	*E. grandis*	Tully, Queensland	T.I. Burgess/G. Pegg	This study	[2]DQ103556	[2]DQ103574
CMW 13511	WAC12539	*L. venezuelensis*	*A. mangium*	Acarigua, Venezuela	S. Mohali	This study	[2]DQ103547	[2]DQ103568
CMW 13512	WAC12540	*L. venezuelensis*	*A. mangium*	Acarigua, Venezuela	S. Mohali	This study	[2]DQ103548	[2]DQ103569
CMW 13513		*L. venezuelensis*	*A. mangium*	Acarigua, Venezuela	S. Mohali	This study	[2]DQ103549	[2]DQ103570

[1] Abbreviations of isolates and culture collections: CBS = Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; CMW = Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; KJ = Jacobs and Rehner 1998; ATCC = American Type Culture Collection, Manassas, USA; WAC = Department of Agriculture Western Australia Plant Pathogen Collection, Perth, Australia.

[2] Sequences obtained in this study.

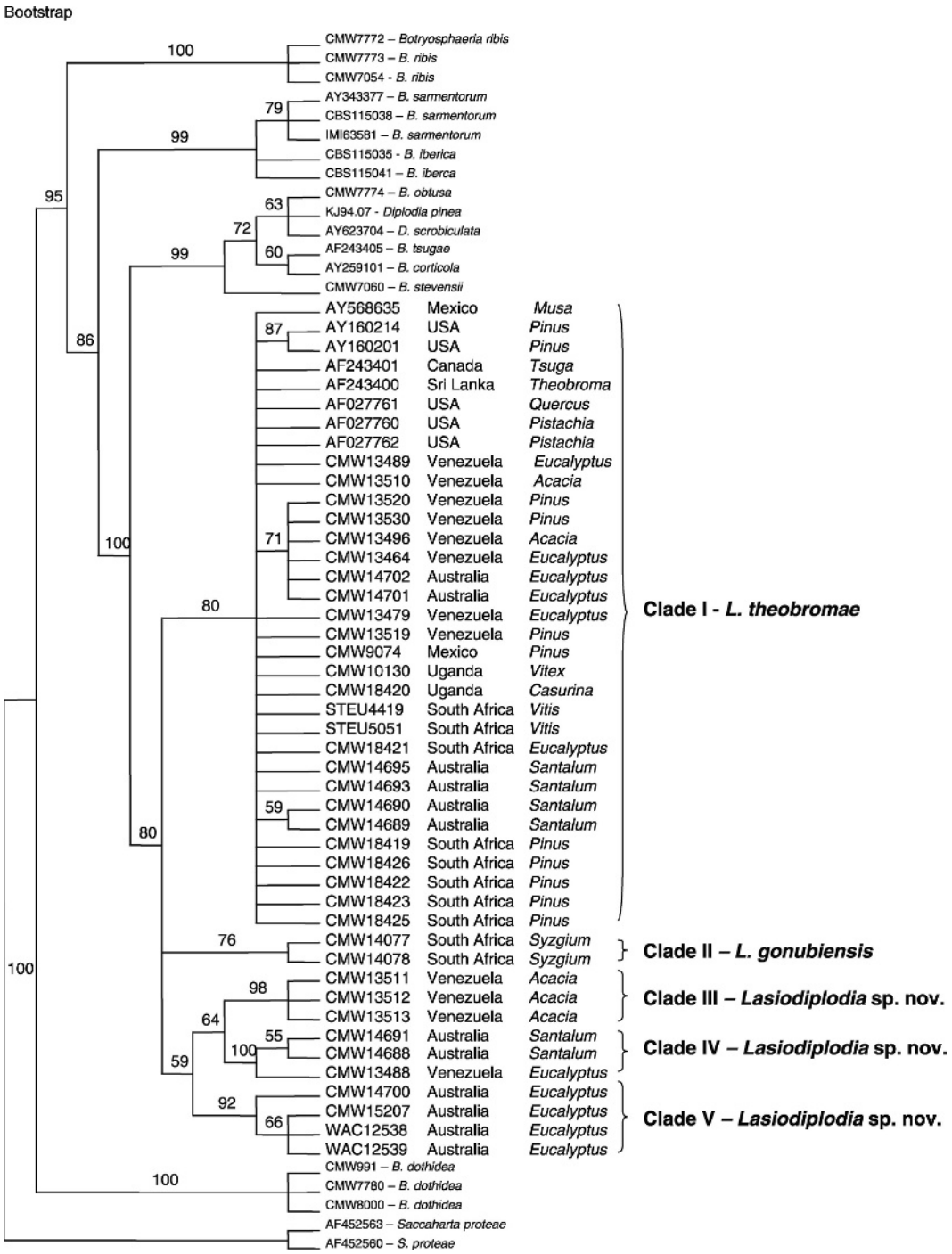

FIG. 1. Bootstrap consensus tree from analysis of ITS sequence data. Bootstrap support (based on 1000 replication) is given above the branches. The sequence of *Botryosphaeria rhodina* from GenBank is compared with isolates sequenced in this study. The locality and host of each isolate is provided next to the culture number or GenBank accession number.

maturitate vel ante germinationem colorascentia uno cum septo, in maturis strias verticales visas.

Pycnidia superficial, mostly solitary, conical, smooth, iron gray (21–23''''k), 0.5–1 mm diam. *Paraphyses* cylindrical, septate, hyaline (21)30–62(66) × 2–3.5(4) µm (average of 50 paraphyses 45.7 × 2.7 µm). *Conidiogenous cells* holoblastic, hyaline, subcylindrical to cylindrical to ampulliform, (6)8–16(19) × 3–7 µm (average of 50 conidiogenous cells 11.8 × 5.0 µm), proliferating percurrently. *Conidia* produced in culture initially hyaline, unicellular, ellipsoid to obovoid, thick-walled (2–3 µm,

