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The pathogenic potential of endophytic Botryosphaeriaceous fungi on *Terminalia* species in Cameroon

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Summary

In Cameroon, native *Terminalia* spp. represent an important component of the forestry industry, but limited information is available regarding the fungal pathogens that affect them. The Botryosphaeriaceae are endophytic fungi and latent pathogens that can result in wood stain, cankers, die-back and death of trees, particularly when trees are under stress. The aim of this study was, therefore, to identify and characterize the Botryosphaeriaceae occurring as endophytes of *Terminalia* spp. in Cameroon, as part of a larger project to identify potential pathogens of these trees in the country. Samples were collected from three *Terminalia* spp. in the Central, Southern and Eastern Regions and the resultant Botryosphaeriaceae were identified using morphology and DNA sequence comparisons for the ITS and *tef 1-α* gene regions. Furthermore, inoculation trials were conducted to consider the relative pathogenicity of the isolates collected. The majority of isolates (88%) represented species of *Lasiodiplodia,* including *L. pseudotheobromae*, *L. theobromae* and *L. parva.* The remaining isolates were identified as *Endomelanconiopsis endophytica.* Pathogenicity trials on young *T. mantaly* and *T. catappa* trees revealed that *L. pseudotheobromae* was the most pathogenic species followed by *L. theobromae*.

1 Introduction

The forestry sector in Cameroon plays an important role in the national economy of the country. Timber is the second most exported product, after petroleum, with wood-based exports generating revenue of US $210 million in 2001 (Anonymous 2005a). The total forest area in Cameroon is estimated to represent ~12.8 million ha of natural forests and about 17 000 ha of planted forests (Anonymous 2005b), made up of a variety of native trees such as *Terminalia* spp.

Species of *Terminalia* currently found in forest plantations in Cameroon include *T. ivorensis* and *T. superba*. These tree species have a well acknowledged commercial value with a total volume of exported logs representing 10% of the national round wood production (Laird 1999). Besides their high commercial value, *Terminalia* spp. are commonly used in agriculture to establish a 'taungya' agri-sylvicultural system in which they provide shade or improve soil fertility for crops (Norgrove and Hauser 2002). Furthermore, species such as *T. ivorensis* are important components for traditional medicine (Kamtchouing et al. 2006). Additional to native *Terminalia* spp., non-native species such as *T. mantaly* and *T. catappa* are frequently encountered as ornamentals in urban areas in Cameroon. The socio-economic importance of *Terminalia* spp. in Cameroon, coupled with their fast growth account for their extensive exploitation in national regeneration programmes.

Fungal pathogens belonging to the family of the Botryosphaeriaceae are among the potential threats to forest tree species. Species in the Botryosphaeriaceae have a worldwide occurrence, causing a wide range of diseases, predominantly die-back, canker and blue stain, on numerous hosts, including trees (Brown and Britton 1986; Denman et al. 1999, 2000; Desprez-Laustaud et al. 2006). This group of fungi commonly exists as endophytes in healthy plant tissues (Smith et al. 1996; Swart et al. 2000; Slippers and Wingfield 2007). Disease symptoms typically appear only after stress caused by abiotic and biotic disturbances (Schoeneweiss 1981; Slippers and Wingfield, 2007). Their occurrence as endophytes makes them especially important in international trade, as they may be spread undetected from one area to another, causing potentially serious damage to hosts that might have no co-evolved resistance (Slippers and Wingfield 2007).

Species of Botryosphaeriaceae contribute directly or indirectly to economic and environmental losses, although the impact of their diseases is difficult to assess in forestry. In South African pine plantations, for instance, up to 55% loss of production has been recorded after hail damage and die-back due to *Diplodia pinea* Fries (Zwolinski et al. 1990). In the USA several tree diseases associated with non-aggressive pathogens belonging to the Botryosphaeriaceae caused extensive mortality of Aspen during the 1930s (Schoeneweiss 1981). Moreover, other reports appear in literature recognizing severe decline of *Quercus* spp. due to species in the Botryosphaeriaceae in 1980 in the Mediterranean Basin (Sanchez et al. 2003).

In Africa, species of *Terminalia* occur in environments ranging from evergreen, primary and secondary forests to open woodlands or wooded savannahs (Carr 1994; Dale and Greenway 1961; Keay 1989; Lebrun and Stork 1991). Although these trees tend to display natural resistance to pests and diseases (Lamb and Ntima 1971; Groulez and Wood 1985), their wide ecological distribution exposes them to highly variable climatic conditions, environmental stress and other negative factors such as human activities (uncontrolled deforestation) and diverse pests and diseases. These factors may play an important role in predisposing *Terminalia* spp. in Africa to infection by species of the Botryosphaeriaceae (Jurskis 2005).

