Morphological and molecular data reveal cryptic speciation in *Lasiodiplodia theobromae*

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Lasiodiplodia theobromae is a common pathogen that occurs on a large number of hosts in the tropics and subtropics. A collection of isolates identified as *L. theobromae* was studied on the basis of sequence data from the ITS regions and EF1-α gene. Phylogenetic analyses identified three well-supported clades within this group of isolates, one corresponding to *L. theobromae* and two others corresponding to potential cryptic species. The distinct phylogenetic position of the two clades is supported by differences in conidial morphology and these are, therefore, described as *Lasiodiplodia pseudotheobromae* sp. nov. and *Lasiodiplodia parva* sp. nov.

Key words: *Botryosphaeriaceae*, cryptic species, *Lasiodiplodia*, phylogeny, taxonomy

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Introduction

Species of *Botryosphaeriaceae* are cosmopolitan and occur on a variety of plant hosts causing dieback and canker diseases (von Arx and Müller, 1954; Barr, 1987). A recent study by Crous *et al*. (2006) showed that *Botryosphaeria* comprised several different phylogenetic lineages that correlated well with morphological features of the anamorphs. Thus, *Botryosphaeria* is now considered to be a relatively small genus consisting of only *B. dothidea* (Moug. : Fr.) Ces. & De Not. (the type species of the genus) and *B. corticis* (Demaree & M.S. Wilcox) Arx & E. Müll. (a species restricted to *Vaccinium* spp., Phillips *et al*., 2006). The remaining lineages within what was known as *Botryosphaeria* consist of the anamorph genera *Diplodia* (including *Sphaeropsis*), *Lasiodiplodia*, *Neofusicoccum*, *Pseudofusicoccum*, *Macrophomina*, *Neoscytalidium* and *Dothiorella* (Crous *et al*., 2006). We recently determined that *Diplodia seriata* De Not. was the correct name for the anamorph of "*Botryosphaeria*" *obtusa* (Phillips *et al*., 2007).

The type species of the genus *Lasiodiplodia*, *L. theobromae* (Pat.) Griffon & Maubl., is geographically widespread but is most common in the tropics and subtropics (Punithalingam, 1980). It is plurivorous and has been associated with approximately 500 hosts (Punithalingam, 1980). This apparently unspecialized plant pathogen has been reported to cause numerous diseases, including dieback, root rot, fruit rots, leaf spot and witches' broom amongst many others (Punithalingam, 1980). It also occurs as an endophyte (Rubini *et al*., 2005; Mohali *et al*., 2005). Less frequently it has been associated with keratomycosis and phaeohyphomycosis in humans (Punithalingam, 1976; Rebell and Forster, 1976; Summerbell *et al*., 2004).

In view of its widespread occurrence, the large number of hosts and its known morphological variability (Punithalingam, 1980) it is possible that *L. theobromae* is composed of a number of cryptic species. Recently, Pavlic *et al*. (2004) described a new species *L. gonubiensis* Pavlic, Slippers & M.J. Wingf. on the basis of conidial morphology and dimensions, and ITS sequence data. In a similar way, Burgess *et al.* (2006) described three new *Lasiodiplodia* species (*L. crassispora*, *L. venezuelensis* and *L. rubropurpurea*) from the tropics on the basis of their ITS and EF1-α sequence data and morphological characters.

The purpose of this work was to determine genetic variability within a collection of isolates previously identified as *L. theobromae* through a study of ITS and EF1-α nucleotide sequences.

Materials and methods

Isolates and morphology

A collection of isolates previously identified as *Lasiodiplodia theobromae* was studied (Table 1). To investigate the effect of temperature on growth, isolates were grown on 2% Difco potato-dextrose agar (PDA). To induce sporulation, isolates were grown on autoclaved poplar twigs on 2% water agar and incubated at room temperature where they received diffused daylight. Structures were mounted in 100% lactic acid and digital images were recorded with a Leica DFC 320 camera on a Leica DMR HC microscope. Measurements were made with the Leica IM500 measurement module. From 50 measurements of each type of structure the mean, standard deviation and 95% confidence intervals were calculated. Dimensions are given as the range of measurements with extremes in parentheses followed by 95% confidence limits and mean ± standard deviation.

DNA isolation, PCR amplification and sequencing

Genomic DNA was extracted from mycelium by 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).

The ITS regions were amplified with primer pairs ITS1 and ITS4 (White *et al*., 1990) as described by Alves *et al*. (2004). The primers EF1-728F and EF1-986R (Carbone and Kohn, 1999) were used to amplify part of the translation elongation factor 1-alpha (EF1-α) as described by Phillips *et al.* (2005). For some isolates amplification of the EF1-α region was impossible with the primer set EF1-728F and EF1-986R. Thus, a new set of primers was designed based on the sequences of the elongation factor 1-alpha gene from *Neurospora crassa* (D45837) and *Aureobasidium pullulans* (U19723). The sequences were aligned and suitable regions for primer design were identified by visual inspection and the following set of primers was chosen: EF1-688F (5'-CGGTCACTTGATCTACAAGTGC-3') and EF1-1251R (5'-CCTCGAACTCACCAGTACCG -3'). The amplification conditions for the EF1-α region using the new set of primers were the same as above.

The amplified PCR fragments were purified with the JETQUICK PCR Purification Spin Kit (GENOMED, Löhne, Germany). Both strands of the PCR products were sequenced with the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit with AmpliTAQ DNA Polymerase (PE Applied Biosystems, Foster City, California, USA) in a Bio-Rad iCycler Thermal Cycler. Cycle sequencing procedure was described elsewhere (Alves *et al.*, 2004).

The sequences were obtained with the ABI PRISM® 310 Genetic Analyzer (PE Applied Biosystems, Foster City, California, USA). The nucleotide sequences were read and edited with Chromas 1.45 (http://www.technelysium.com.au/chromas.html). All sequences were checked manually and nucleotide arrangements at ambiguous positions were clarified using both primer direction sequences. New sequences were deposited in GenBank. Sequences of both DNA regions of additional isolates and species were retrieved from GenBank (Table 1).

Table 1. Isolates studied.