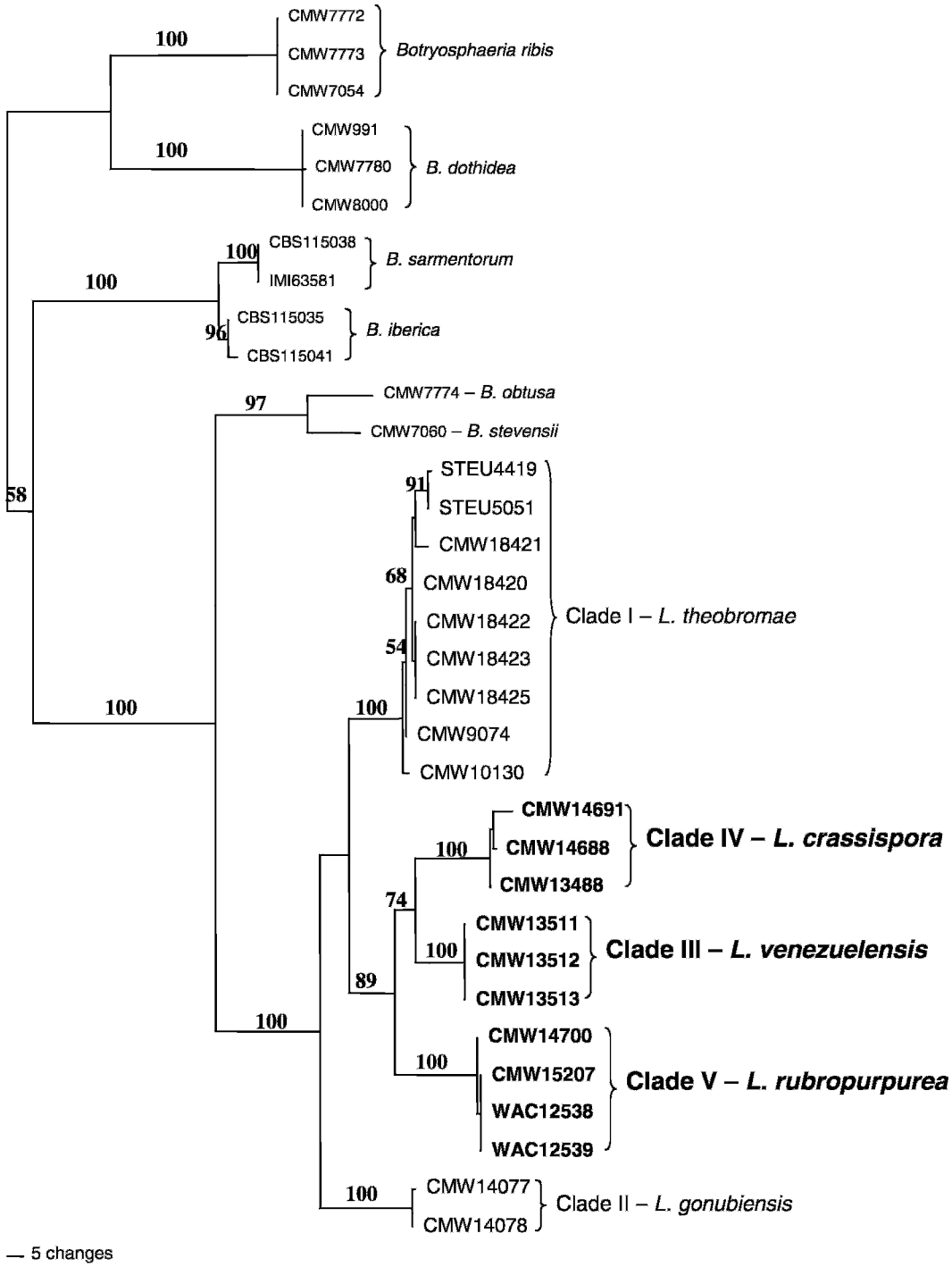

FIG. 2. A phylogram of one of the eight most parsimonious trees obtained from the combined ITS and EF-1α sequence data of *Botryosphaeria* isolates. The three new *Lasiodiplodia* spp. are in bold. Branch support (bootstrap values) is given above the branches based on 1000 bootstrap replicates. The tree is rooted to *Botryosphaeria ribis*.

average of 50 conidia 2.6 μm) with granular content, round at apex, occasionally truncate at base, becoming pigmented with one septa when mature or before germination, vertical striations observed at maturation, 27–30(–33) ×14–17 μm (average of 75 conidia, 28.8 × 16.0, l/w 1.8). *Cultural characteristics.* Moderately dense, appressed mycelial mat. Aerial mycelia/colonies initially white to buff turning pale olivaceous gray (21’’’’d) within 7 d and becoming olivaceous gray (21’’’’i) with age. At 7 d the submerged mycelia are olivaceous gray (21’’’’i), becoming iron gray (21–23’’’’k) to black with age. Optimum temperature for

TABLE II. Conidial dimensions of *Lasiodiplodia* species examined in the present study and described previously in the literature

Identity	Conidial size (μm)	L/W	paraphyses	Source of data
L. theobromae	15–35 × 10–15			Cited in Pavlic et al (2004)
	17–33 × 10–15 [Av. 22.6 × 12.2]	1.9	aseptate	This study
L. gonubiensis	(28–)32–36(–39) × (14–)16–18.5(–21) [Av. 33.8 × 17.3]	1.9	aseptate	Pavlic et al (2004)
L. venezuelensis	26–33 × 12–15 [Av. 28.4 × 13.5]	2.1	septate	This study
L. crassispora	27–30(–33) × 14–17 [Av. 28.8 × 16.0]	1.8	septate	This study
L. rubropurpurea	24–33 × 13–17 [Av. 28.2 × 14.6]	1.9	aseptate	This study