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The aim of this study was to identify species in the Botryosphaeriaceae that occur on *Terminalia* trees in Cameroon. This information will be valuable in the management of the health of these trees, because the Botryosphaeriaceae includes a key group of pathogens that generally affect forest trees and that will negatively affect these trees given projected changes in weather patterns. Identifications were done using a combination of morphological and DNA sequence data of the ITS and *tef 1-α* gene regions. Furthermore, inoculation trials using species of the Botryosphaeriaceae from *Terminalia* spp. were conducted to determine their potential pathogenicity.

2 Materials and methods

2.1 Sample collection and fungal isolation

Plant material was collected in 2007 and 2008 from two species of native and one non-native *Terminalia* in Cameroon. The tree species sampled were the non-native *T. mantaly* and native *T. ivorensis* and *T. superba.* Four sites, located in three regions, were chosen for sampling (Table 1). Depending on the availability of trees at each location, at least 15 trees per species were randomly chosen for sampling without considering either their size or age. In each area, samples from healthy twigs or bark were collected and placed in paper bags and transferred to the laboratory where they were processed within a few days.

For each sample, two pieces of twig or bark (1 cm in length) were split longitudinally. Samples were surface disinfested by sequential soaking in 70% ethanol (1 min), undiluted bleach (3.5% sodium hypochlorite for 1 min), 70% ethanol (1 min), rinsed in sterile water and allowed to dry under sterile conditions. Three disinfested pieces were placed onto 2% malt extract agar (MEA) (2% malt extract, 1.5% agar; Biolab, Midrand, Johannesburg, South Africa) supplemented with 1 mg ml^{-1} streptomycin (Sigma, St Louis, MO, USA) to suppress bacterial growth. The Petri plates were sealed with Parafilm (Pechiney Plastic Packaging, Chicago, USA) and incubated at 20°C under continuous near-UV light for 1 week. Single hyphal tips growing from the plant tissues were transferred to new Petri plates containing MEA. After 2 weeks of incubation under near UV-light, cultures resembling species of the Botryosphaeriaceae (fast growth, mycelium white originally, turning dark greenish-grey or greyish within few days) were selected and transferred to new Petri dishes containing MEA.

2.2 Morphology and cultural characteristics

To encourage formation of fruiting structures, isolates were inoculated onto sterile pine needles on 1.5% water agar (WA) (Biolab) as described previously (Slippers et al. 2004). The plates were incubated at 25°C under near UV-light for 4–6 weeks. Microscope slides of conidia from pycnidia formed on the pine needles were prepared in lactic acid for morphological observations. Conidial dimensions were taken from digital images using a HRc Axiocam digital camera and accompanying Axiovision 3.1 (Carl Zeiss Ltd, München, Germany) microscope. For each isolate, 15 measurements of both conidial length and width were made. Colony appearance of cultures growing on 2% MEA at 25°C under near UV-light for 2 weeks was described and colours of the colonies were recorded. Cultures are maintained in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

2.3 DNA extraction, PCR reactions and DNA sequencing

Procedures and protocols for genomic DNA extraction and sequencing of representative isolates of the Botryosphaeriaceae were the same as those described in Begoude et al. (2010), using two gene regions. The entire Internal Transcribed Spacer region (ITS) of the rDNA, including the 5.8S gene, was amplified by polymerase chain reaction (PCR), for all isolates collected, using the primers ITS1 and ITS4 (White et al. 1990). A part of the Translation Elongation Factor-1α (*tef 1-α*) gene was amplified for selected isolates using the primers EF1F and EF1R (Jacobs et al. 2004).

2.4 Sequence analyses

Sequences of the Botryosphaeriaceae generated in this study were edited using mega version 4 (Tamura et al. 2007). For the phylogenetic analyses, DNA sequences from this study, together with those retrieved from published sequences in GenBank (http://www.ncbi.nlm.gov) were aligned online using MAFFT (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/) version 6 (Katoh et al. 2005). The aligned sequences were transferred to phylogenetic analysis using parsimony (PAUP) v. 4.0b10 (Swofford 2001) where a final manual alignment was made.