Isolate number[1]	Species	Host	Locality	Collector	GenBank	
					ITS[2]	EF1-α
CBS 112553	*D. mutila*	*Vitis vinifera*	Portugal	A.J.L. Phillips	*AY259093*	*AY573219*
CBS 230.30	*D. mutila*	*Phoenix dactylifera*	U.S.A	L.L. Huillier	*DQ458886*	*DQ458869*
CBS 418.64	*'B.' tsugae*	*Tsuga heterophylla*	Canada	A. Funk	*DQ458888*	*DQ458873*
CBS 112547	*D. corticola*	*Quercus ilex*	Spain	M.E. Sanchez	*AY259110*	*DQ458872*
CBS 112549	*D. corticola*	*Quercus suber*	Portugal	A. Alves	*AY259100*	*AY573227*
CBS 112555	*D. seriata*	*Vitis vinifera*	Portugal	A.J.L. Phillips	*AY259094*	*AY573220*
CBS 119049	*D. seriata*	*Vitis* sp.	Italy	L. Mugnai	*DQ458889*	*DQ458874*
CBS 393.84	*D. pinea*	*Pinus nigra*	Netherlands	H.A. van der Aa	*DQ458895*	*DQ458880*
CBS 109727	*D. pinea*	*Pinus radiata*	South Africa	W.J. Swart	*DQ458897*	*DQ458882*
CBS 109725	*D. pinea*	*Pinus patula*	South Africa	M.J. Wingfield	*DQ458896*	*DQ458881*
CBS 109943	*D. pinea*	*Pinus patula*	Indonesia	M.J. Wingfield	*DQ458898*	*DQ458883*
CBS 109944	*D. scrobiculata*	*Pinus greggii*	Mexico	M.J. Wingfield	*DQ458899*	*DQ458884*
CBS 113423	*D. scrobiculata*	*Pinus greggii*	Mexico	M.J. Wingfield	*DQ458900*	*DQ458885*
CBS 168.87	*D. cupressi*	*Cupressus sempervirens*	Israel	Z. Solel	*DQ458893*	*DQ458878*
CBS 261.85	*D. cupressi*	*Cupressus sempervirens*	Israel	Z. Solel	*DQ458894*	*DQ458879*
CBS 124.13	*L. theobromae*	Unknown	U.S.A.	J.J. Taubenhaus	*DQ458890*	*DQ458875*
CBS 164.96	*L. theobromae*	Fruit on coral reef coast	New Guinea	A. Aptroot	*AY640255*	*AY640258*
CBS 175.26	*L. theobromae*	Unknown	Unknown	K.B. Boedijn	EF622067	EF622047
CBS 190.73	*L. theobromae*	*Persea americana*	Tanzania	W.S. Bos	EF622068	EF622048
CBS 287.47	*L. theobromae*	*Musa sapientum*	Unknown	K. Sys	EF622069	EF622049
CBS 289.56	*L. theobromae*	sail-cloth	New Guinea	M.B. Schol-Schwartz	EF622070	EF622050
CBS 306.58	*L. theobromae*	*Cocos nucifera*	Unknown	G.H. Boerema	EF622071	EF622051
CBS 339.90	*L. theobromae*	phaeohyphomycotic cyst	Canada	R.C. Summerbell	EF622072	EF622052
CBS 559.70	*L. theobromae*	*Zea mays*	Unknown	H.A. van der Aa	EF622073	EF622053
CBS 111530	*L. theobromae*	Unknown	Unknown	Unknown	EF622074	EF622054
CBS 112874	*L. theobromae*	*Vitis vinifera*	South Africa	F. Halleen	EF622075	EF622055
CBS 113520	*L. theobromae*	Unknown	Unknown	Unknown	EF622076	EF622056
CAA 006	*L. theobromae*	*Vitis vinifera*	USA	T.J. Michailides	*DQ458891*	*DQ458876*
CMW 9074	*L. theobromae*	*Pinus* sp.	Mexico	T. Burgess	*AY236952*	*AY236901*
CMW 10130	*L. theobromae*	*Vitex doniana*	Uganda	J. Roux	*AY236951*	*AY236900*
STE-U 5051	*L. theobromae*	*Vitis vinifera*	Argentina	M. Gatica	*AY343483*	*AY343369*
CBS 110492	*L. crassispora*	Unknown	Unknown	Unknown	EF622086	EF622066
CBS 118741	*L. crassispora*	*Santalum album*	Australia	T.I. Burgess/B. Dell	*DQ103550*	*DQ103557*
CMW 13488	*L. crassispora*	*Eucalyptus urophylla*	Venezuela	S. Mohali	*DQ103552*	*DQ103559*
CBS 118739	*L. venezuelensis*	*Acacia mangium*	Venezuela	S. Mohali	*DQ103547*	*DQ103568*
CMW 13512	*L. venezuelensis*	*Acacia mangium*	Venezuela	S. Mohali	*DQ103548*	*DQ103569*
CBS 118740	*L. rubropurpurea*	*Eucalyptus grandis*	Queensland	T.I. Burgess/G. Pegg	*DQ103553*	*DQ103571*
CMW 15207	*L. rubropurpurea*	*Eucalyptus grandis*	Queensland	T.I. Burgess/G. Pegg	*DQ103554*	*DQ103572*
CBS 115812	*L. gonubiensis*	*Syzygium cordatum*	South Africa	D. Pavlic	*DQ458892*	*DQ458877*
CBS 116355	*L. gonubiensis*	*Syzygium cordatum*	South Africa	D. Pavlic	*AY639594*	*DQ103567*
CBS 304.79	*L. pseudotheobromae*	*Rosa* sp.	Netherlands	Unknown	EF622079	EF622061
CBS 374.54	*L. pseudotheobromae*	*Coffea* sp.	Zaire	Unknown	EF622080	EF622059
CBS 447.62	*L. pseudotheobromae*	*Citrus aurantium*	Suriname	C. Smulders	EF622081	EF622060
CBS 116459	*L. pseudotheobromae*	*Gmelina arborea*	Costa Rica	J. Carranza-Velásquez	EF622077	EF622057
CBS 116460	*L. pseudotheobromae*	*Acacia mangium*	Costa Rica	J. Carranza-Velásquez	EF622078	EF622058
CBS 356.59	*L. parva*	*Theobroma cacao*	Sri Lanka	A. Riggenbach	EF622082	EF622062
CBS 456.78	*L. parva*	Cassava-field soil	Colombia	O. Rangel	EF622083	EF622063
CBS 494.78	*L. parva*	Cassava-field soil	Colombia	O. Rangel	EF622084	EF622064
CBS 495.78	*L. parva*	Cassava-field soil	Colombia	O. Rangel	EF622085	EF622065
CBS 115476	*B. dothidea*	*Prunus* sp.	Switzerland	B. Slippers	*AY236949*	*AY236898*
CBS 110299	*N. luteum*	*Vitis vinifera*	Portugal	A.J.L. Phillips	*AY259091*	*AY573217*