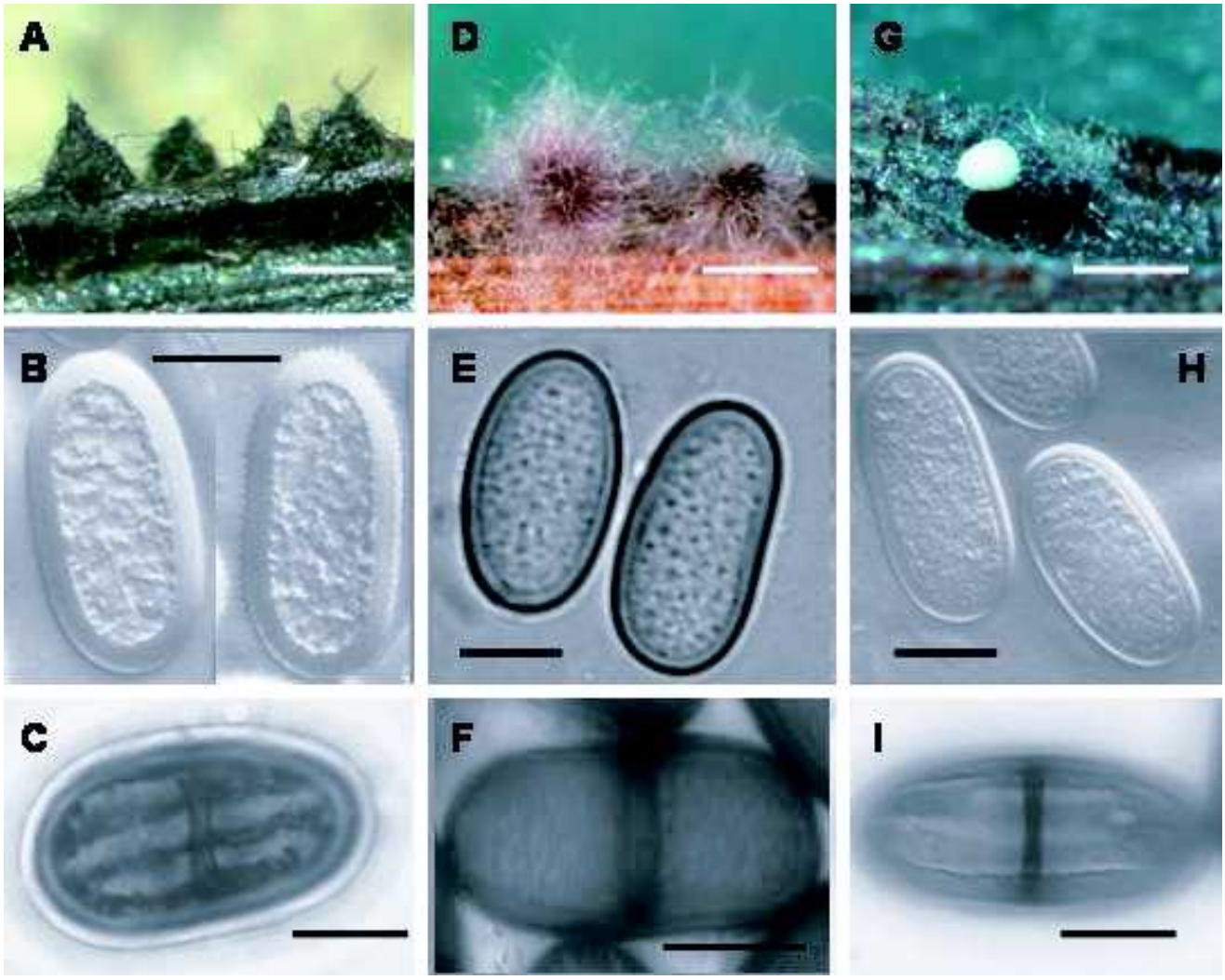

FIG. 3. Micrographs of fruiting structures of (a) *Lasiodiplodia crassispora*, conical pycnidia formed in culture on pine needles; (b) *Lasiodiplodia crassispora*, immature conidia with thick walls; (c) *Lasiodiplodia crassispora*, mature conidia with melanized banding; (d) *Lasiodiplodia rubropurpurea*, pycnidia formed in culture on pine needles covered with mycelium; (e) *Lasiodiplodia rubropurpurea*, immature conidia; (f) Lasiodiplodia rubropurpurea, mature conidia; (g) *Lasiodiplodia venezuelensis*, pycnidia formed in culture on pine needles oozing immature conidia; (h) *Lasiodiplodia venezuelensis* immature conidia; (i) *Lasiodiplodia venezuelensis* mature conidia with apparent vertical striations. Bar = 0.5 mm (a, d, g) or 10 μm (b–c, e–f, h–i).