Table 1. Locations and characteristics of sites from where *Terminalia* trees were sampled for Botryosphaeriaceae in Cameroon.

Site			
Region	Locality	GPS coordinates	Tree species sampled
Central	Mbalmayo	N3 26.034 E11 29.344	*T. superba, T. ivorensis*
South	Nkoemvone	N2 49.045 E11 07.577	*T. superba*
	Kribi	N2 58.064 E9 54.904	*T. mantaly*
Eastern	Belabo	N4 57.376 E13 19.433	*T. superba, T. ivorensis, T. mantaly*

A phylogenetic analysis was run for each of the ITS and *tef 1-α* data sets, followed by combined analyses of these data sets for core isolates. A partition homogeneity test (Farris et al. 1995) was conducted in PAUP v. 4.0b10 (Swofford 2001) to assess the possibility of combining the ITS and *tef 1-α* data sets. In all analyses, gaps were treated as fifth character and characters were unordered and of equal weight. The phylogenetic analyses for the datasets were performed using the maximum parsimony (MP) option, with trees generated by heuristic searches with random stepwise addition in 1000 replicates, tree bisection and reconnection (TBR) as branch swapping algorithm and random taxon addition of sequences for the construction of most parsimonious trees. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. *Guignardia mangiferae* A.J. Roy (strain no. 1095) was used as the outgroup in the analyses of ITS and *tef 1-α* data sets. The support for branches of the most parsimonious trees was assessed using 1000 bootstrap replicates (Felsenstein 1985). Other measures considered were the tree length (TL), consistency index (CI), rescaled consistency index (RC) and retention index (RI) (Hillis and Huelsenbeck 1992).

Bayesian phylogenetic analyses using Markov chain Monte Carlo (MCMC) were performed in MrBayes 3.1.2. (Huelsenbeck and Ronquist 2001) for all three data sets described above. Version 2.2 of MrModeltest (Nylander 2004) was used to select the model that best fits each of the partitions. The Likelihood settings from best-fit models, SYM + I + G and HKY + G, were selected based on the Akaike Information Criteria (AIC) for ITS and *tef 1-α* respectively. Bayesian analyses were performed for one million generations, with four independent chains and sampled every 100th tree. The first 1000 trees were graphically identified as the burn-in and deleted when constructing consensus trees and calculating posterior probabilities. A total of 9001 trees were imported into MEGA v. 4 to construct a 50% majority-rule consensus tree.

2.5 Pathogenicity tests

Plants of native species of *Terminalia* are rare and could not be obtained. Pathogenicity tests were consequently carried out on 1-year-old non-native *T. mantaly* and *T. catappa* trees grown in the Yaoundé Urban Council nursery, Cameroon. These trials were conducted between October–December 2008. This period falls at the end of the rain season and the beginning of the dry season, with average day and night temperatures of 26°C. The trees were maintained under shade in 15 cm diameter plastic bags and watered daily. At the time of inoculation, the stem diameters were approximately 10 mm and the trees varied from 15–30 cm in height. For inoculations, 14 isolates of the Botryosphaeriaceae, representing all the species identified in the study, were grown on 2% MEA for 10 days prior to inoculation.

To inoculate trees, wounds were made on the stems, half way between the soil level and the first branch by removing the outer bark with a 5 mm diameter cork-borer. A 5 mm-diameter plug of each isolate was placed into each wound, with the mycelium facing the cambium and wrapped with a strip of Parafilm to prevent desiccation and cross contamination of the wounds and inoculum. The trees were divided into four separate blocks and within each block, six trees arranged in a completely randomized design, were used for inoculation with each isolate. The entire trial was repeated once. For the controls, sterile MEA plugs were used in place of the fungal cultures.

After 6 weeks, the lengths of the lesions produced in the cambium, including the inoculation point, were measured to obtain an indication of the virulence of the isolates tested. Furthermore, a small piece of necrotic tissue was cut from the edges of all lesions and placed on MEA for isolations to show that the inoculated fungus was associated with the lesions. As no significant differences were noticed between results obtained for the two replications of the experiment ($p > 0.05$), the data for all isolates were pooled in a single dataset for analyses. Variations in the extent of the lesions were assessed through a one-way analysis of variance (ANOVA) using SAS (SAS systems, v. 8.2; SAS Institute).