[1]Acronyms of culture collections: CAA – A. Alves, Universidade de Aveiro, Portugal; CBS – Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CMW – M.J. Wingfield, FABI, University of Pretoria, South Africa; STE-U – Department of Plant Pathology, University of Stellenbosch, South Africa. Isolates in bold are cultures ex-type.
[2]Sequence numbers in italics were retrieved from GenBank. All others were obtained in the present study.

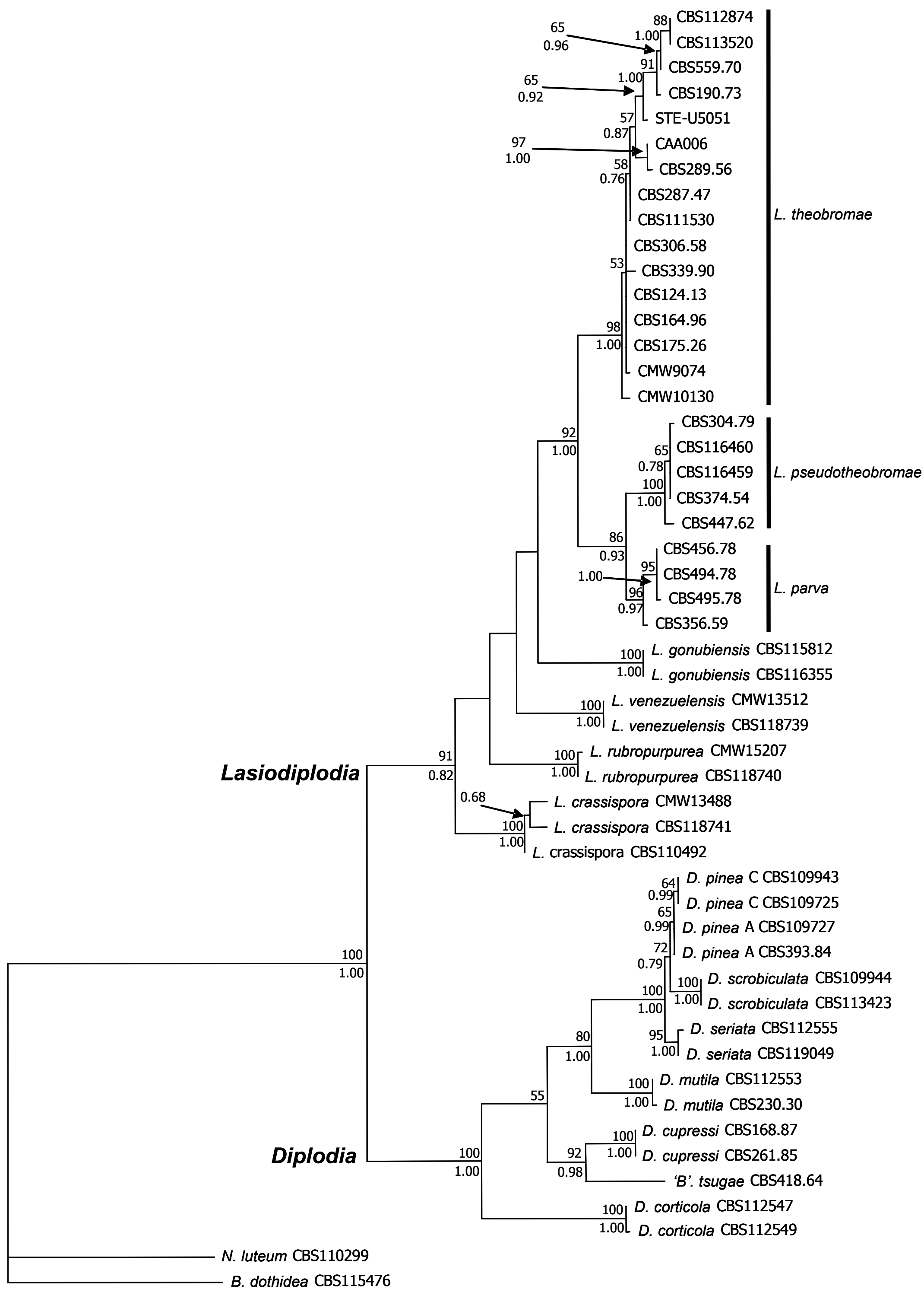


Fig. 1. One of five most parsimonious trees obtained from the combined analysis of ITS and EF1-α sequence data (tree length = 550 steps; CI = 0.7527; RI = 0.9169; RC = 0.6902; HI = 0.2845). Bootstrap support values from 1000 replications are shown above the branches and Bayesian posterior probabilities below the branches.

Phylogenetic analyses

The sequences were aligned with ClustalX version 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* version 4.0b10 (Swofford, 2003) for Maximum-parsimony (MP) analyses and Mr Bayes v3.0b4 (Ronquist and Huelsenbeck, 2003) for Bayesian analyses. Trees were rooted to *Neofusicoccum luteum* and *Botryosphaeria dothidea* and visualized 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 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.

In this study we assessed the possibility of combining the ITS and EF1-α data sets 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).