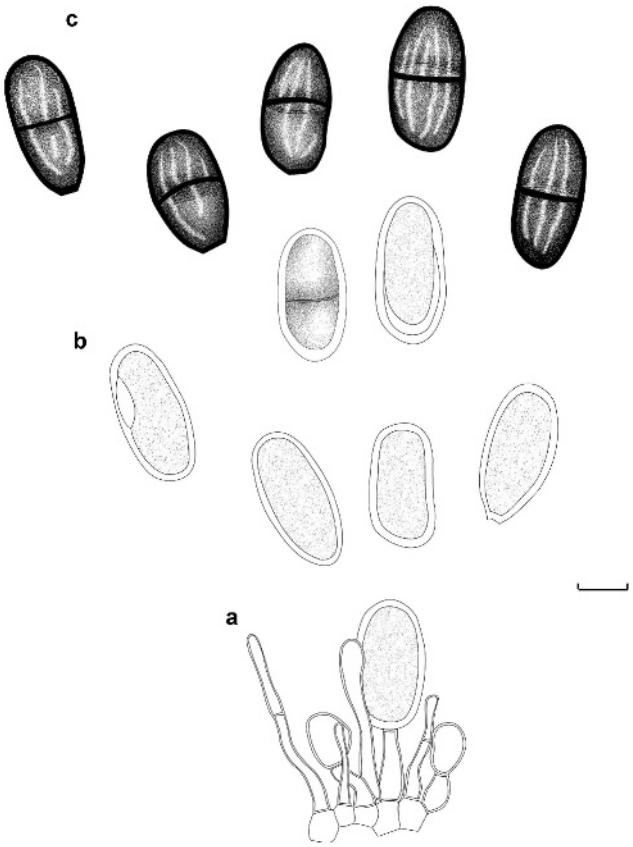

FIG. 4. *Lasiodiplodia crassispora*, (a) conidiogenous cells and paraphyses, (b) immature conidia, (c) mature conidia. Bar = 10 µm.

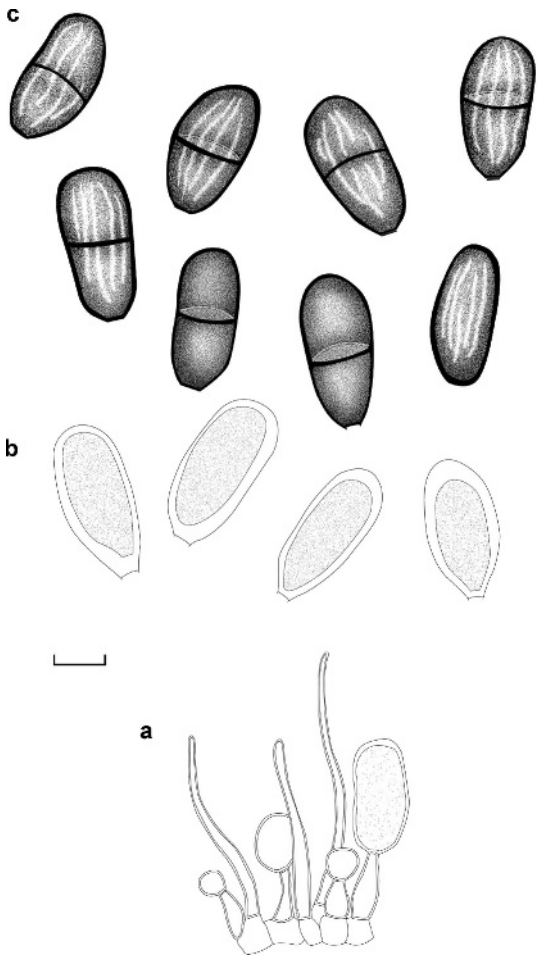

FIG. 5. *Lasiodiplodia rubropurpurea*, (a) conidiogenous cells and paraphyses, (b) immature conidia (c) mature conidia. Bar = 10 µm.

growth 30 C, reaching 74 mm diam on PDA after 3 d at 30 C in the dark.

Teleomorph. ***Botryosphaeria*** sp. (based on phylogenetic inferences, but unknown)

Etymology. Having thick-walled spores

Specimens examined. AUSTRALIA, Western Australia: Kununurra from canker of *Santalum album*, Dec 2003, T.I. Burgess (HOLOTYPE MURU 407) (culture WAC12533); Kununurra, *S. album*, T.I. Burgess (MURU 408) (culture WAC12534): VENEZUELA, Portuguesa State: Acarigua from wood of living *Eucalyptus urophylla*, Oct 2003, S. Mohali (culture CMW13448)

Lasiodiplodia rubropurpurea MB500236 Burgess, Barber, Pegg, sp.nov. FIGS. 3D–F, 5

Pycnidia superficialia, globosa, atrocarminea vel atrovinosa, pro parte maxima solitaria, 0.5–1 mm diam, mycelio tecta. Paraphyses cylindricae, aseptatae, hyalinae. Cellulae conidiogenae holoblasticae, hyalinae, subcylindricae vel ampuliformes, percurrenter proliferantes cum una annelatione. Conidia (statura media 28.2 × 14.6 µm, longitudo/latitudo 1.90) primo hyalina, unicellularia, ellipsoidea vel obovoidea, parietibus crassis cum contento granulari, cum maturitate vel ante germinationem colorascentia uno cum septo, in maturis strias verticales visas.