3 Results

3.1 Isolates and morphology

A total of 115 trees were sampled at four localities. These included 35 *T. ivorensis* trees, 50 *T. superba* and 30 *T. mantaly* trees. Isolates of Botryosphaeriaceae were obtained from 55 of the 115 trees sampled. In total, 43 isolates were obtained from 35 *T. ivorensis* trees, 20 isolates from seven *T. superba* trees and 27 isolates from 11 *T. mantaly* trees. No sign of disease, caused by fungi in the Botryosphaeriaceae, was observed on any trees at the time of collection. It was thus assumed that all isolates were from healthy trees.

The isolates obtained were assigned to two groups based on colony and conidial morphology. The majority of isolates collected (82 isolates) produced aerial mycelium that was white at first, turning dark grey-green or grey after 4–5 days at 25°C under near UV-light. These isolates produced thick-walled, hyaline conidia that turned dark with age (Fig. 1). The conidia were aseptate when young, becoming uniseptate with age. Conidia were ovoid in shape and some developed longitudinal striations as they aged. These isolates were identified as belonging to species of *Lasiodiplodia* based on their conidial morphology. The second group of isolates (eight isolates) were characterized by dark grey or green to black mycelium, producing small dark brown conidia (Fig. 1) and resembling *Endomelanconiopsis endophytica* (Rojas et al. 2008).

No sexual fruiting structures were produced on pine needles by any of the isolates from *Terminalia* spp. in Cameroon. Isolates from the *Lasiodiplodia* group were found at all the localities sampled and from all three host species. Isolates residing in the second group were found only in three locations (Belabo, Nkoemvone and Mbalmayo) and only on *T. ivorensis* and *T. superba*. The Botryosphaeriaceae occurring on *Terminalia* spp. in Cameroon were compared to similar species described in previous studies (Table 3).

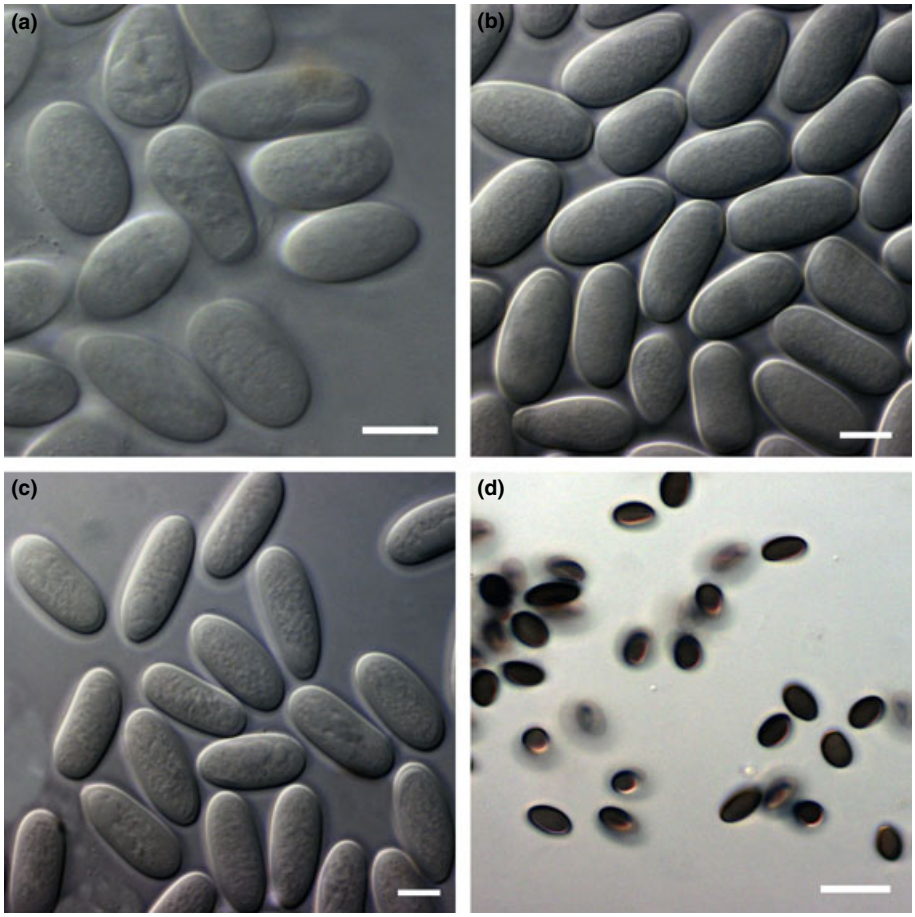

Fig. 1. Conidia of species of the Botryosphaeriaceae from Terminalia spp. (a) young hyaline thick-walled conidia of Lasiodiplodia theobromae (CMW28550), (b) *L. pseudotheobromae* (CMW28297), (c) *L. parva* (CMW28333) (d) dark brown conidia of *E. endophytica* (CMW28618). Bars: 10 μm.