Results

Phylogenetic analyses

For all our ingroups, approximately 550 and 300 bases were determined for the ITS and EF1-α genes respectively. The new set of primers was able to amplify the EF1-α region from those isolates that did not form any amplicon with the EF1-728F and EF1-986R set of primers. The sequences of the amplified region showed that lack of amplification was due to several mismatched bases in the primer EF1-728F. The new forward primer EF1-688F worked equally well with either of the reverse primers. However, the best sequencing results were obtained with the primer pair EF1-688F/EF1-986R, and this was the one that was used for amplification and sequencing of the isolates.

New sequences were deposited in GenBank (Table 1) and the alignments in TreeBase (S1940). Sequences of the two genes were aligned and analysed separately by Maximum parsimony and Bayesian analyses, and the resulting trees were compared. No major conflicts were detected between single gene phylogenies indicating that the ITS and EF1-α datasets could be combined.

After alignment, the ITS and EF1-α dataset consisted of 548 characters for the ITS regions, and 326 characters for EF1-α,

Table 2. Conidial size and septation of selected *Lasiodiplodia* species.

Species	Conidia		References
	septation	size (μm)	
Lasiodiplodia gonubiensis Pavlic *et al*.	1-3-septate	32-36 × 16-18.5	Pavlic *et al*. (2004)
Lasiodiplodia nigra K.R. Appel & Laubert	1-septate	28-32 × 18-21	Pavlic *et al*. (2004)
Lasiodiplodia theobromae (Pat.) Griffon & Maubl.	1-septate	26.2-27 × 14-14.4	Present study
Lasiodiplodia triflorae B.B. Higgins (= *L. theobromae*)	1-septate	22-25 × 13-16.5	Pavlic *et al*. (2004)
Lasiodiplodia tubericola Ellis & Everh. (= *L. theobromae*)	1-septate	18-22 ×11-14	Pavlic *et al*. (2004)
Lasiodiplodia crassispora T. Burgess & Barber	1-septate	27-30 × 14-17	Burgess *et al*. (2006)
Lasiodiplodia rubropurpurea T. Burgess *et al*.	1-septate	24-33 × 13-17	Burgess *et al*. (2006)
Lasiodiplodia venezuelensis T. Burgess *et al*.	1-septate	26-33 × 12-15	Burgess *et al*. (2006)
Lasiodiplodia pseudotheobromae A.J.L. Phillips *et al*.	1-septate	23.5-32 × 14-18	Present study
Lasiodiplodia parva A.J.L. Phillips *et al*.	1-septate	16-23.5 × 10.5-13	Present study

including alignment gaps (data not shown). Indels were coded separately and added to the end of the alignment as characters 875-956. Gaps were treated as missing data.

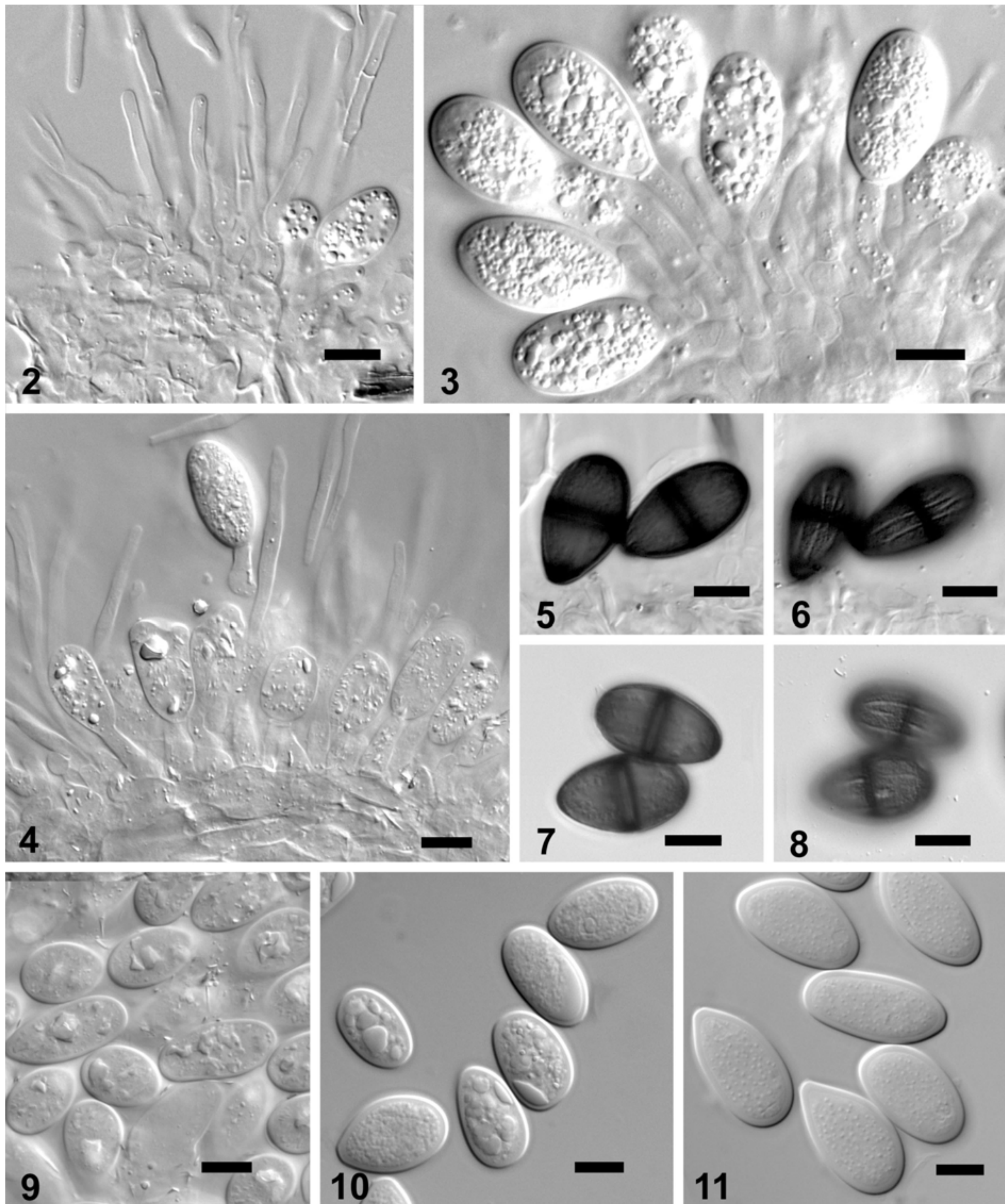
The combined dataset contained 956 characters, of which 264 were parsimony informative, 67 were variable and parsimony uninformative and 625 were constant. Maximum parsimony analysis of the combined dataset resulted in 5 equal, most parsimonious trees (tree length = 550 steps; CI = 0.7527; RI = 0.9169; RC = 0.6902; HI = 0.2845). The trees differed only in the terminal arrangement of taxa and showed a topology identical to the 50% majority-rule consensus bootstrap tree. In addition, the 50% majority-rule consensus tree of 10,000 trees sampled during the Bayesian analysis was identical in topology. One of the 5 most parsimonious trees is presented in Fig. 1 with bootstrap support above and Bayesian posterior probabilities below the branches.