Pycnidia superficial, globose, livid red (69''i) to dark vinaceous (69''m), mostly solitary, 0.5–1.5 mm diam and covered with mycelium. *Paraphyses* cylindrical, aseptate, hyaline (30)32–52(58) × 1.5–3.5 µm (average of 50 paraphyses 42.4 × 2.6 µm). *Conidiogenous cells* holoblastic, hyaline, subcylindrical to ampulliform, 7–13(15) × 3–5 µm (average of 50 conidiogenous cells 10.2 × 4.0 µm), proliferating percurrently with up to 1 annellation. *Conidia* initially hyaline, unicellular, ellipsoid to obovoid, thick-walled (1 µm, average of 50 conidia = 1 µm) with granular content, round at apex, occasionally truncate at base, initially hyaline and unicellular, becoming pigmented with one septa when mature or before germination, vertical striations observed at maturation, 24–33 × 13–17 µm (average of 100 conidia 28.2 × 14.6, l/w 1.9). *Cultural characteristics*. Moderately dense, appressed mycelial mat. Aerial mycelia/colonies initially

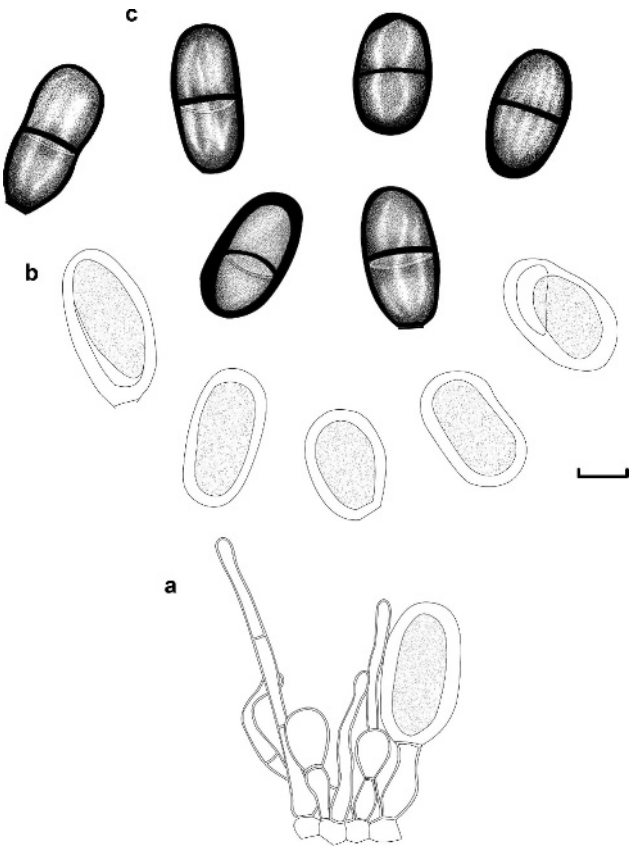

FIG. 6. *Lasiodiplodia venezuelensis*, (a) conidiogenous cells and paraphyses, (b) immature conidia, (c) mature conidia. Bar = 10 µm.

white to buff turning smoke gray (21''''d) to pale olivaceous gray (21''''d) within 7 d and becoming gray olivaceous (21''''b) to olivaceous gray (21''''i) with age. At 7 d the submerged mycelia are gray olivaceous (21''''b) to olivaceous gray (21''''i), becoming iron gray (23''''k) to black with age. Optimum temperature for growth 25–30 C, reaches 76 mm diam on PDA after 3 d at both 25 C and 30 C in the dark.

Teleomorph. ***Botryosphaeria*** sp. (based on phylogenetic inferences, but unknown)

Etymology. Refers to the reddish-purple pycnidia

Specimens examined. AUSTRALIA. Queensland: Tully, from canker of *Eucalyptus grandis*, May 2003, T.I. Burgess (HOLOTYPE MURU 409) (culture WAC12535); Tully, *E. grandis*, T.I. Burgess (MURU 410) (culture WAC12536); Tully, *E. grandis*, T.I. Burgess (MURU 411) (culture WAC12537); Tully, *E. grandis*, T.I. Burgess (MURU 412) (culture WAC12538)

Lasiodiplodia venezuelensis MB500237 Burgess, Barber, Mohali, sp. nov. FIGS. 3H–J, 6

Pycnidia superficialia, chalybea, laeves, cylindrica, pro parte maxima solitaria, 0.5–1 mm diam, saepe conidia immatura stillantia. Paraphyses cylindricae, septatae, hyalinae. Cellulae conidiogenae holoblasticae, hyalinae, subcylindricae vel cylindricae vel ampulliformes, percurrenter proliferantes. Conidia (statura media 28.4 × 13.5 µm, longitudo/latitudo 2.1) primo hyalina, unicellularia, ellipsoidea vel obovoidea, parietibus crassis cum contento granulari, apice rotundata, interdum basin truncata, cum maturitate vel ante germinationem colorascentia uno cum septo, in maturis strias verticales visas.