3.2 DNA extraction and PCR amplification

A total of 55 isolates, each originating from a single *Terminalia* tree, were selected for sequencing of their ITS and 5.8S rDNA regions to obtain an indication of their identities and to select isolates for the data sets used in the final analyses. These comprised 51 isolates from the morphological group resembling *Lasiodiplodia* and four from the group resembling *Endomelanconiopsis.* Based on results of the ITS sequences, fourteen isolates were selected for sequencing of the *tef 1-α* gene region and considered in the final analyses. PCR fragments for the ITS and 5.8S gene were ~580 bp and for *tef 1-α* gene region were ~710 bp in size. The *tef 1-α* sequences obtained were larger than those retrieved from GenBank, which spanned 244–500 bp and only the corresponding regions were used in the phylogenetic analyses.

3.3 Phylogenetic analyses

A BLAST search against the GenBank database, using ITS sequences obtained from *Terminalia* spp. in Cameroon, showed that isolates resembling species of *Lasiodiplodia* were most closely related to *L. theobromae* (Pat.) Griff. & Maubl. and *L. pseudotheobromae* A.J.L. Phillips, A. Alves & Crous. Isolates from the second group, with small dark brown conidia, were identified as *Endomelanconiopsis endophytica* Rojas & Samuels.

3.3.1 ITS phylogeny

The ITS dataset comprised 91 sequences including 55 from *Terminalia* spp. sampled in Cameroon and 36 sequences were retrieved from GenBank. After alignment, the ITS sequence data set consisted of 575 characters of which 313 were constant, 112 were parsimony uninformative and 150 were parsimony informative. The MP analyses generated 100 trees with identical topologies with respect to the major clades (TL = 563, CI = 0.627, RI = 0.868, RC = 0.544).

The MP analyses of the ITS gene region resulted in two main clades that contained isolates from *Terminalia* spp. These clades represented the two genera *Lasiodiplodia* [Bootstrap support (BS) = 100% and Bayesian posterior probabilities (BPP) = 0.99] and *Endomelanconiopsis* (BS = 100% and BPP = 1) (Fig. 2). In the *Endomelanconiopsis* group, except for two isolates