Two main clades were identified. One corresponded to the anamorphic genus *Diplodia* (MP bootstrap = 100%; posterior probability = 1.00) and the other corresponding to the anamorphic genus *Lasiodiplodia* (MP bootstrap = 91%, posterior probability = 0.82). Within these two clades several sub-clades supported by high bootstrap values and Bayesian posterior probabilities could be identified that correspond to known species. Thus, within the *Diplodia* clade, seven sub-clades corresponding to known species could be distinguished. Bootstrap and posterior probabilities for the clades were generally high, but the complex of *D. pinea*, and *D. scrobiculata* was only partially resolved.

The *Lasiodiplodia* clade was resolved into five sub-clades corresponding to *L. gonubiensis, L. crassispora*, *L. rubropurpurea*, *L. venezuelensis* and a large clade containing isolates previously identified as *L. theobromae*. This latter clade was further subdivided into three sub-clades (Fig. 1).

Morphology

All isolates previously identified as *L. theobromae* that were induced to sporulate in culture corresponded with the typical *Lasiodiplodia* morphology. Thus, paraphyses were present within the conidiomata and the conidia were initially hyaline and aseptate, but in time a single median septum formed, the wall became dark brown and melanin granules deposited longitudinally on the inner surface of the wall gave the conidia a striate appearance. Isolates in the three sub-clades within *Lasiodiplodia theobromae* as revealed by combined ITS and EF1-α sequence data analysis were morphologically distinct (Table 2). Therefore, these three sub-clades were considered to represent three distinct species, two of which are described as new. Conidia of *L. theobromae* isolates (Figs 3-12) were 21-31 × 13-15 µm ($\bar{x}$ = 26.2 × 14.2 µm), and corresponded to previous reports for the species (Punithalingam, 1976; Pavlic *et al.*, 2004). Conidia of *L. pseudotheobromae* (Figs 13-20) were somewhat larger (23.5-32 × 14-18 µm, $\bar{x}$ = 28 × 16 µm), while those of *L. parva* (Figs 21-25) were smaller (16-23.5 × 10.5-13 µm, $\bar{x}$ = 20.2 × 11.5).



Figs 2-11. *Lasiodiplodia theobromae*. **Figs 2, 3, 5, 6, 10, 11.** CBS164.96. **2.** Paraphyses. **3, 4.** Conidiogenous cells and young conidia. **5, 6.** Mature conidia in two different focal planes to show the longitudinal striations. **10, 11.** Hyaline, immature conidia. **Figs 4, 7, 8, 9,** K118158. **4.** Conidiogenous layer with paraphyses. **7, 8.** Mature conidia in two different focal planes to show the longitudinal striations. **9.** Conidia. Bars = 10 µm.

Lasiodiplodia theobromae (Pat.) Griffon & Maubl., Bull. trimest. Soc. Mycol. Fr. 25: 57 (1909)

≡ *Botryodiplodia theobromae* Pat., Bull. Soc. Mycol. Fr. 8: 136 (1892).

= *Diplodia gossypina* Cooke, Grevillea 7: 95 (1879). (Figs 2-11)

Conidiomata formed on poplar twigs in culture pycnidial, uniloculate, dark brown to black, immersed in the host becoming erumpent when mature. *Paraphyses* hyaline, cylindrical, septate, occasionally branched, ends rounded, up to 55 µm long, 3-4 µm wide. *Conidiogenous cells* hyaline, thin-walled, smooth, cylindrical, holoblastic, proliferating percurrently to form one or two annellations, or proliferating at the same level giving rise to periclinal thickenings. *Conidia* subovoid to ellipsoid-ovoid, apex broadly rounded, tapering to truncate base, widest in middle to upper third, thick-walled, contents granular, initially hyaline and aseptate, remaining hyaline for a long time, finally becoming dark brown and one-septate but only after discharge from the pycnidia, with melanin deposits on the inner surface of the wall arranged longitudinally giving a striate appearance to the conidia, (19-)21-31(-32.5) × (12-)13-15.5(-18.5) µm, 95% confidence interval = 26.2-27 × 14-14.4 µm ($\bar{x}$ ± S.D. = 26.2 ± 2.6 × 14.2 ± 1.2 µm, l/w ratio = 1.9 ± 0.2).

Teleomorph: ?"*Botryosphaeria*" *rhodina* (Cooke) Arx, Gen. Fungi Sporul. Cult. (Lehr): 143 (1970).

≡ *Physalospora rhodina* Cooke, Grevillea 17: 92 (1889).

Habitat: plurivorous.

Known distribution: widespread in tropical and subtropical regions.

Material examined: CBS164.96, CBS111530, K118158 (**holotype** of *D. gossypina*). Other isolates studied are listed in Table 1.

Notes: The connection between *L. theobromae* and its teleomorph has not been proven absolutely. Stevens (1925) made single ascospore cultures from a fungus he referred to as *Physalospora gossypina* on cotton stems in Florida, and from *Hicoria, Ilex, Liquidambar, Quercus* and *Vitis*. In all cases the conidia formed in these cultures were morphologically identical to those of *L. theobromae*. Stevens (1926) then determined that the fungus he called *P. gossypina* was in fact the same as *Physalospora rhodina* Cooke, which was later transferred by von Arx (1970) to *Botryosphaeria* as *B. rhodina* (Cooke) Arx. However, there have been no subsequent reports to confirm this connection.