Pycnidia. superficial, iron gray (21–23'''''k), smooth, cylindrical, mostly solitary, 0.5–1 mm diam often oozing immature conidia. *Paraphyses* cylindrical, septate, hyaline (12)16–41(45) × (1.5)2–5 (average of 50 paraphyses 28.3 × 3.5 µm). *Conidiogenous cells* holoblastic, hyaline, subcylindrical to cylindrical to ampulliform, (5)7–14 (15) × 3–4.5(5) µm (average of 50 conidiogenous cells 10.4 × 3.7 µm), proliferating percurrently. *Conidia* initially hyaline, unicellular, ellipsoid to obovoid, thick-walled (1.5–2.5(3) µm, average of 50 conidia = 1.96 µm) with granular content, round at apex, occasionally truncate at base, becoming pigmented with one septa when mature or before germination, vertical striations observed at maturation, 26–33 × 12–15 µm (average of 75 conidia 28.4 × 13.5, l/w 2.1),. *Cultural characteristics*. Moderately dense, appressed mycelial mat. Aerial mycelia/colonies initially white to buff turning pale olivaceous gray (21'''''d) within 7 d and becoming olivaceous gray (21'''''i) with age. At 7 d the submerged mycelia are olivaceous gray (21'''''i), becoming iron gray (23'''''k) to black with age. Optimum temperature for growth 25 C, reaching 75 mm diam on PDA after 3 d at 25 C in the dark.

Teleomorph. ***Botryosphaeria*** sp. (based on phylogenetic inferences, but unknown)

Etymology. Country of origin, Venezuela

Specimens examined. VENEZUELA, Estado Portuguesa: Acarigua from wood of living *Acacia mangium*, Oct 2003, S. Mohali (HOLOTYPE MURU 413) (culture WAC12539); Acarigua, *A. mangium*, S. Mohali (MURU 414) (culture WAC12540); Acarigua, *A. mangium*, S. Mohali (culture CMW13513)

KEY TO *LASIODIPLODIA* SPP.

1. Paraphyses septate 2
1. Paraphyses aseptate 3
2. spore wall of immature conidia thin (<2 µm), striations in mature conidia narrow . . . . . ***L. venezuelensis***
2. spore wall of immature conidia thick (>2 µm), striations in mature conidia wide . . . . . ***L. crassispora***

3. conidia on average <30 um long 4
3. conidia on average >30 um long . . . . ***L. gonubiensis***
4. conidia on average <25 um long, pycnidia smooth and dark with oozing spores. . . . . ***L. theobromae***
4. conidia on average >25 um long, pycnidia fluffy and reddish-purple. . . . . ***L. rubropurpurea***

DISCUSSION

Recognition of new *Lasiodiplodia* species in this study highlights both the underestimation of fungal species numbers in well studied groups and the importance of combining DNA techniques with classical taxonomy. Discovery of new *Lasiodiplodia* spp. is perhaps not surprising because more than 10 species were assigned to this genus before the monograph of Punithalingam (1976), which reduced them to synonymy. This study supports the views of Pavlic et al (2004), who suggested that undescribed species of *Lasiodiplodia* are likely to emerge through phylogenetic studies based on DNA sequence comparisons.

Consideration must be given to the possibility that some of the *Lasiodiplodia* spp. described before the study of Punithalingam (1976) are the same as those emerging from DNA sequence comparisons. We do not believe this to be the case because Punithalingam (1976) was able to merge all species based on a lack of morphological differences. Pavlic et al (2004) sought herbarium specimens for *L. theobromae* to compare with *L. gonubiensis*, but neither cultures nor herbarium specimens pertaining to the original host and location (*Theobroma cacao* L. in Ecuador) could be located. Thus, until original material can be located or an epitype specimen assigned, it is necessary to rely on descriptions from the literature.

The species described in this study are morphologically and phylogenetically distinct from both *L. theobromae* and *L. gonubiensis*. It is not possible to test the genetic relatedness of the new species to other previously described species due to the absence of cultures. In recent studies we have examined more than 200 isolates from pines, eucalypts, acacia, and sandalwood in Australia, South Africa, Venezuela and Mexico, and among all these isolates only 10 (less than 5%) were found to be distinct from *L. theobromae*. In our opinion *L. theobromae* is a common species in tropical parts of the world but other less common species of this genus are yet to be described.