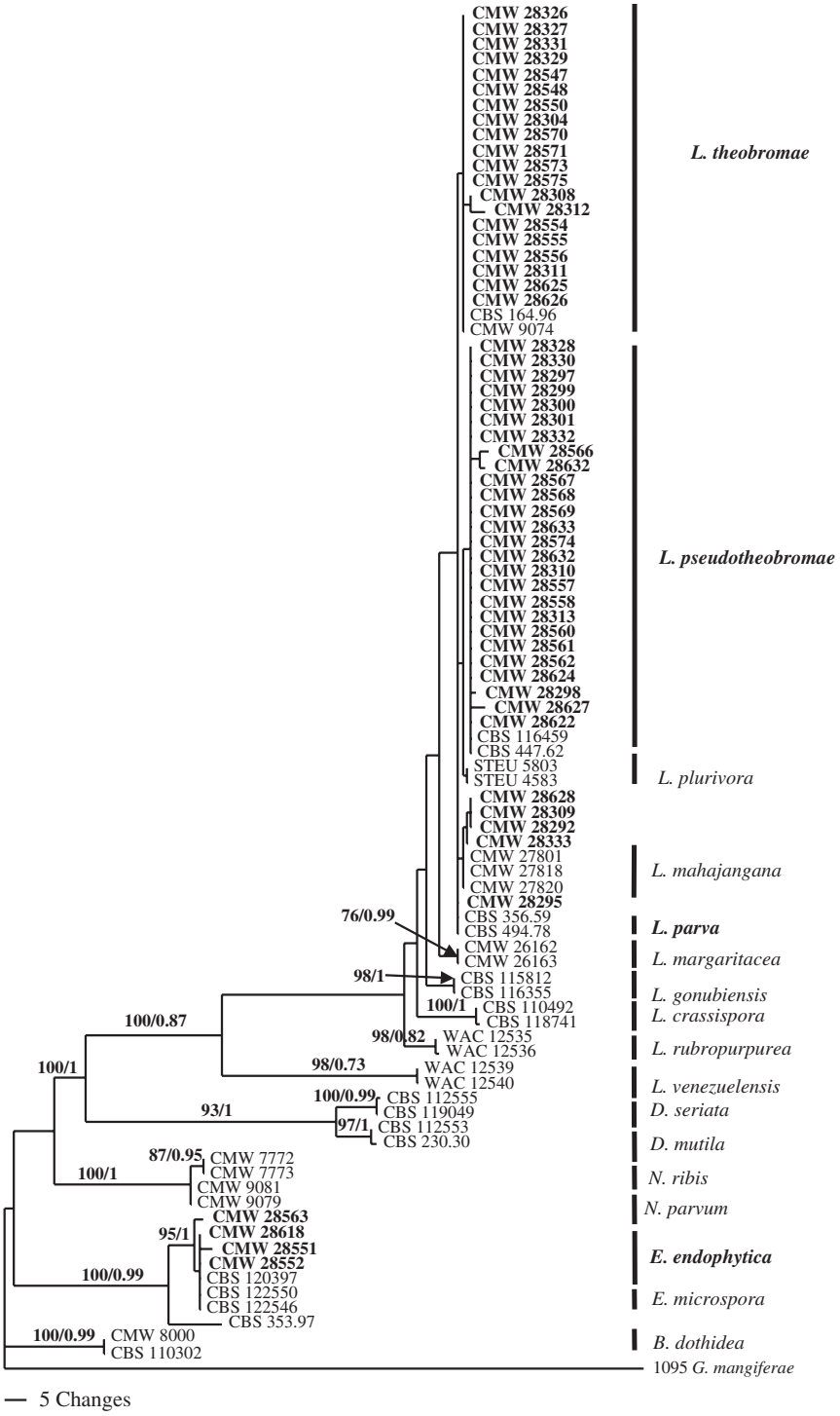

Fig. 2. One of the most parsimonious trees obtained from analyses of the ITS sequence data of the Botryosphaeriaceae from *Terminalia spp.* Bootstrap support (%) followed by Posterior probabilities from 1000 replications are given on the branches (BS/PP). Isolates marked in bold represent those obtained from *Terminalia spp.* in Cameroon.

(CMW28551, CMW28563) where very little divergence (two to three base pairs) was observed, sequences from *Terminalia* spp. in Cameroon were identical to *E. endophytica* and clustered with isolates from Panama (see Table 2). The *Lasiodiplodia* group included most of the isolates obtained in this study and was divided into three sub-clades with no clear Bootstrap support. The first sub-clade (20 isolates) consisted of isolates grouping with *L. theobromae*. Except for two isolates, no sequence variation was detected between isolates in this clade. The second sub-clade (25 isolates) accommodated isolates clustering with *L. pseudotheobromae*. Small sequence variations were observed in a few isolates of this group. The third group,

Table 2. Botryosphaeriaceae used in phylogenetic analyses in this study.