Lasiodiplodia pseudotheobromae A.J.L. Phillips, A. Alves & Crous, **sp. nov.** (Figs 12-18)

MycoBank: 510941.

Etymology: Named for its resemblance to *L. theobromae*.

Conidiomata in contextu hospitis inclusa, solitaria, stromatiformia, globosa. *Cellulae conidiogenae*, holoblasticae, hyalinae, subcylindricae, percurrenter cum 1-2 proliferationibus prolificentes, vel in plano eodem periclinaliter incrassatae. *Conidia* 27.5-28.5 × 15.5-16.5 µm, unicellulares, parietibus crassis, ovoidea, apicibus obtuse rotundato, in fundo obtuse rotundato, primaria hyalinae, cum maturitate cinnamomescentia vel brunnescentia, longitudinaliter striata et unum septa formantia.

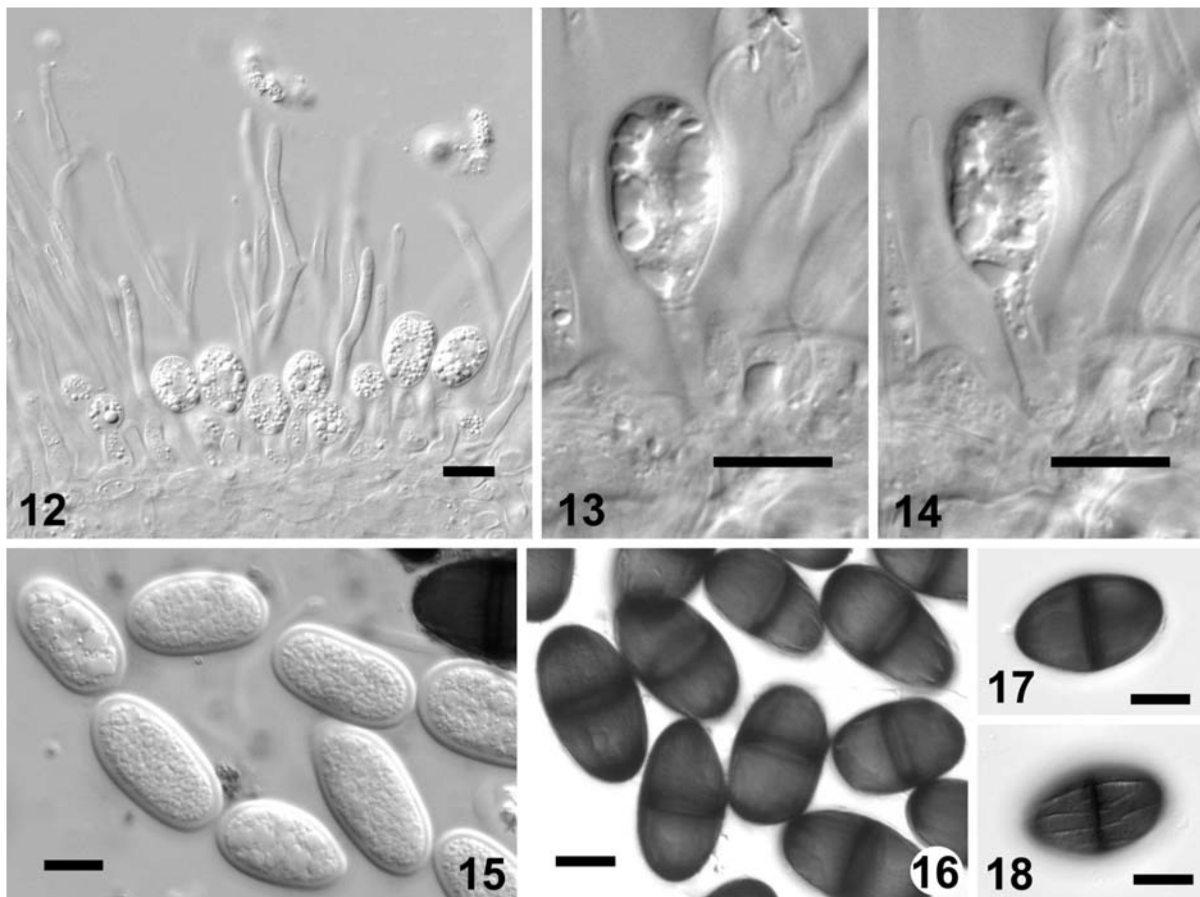
Conidiomata formed on poplar twigs in culture pycnidial, uniloculate, dark brown to black, immersed in the host becoming erumpent when mature. *Paraphyses* hyaline, cylindrical, mostly aseptate, sometimes branched, ends rounded, up 58 µm long, 3-4 µm wide arising amongst the conidiogenous cells. *Conidiogenous cells* hyaline, smooth, cylindrical, slightly swollen at the base, holoblastic, proliferating percurrently to form one or two closely spaced annelations. *Conidia* ellipsoidal, apex and base rounded, widest at the middle, thick-walled, initially hyaline and aseptate and remaining so for a long time, becoming one-septate and dark brown only some time after release from the conidiomata, with melanin deposits on the inner surface of the wall arranged longitudinally giving a striate appearance to the conidia, (22.5-)23.5-32(-33) × (13.5-)14-18(-20) µm, 95% confidence limits = 27.5-28.5 × 15.5-16.5 µm ($\bar{x}$ ± S.D. = 28 ± 2.5 × 16 ± 1.2 µm, l/w ratio = 1.7 ± 0.2).

Teleomorph: unknown

Habitat: *Acacia mangium, Citrus aurantium*, *Coffea* sp., *Gmelina arborea*, *Rosa* sp.

Known distribution: Costa Rica, Netherlands, Suriname, Zaire

Material examined: COSTA RICA, San Carlos, J. Carranza-Velásquez, *Gmelina arborea*, (CBS-H 19916; **holotype**, culture ex-type CBS116459). Other isolates are listed in Table 1.