In addition to the clear phylogenetic differences between the newly described species, these fungi differ from each other and existing species based on septation of the paraphyses, the size of the spores, thickness of spore walls and the color of pycnidia. All species described as new in this study have larger conidia than those of *L. theobromae* but smaller than *L. gonubiensis*. *Lasiodiplodia venezuelensis* and *L. crassispora* have septate paraphyses, while they are aseptate in other species. *Lasiodiplodia crassispora* has notably thicker cell walls in the immature spores and the striations appear to be wider and the cytoplasm wart-like in appearance, which is different from all other species. *Lasiodiplodia rubropurpurea* is unique in having red-purple pycnidia.

The three new species, *L. crassispora, L. rubropurpurea* and *L. venezuelensis*, have been recognized as residing in *Lasiodiplodia*, based on size and shape of conidia and the presence of vertical striations, which are characteristic of this genus. No teleomorph structures were found for these new species but phylogenetic inference leads us to conclude that they are species of the teleomorph genus *Botryosphaeria*. *Botryosphaeria* is a large genus with clear monophyletic groups emerging from phylogenetic studies based on DNA sequence data. Thus clear subdivisions have been recognized between those species that have *Diplodia* anamorphs with primarily dark-colored conidia and those that have hyaline conidia and *Fusicoccum* anamorphs (Denman et al 2000, Zhou and Stanosz 2001). These might be assigned to new genera, and if that is the case the generic placement of *Lasiodiplodia* will come into question.

In this study we have chosen to retain the name *Lasiodiplodia* and not reduce it to synonymy with *Diplodia* as had been considered (Denman et al 2000, Zhou and Stanosz 2001). This decision was made because all species of *Lasiodiplodia* group together in a highly supported (100% bootstrap) clade related to but distinct from the clade encompassing *Diplodia* and *Dothiorella* species. Through the addition of three new species the cohesiveness and separate nature of this clade is enhanced. Phillips et al (2005) resurrected *Dothiorella* to encompass two new *Botryosphaeria* spp. with dark conidia. Based on a combined ITS and EF-1α phylogeny, these new species formed a clade, which like the *Lasiodiplodia* clade is close to but distinct from the *Diplodia* clade (Phillips et al 2005).

Species in *Diplodia, Dothiorella* and *Lasiodiplodia* are clearly separated from those in *Fusicoccum* by thick-walled conidia with a much smaller length to width ratio (Luque et al 2005, Phillips et al 2005). When mature the conidia of most *Diplodia, Dothiorella* and *Lasiodiplodia* species are dark and septate, however conidia of *B. stevensii* and *B. corticola* are mostly hyaline and often aseptate and can germinate before darkening (Denman et al 2000, Zhou and Stanosz 2001, Alves et al 2004). Conidia of *D. pinea* and *B. obtusa* while dark are often aseptate (de Wet et

al 2003, Alves et al 2004). Conidia of the newly described *B. iberica, B. sarmentorum* and *B. viticola* become brown and 1-septate early in their development (Luque et al 2005, Phillips et al 2005). *Diplodia, Dothiorella* and *Lasiodiplodia* spp. conidia sizes overlap, although in general those of *Lasiodiplodia* are wider and more obovoid. The other distinguishing feature of *Lasiodiplodia* spp. is the obvious vertical striations in mature conidia (von Arx 1974). We believe there is a reasonable argument to retain *Lasiodiplodia* as distinct from *Diplodia* and *Dothiorella* and expect that if the genus *Botryosphaeria* is subdivided those species with *Lasiodiplodia* anamorphs will be retained in a discrete teleomorph genus.

All new species described in this study were isolated from cankers on various tree species. In each case they were associated with *L. theobromae* and other opportunistic pathogens such as *Cytospora eucalyptticola sensu lato* and *B. ribis*. Their distribution and host range currently is limited, especially compared with *L. theobromae* which has more than 500 host species and a global distribution (Punithalingam 1976, 1980). Pathogenicity studies were not conducted, but it would be interesting to compare the pathogenicity of the new species with that of *L. theobromae*. Although the new species were isolated from cankers, it is likely they are also endophytes and latent pathogens and not primary pathogens as is the case for many *Botryosphaeria* spp. (von Arx 1987, Smith et al 1996, Burgess et al 2001, Pavlic et al 2004).

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