Species	Culture number[1]	Origin	Host	Collectors	GenBank accession no.		
					ITS	*tef* 1-α	*β-tub*
Botryosphaeria dothidea	CMW7999	Switzerland	*Ostrya* spp.	B. Slippers	AY236948	AY236897	
	CMW8000	Switzerland	*Prunus* spp.	B. Slippers	AY236949	AY236898	
Diplodia mutila	CBS112553	Portugal	*Vitis vinifera*	A.J.L. Phillips	AY259093		
	CBS230.30	USA	*P. dactylifera*	L.L. Huilllier	DQ458886		
D. seriata	CMW7774	USA	*Ribes* spp.	B. Slippers/G.Hudler	EF445343	EF445382	
	CMW7775	USA	*Ribes* spp.	B. Slippers/G.Hudler	EF445344	EF445383	
Endomelanconiopsis endophytica	**CMW28618**	Cameroon	*Terminalia ivorensis*	D. Begoude	GQ469966	GQ469906	
	CMW28551	Cameroon	*T. superba*	D. Begoude/J. Roux	GQ469967	GQ469907	
	CMW28552	Cameroon	*T. superba*	D. Begoude/J. Roux	GQ469968	GQ569908	
	CMW28563	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469965	EU683637	
	CBS120397	Panama	*Theobroma cacao*	E. Rojas/L.Mejia/Z. Maynard	EU633656	EU683642	
	CBS122546	Panama	*Th. cacao*	E. Rojas/L.Mejia/Z. Maynard	EU683661	EU683645	
	CBS122550	Panama	*Th. cacao*	E. Rojas/L.Mejia/Z. Maynard	EU683664	EU683636	
E. microspora	CBS353.97	Panama	Soil	E. Rojas/L.Mejia/Z. Maynard	EU683655	EU683652	
Guignardia mangiferae	1095	Panama	*Th. cacao*	E. Rojas/L.Mejia/Z. Maynard	EU683671		
Lasiodiplodia crassispora	WAC12533	Venezuela	*Eucalyptusurophylla*	S. Mohali	DQ103552	DQ10355	
	WAC12534	Australia	*Santalum album*	T.I. Burgess/B. Dell	DQ103550	DQ103557	
	WAC12535	Australia	*S. album*	T.I. Burgess/B. Dell	DQ103551	DQ103558	
L. gonubiensis	CBS115812	South Africa	*S. cordatum*	D. Pavlic	DQ458892	DQ458877	
	CBS116355	South Africa	*S. cordatum*	D. Pavlic	AY639594	DQ103567	
L. mahajangana	CMW27801	Madagascar	*T. catappa*	J. Roux	FJ900595	FJ900641	
	CMW27818	Madagascar	*T. catappa*	J. Roux	FJ900596	FJ900642	
	CMW27820	Madagascar	*T. catappa*	J. Roux	FJ900597	FJ900643	
L. margaritacea	CMW26162	Australia	*A. gibbosa*	D. Pavlic	EU144050	EU144065	
	CMW26163	Australia	*A. gibbosa*	D. Pavlic	EU144051	EU144066	
L. parva	CBS356.59	Sri Lanka	*T. cacao*	A. Riggenbach	EF622082	EF622062	GQ469892
	CBS494.78	Colombia	Cassava-field soil	O. Rangel	EF622084	EF622064	GQ469894
	CMW28333	Cameroon	*T. superba*	D. Begoude/J. Roux	GQ469961	GQ469903	GQ469893
	CMW28309	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469962	GQ469904	
	CMW28292	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469963	GQ469905	
	CMW28295	Cameroon	*T. mantaly*	D. Begoude/J. Roux	GQ469964		
		CMW28628	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469960	
L. plurivora	STEU	South Africa	*P. salicina*	U.Damm	EF445362	EF445395	
	STEU	South Africa	*V. vinifera*	F.Halleen	AY343482	EF445396	
L. pseudotheobromae	**CMW28297**	Cameroon	*T. mantaly*	D. Begoude/J. Roux	GQ469937	GQ469899	
	CMW28300	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469939	GQ469900	
	CMW28574	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469947	GQ469901	
	CMW28624	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469956	GQ469902	
	CMW28328	Cameroon	*T. mantaly*	D. Begoude/J. Roux	GQ469935		
	CMW28330	Cameroon	*T. mantaly*	D. Begoude/J. Roux	GQ469936		
	CMW28299	Cameroon	*T. superba*	D. Begoude/J. Roux	GQ469938		
	CMW28301	Cameroon	*T. superba*	D. Begoude/J. Roux	GQ469940		
	CMW28332	Cameroon	*T. superba*	D. Begoude/J. Roux	GQ469941		
	CMW28566	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469942		
	CMW28314	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469943		
	CMW28568	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469944		
	CMW28569	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469945		

Table 2. (Continued)