Figs 12-18. *Lasiodiplodia pseudotheobromae* CBS116459. **12.** Conidiogenous layer with paraphyses. **13, 14.** Percurrently proliferating conidiogenous cell at two different focal planes. **15.** Hyaline, aseptate conidia. **16.** Septate, dark-walled conidia. **17, 18.** Mature conidium at two focal planes to show striations. Bars = 10 µm.

Lasiodiplodia parva A.J.L. Phillips, A. Alves & Crous, **sp. nov.** (Figs 19-23)
MycoBank: 510942.

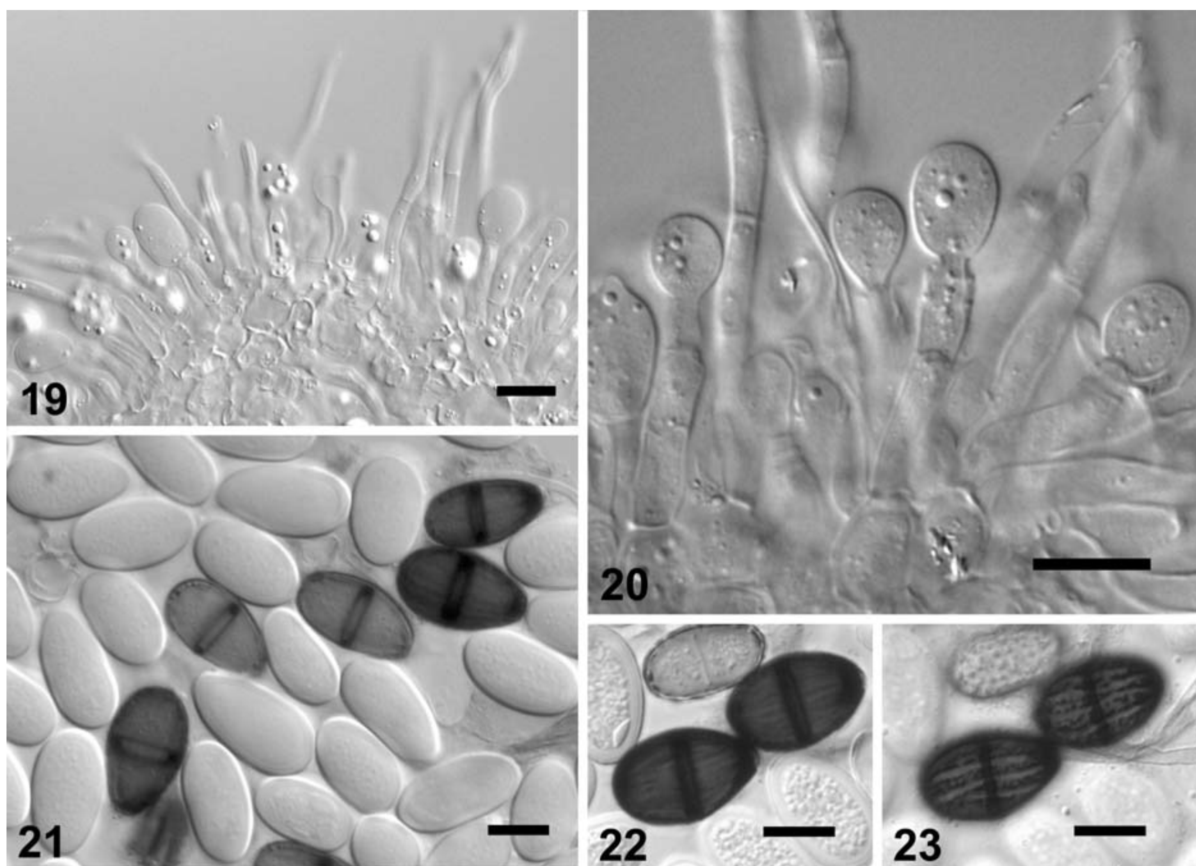
Etymology: Named for the small size of conidia.

Conidiomata in contextu hospitis inclusa, solitaria, stromatiformia, globosa. *Cellulae conidiogenae*, holoblasticae, hyalinae, subcylindricae, percurrenter cum 1-2 proliferationibus prolificentes, vel in plano eodem periclinaliter incrassatae. *Conidia* 19.8-20.5 × 11.4-11.7 µm, unicellulares, parietibus crassis, ovoidea, apicibus obtuse rotundato, in fundo obtuse rotundato, primaria hyalinae, cum maturitate cinnamomescentia vel brunnescentia, longitudinaliter striata et unum septa formantia.

Conidiomata formed on poplar twigs in culture pycnidial, uniloculate, dark brown to black, immersed in the host becoming erumpent when mature. *Paraphyses* hyaline, cylindrical, septate, ends rounded, up 105 µm long, 3-4 µm wide arising amongst the conidiogenous cells. *Conidiogenous cells* hyaline, smooth, cylindrical, slightly swollen at the base, holoblastic, proliferating percurrently to form one or two annelations, or proliferating at the same level giving rise to periclinal thickenings. *Conidia* ovoid, apex broadly rounded, base rounded or truncate, widest in the middle or upper third, thick-walled, initially hyaline and aseptate and remaining so for a long time, becoming one-septate and dark walled only some time after release from the conidiomata, with melanin deposits on the inner surface of the wall arranged longitudinally giving a striate appearance to the conidia, (15.5-)16-23.5(-24.5) × (10-)10.5-13(-14.5) µm, 95% confidence limits = 19.8-20.5 × 11.4-11.7 µm ($\bar{x}$ ± S.D. = 20.2 ± 1.9 × 11.5 ± 0.8 µm, l/w ratio = 1.8 ± 0.1).

Teleomorph: unknown

Habitat: Cassava-field soil, *Theobroma cacao*.



Figs 19-23. *Lasiodiplodia parva* CBS456.78. **19.** Conidiogenous layer with paraphyses. **20.** Percurrently proliferating conidiogenous cells. **21.** Hyaline, aseptate conidia and dark-walled, septate conidia. **22, 23.** Mature conidia at two focal planes to show striations. Bars = 10 µm.

Known distribution: Colombia, Sri Lanka.

Material examined: COLOMBIA, Dep. Meta, Villavicencio, cassava field soil, 1978, O. Rangel, (CBS-H 19915; **holotype**, culture ex-type CBS456.78). Other isolates examined are listed in Table 1.

Discussion

Phylogenetic relationships within a collection of isolates originally identified as *L. theobromae* was determined through a study of ITS and EF1-α nucleotide sequences. Morphological variability of conidial characters was also assessed within this collection.

Lasiodiplodia pseudotheobromae and *L. parva* are recognized as two new species in the genus *Lasiodiplodia*, closely related to *L. theobromae*. Both species possess morphological features typical of the genus, namely slowly maturing conidia with thick walls and longitudinal striations resulting from melanin deposition on the inner surface of the wall (Punithalingam, 1976; 1980). Although *L. pseudotheobromae* and *L. parva* resemble *L. theobromae*, they can be separated on the size and shape of their conidia. Thus, mature conidia of *L. pseudotheobromae* are larger, more ellipsoid and do not taper as strongly towards the base as in *L. theobromae*. Also, *L. parva* is easily distinguished from the other two species on account of its smaller conidia. *Lasiodiplodia pseudotheobromae* and *L. parva* produce a dark pink pigment in PDA cultures incubated at 35ºC, a feature that is not observed in *L. theobromae*. Moreover, *L. pseudotheobromae* is capable of growing at 10ºC, while *L. parva* and *L. theobromae* cannot. These conidial (size, shape, and septation) and cultural (colony morphology, chromogenicity, and temperature effects on mycelial growth) characters have been used to distinguish closely related species within "*Botryosphaeria*" anamorphs (Slippers *et al.*, 2004a, b; Phillips *et al.*, 2005; Alves *et al.*, 2006). In this way

Pavlic *et al*. (2004) distinguished *L. gonubiensis* from *L. theobromae* by its larger and multiseptate conidia. *Lasiodiplodia crassispora, L. venezuelensis* and *L. rubropurpurea* are also distinguished on the basis of their conidial morphology (Burgess *et al*., 2006).

Phylogenetic analyses of combined ITS and EF1-α nucleotide sequence data clearly separated all the species in *Lasiodiplodia*. These results showed that the new *Lasiodiplodia* species described are closely related, but distinct from *L. theobromae*. In phylogenetic terms, *L. pseudotheobromae* and *L. parva* are more closely related to each other than they are to *L. theobromae*.

The teleomorph of *L. theobromae* is frequently referred to as *Botryosphaeria rhodina* (Berk. & M.A. Curtis) Arx (von Arx, 1970, 1981). However, the correct name to use for the sexual state of *L. theobromae* is in need of clarification. In 1867 Curtis collected the type material of *Physalospora rhodina* on branches of *Rosa rubiginosa* in South Carolina, USA, and listed the fungus as *Sphaeria rhodina* B. & C., on page 148 of a catalogue published in the *Geographical and Natural History Survey of North Carolina*, Raleigh 1867. Subsequently, Cooke (Grevillea 17: 92, 1889) published a formal description of this fungus under the name *Physalospora rhodina* and attributed it to Berkeley & Curtis. However, because Cooke was the first one to provide a formal description of this fungus under the name *Physalospora rhodina*, and that Curtis merely listed the fungus (without a description) under the name *Sphaeria rhodina,* the species *P. rhodina* must be attributed to Cooke alone. Finally Von Arx (1970) transferred this species to the genus *Botryosphaeria*. Thus, the correct name that should be applied to this fungus is *B. rhodina* (Cooke) Arx. However, Crous *et al.* (2006) restricted *Botryosphaeria* to *B. dothidea* and *B. corticis* and so the genus name *Botryosphaeria* can no longer be applied to the teleomorph of *Lasiodiplodia* species.

Another important subject is related to the teleomorph-anamorph connection. As stated by Sivanesan (1984), the connection between the two states has yet to be absolutely proven, as there are some inconclusive reports in the literature. The connection between the anamorph and teleomorph was determined by Stevens (1925, 1926). Stevens (1925) made single ascospore isolations from a fungus he tentatively referred to as *Physalospora gossypina* on cotton stems in Florida. He made further isolations from a similar ascomycete found on *Hicoria*, *Ilex*, *Liquidambar*, *Quercus* and *Vitis* collected in the USA. In all cases, conidia in cultures generated from single ascospores were reportedly identical to those of *L. theobromae* (= *D. gossypina*). Therefore Stevens (1926) determined that the fungus he called "*Physalospora gossypina*" was in fact the same as *Physalospora rhodina* Cooke. However, there have been no subsequent reports that confirm this connection.

Stevens (1926) studied the perfect stage of the *Citrus* stem-end rot *Diplodia* and determined it to be a *Physalospora* species. He (Stevens, 1926) isolated the same fungus from *Persea* and *Rosa*. Cultures derived from single ascospores of the material from *Citrus* stem-end rot gave rise to conidia that agreed closely in appearance with *L. theobromae* (= *D. gossypina*). The cultures obtained from *Citrus*, *Persea* and *Rosa* exhibited good growth and chromogenesis on potato-dextrose agar at 36-37ºC (Stevens, 1926). However, not all isolates of the *Diplodia* from *Citrus* and other hosts that were able to grow at 36-37ºC showed chromogenesis at this temperature. Furthermore, isolates unable to grow at this temperature also occur on *Citrus* as well as other hosts (Stevens, 1926). Thus, according to Stevens (1926), the high temperature fungus should be considered merely as a variety within a single species (*Physalospora rhodina*), which is common on many hosts.

In the present work we have shown that the fungus previously known as *L. theobromae* is a complex of different cryptic species. In fact, due to the large number of hosts it has been associated with and the number of synonyms listed for this species it was reasonable to assume that it would constitute a complex of species. More importantly, in view of these facts, it is impossible to determine exactly to which species the teleomorph name

B. rhodina (Cooke) Arx should be attributed. In order to clarify this issue it is imperative to collect fresh material that can be used as epitype, and to prepare ex-epitype cultures that in turn can be used to fully characterise the species in terms of its morphology and its phylogenetic relationships with known species.

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