Species	Culture number[1]	Origin	Host	Collectors	GenBank accession no.		
					ITS	*tef* 1-α	*β-tub*
	CMW28633	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469946		
	CMW28632	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469948		
	CMW28310	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469949		
	CMW28557	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469950		
	CMW28558	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469951		
	CMW28313	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469952		
	CMW28560	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469953		
	CMW28561	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469954		
	CMW28562	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469955		
	CMW28298	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469957		
	CMW28627	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469958		
	CMW28622	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469959		
	CBS116459	Costa Rica	*Gmelinea arborea*	J.Carranza/Velásquez	EF622077	EF622057	
	CBS447.62	Suriname	*Citrus aurantium*	C. Smulders	EF622081	EF622060	
L. rubropupurea	WAC12535	Australia	*E. grandis*	T.I. Burgess/G.Pegg	DQ103553	DQ103571	
	WAC12536	Australia	*E. grandis*	T.I. Burgess/G.Pegg	DQ103554	DQ103572	
L. theobromae	**CMW28550**	Cameroon	*T. mantaly*	D. Begoude/ J.Roux	GQ469921	GQ469895	
	CMW28570	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469923	GQ469896	
	CMW26571	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469924	GQ469897	
	CMW27311	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469932	GQ469898	
	CMW28326	Cameroon	*T. mantaly*	D. Begoude/J. Roux	GQ469915		
	CMW28327	Cameroon	*T. mantaly*	D. Begoude/J. Roux	GQ469916		
	CMW28329	Cameroon	*T. mantaly*	D. Begoude/J. Roux	GQ469918		
	CMW28547	Cameroon	*T. mantaly*	D. Begoude/J. Roux	GQ469919		
	CMW28548	Cameroon	*T. mantaly*	D. Begoude/J. Roux	GQ469920		
	CMW28573	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469925		
	CMW28575	Cameroon	*T. superba*	D. Begoude/J. Roux	GQ469926		
	CMW28308	Cameroon	*T. superba*	D. Begoude/J. Roux	GQ469927		
	CMW28312	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469928		
	CMW28554	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469929		
	CMW28555	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469930		
	CMW28556	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469931		
	CMW28625	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469933		
	CMW28626	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469934		
	CMW9074	Mexico	*Pinus* sp.	T. Burgess	EF622074	EF622054	
	CBS164.96	New Guinea	Fruit along coral reef coast	Unknown	AY640255	AY640258	
L. venezuelensis	WAC12539	Venezuela	*Acacia mangium*	S. Mohali	DQ103547	DQ103568	
	WAC12540	Venezuela	*A. mangium*	S. Mohali	DQ103548	DQ103569	
Neofusicoccum parvum	CMW9081	New Zealand	*P. nigra*	G.J. Samuels	AY236943	AY236888	
	CMW9079	New Zealand	*A. deliciosa*	S.R. Pennicook	AY236940	AY236885	
N. ribis	CMW7772	USA	*Ribes* sp.	B. Slippers/G.Hudler	AY236935	AY236877	
	CMW7773	USA	*Ribes* sp.	B. Slippers/G Hudler	AY236936	AY236878	

[1]Isolates sequenced in this study appear in bold. All other sequences were obtained from GenBank.

consisting of five isolates from Cameroon was not clearly resolved and clustered close to *L. mahajangana* Begoude, Jol. Roux, & Slippers. and *L. parva* A.J.L. Phillips, A. Alves & Crous. No statistical support was observed for any of these sub-clades. For this reason, representative isolates from the *Endomelaconiopsis* clade and the three sub-clades in the *Lasiodiplodia* group were selected for *tef 1-α* gene region sequencing.

3.3.2 Combined ITS and tef 1-α analyses

The partition homogeneity test indicated congruence between the ITS and *tef 1-α* partitions (p = 0.355) suggesting that the data sets could be combined. The combined dataset consisted of 48 isolates with 887 characters of which, 377 were constant, 146 were parsimony uninformative and 364 were parsimony informative. Gaps were treated as a fifth character. After heuristic searches, 42 most parsimonious trees were obtained (TL = 1068; CI = 0.738, RI = 0.914, RC = 0.674; TreeBase Accession No. SN4630) and one of them was chosen for presentation (Fig. 3). All 42 trees displayed the same topology with regard to the identified clades. The topology of the tree generated from the combined analyses with MP, as well as with the 50% majority rule consensus tree from the trees obtained through Bayesian analysis, was congruent with the trees obtained with the individual analyses of ITS and *tef 1-α*, identifying the same clades.

All the isolates collected in this study grouped with previously described species of *Lasiodiplodia* and *Endomelanconiopsis*, strongly supported with Bootstrap and Bayesian posterior probability values (Fig. 3). Similar to results obtained for the ITS gene region, isolates from Cameroon could be identified as *L. theobromae* (BS = 100%, BPP = 1), *L. pseudotheobromae* (BS = 100%; BPP = 1). The third group of *Lasiodiplodia* isolates clustered with *L. parva* (BS = 97%; BPP = 1), but one base pair difference in the *tef 1-α* sequences was noticed among isolates in this later group. The fourth group of isolates consisted of *E. endophytica* from *Terminalia* spp. in Cameroon which formed a well supported clade (BS = 100% and BPP = 1) with sequences from authentic isolates of this species from GenBank (Fig. 3).

Isolates of Botryosphaeriaceae found on *Terminalia* spp. that were phylogenetically related to *L. parva* based on ITS and *tef 1-α* sequence comparisons, mostly conformed to the description of *L. parva* (Alves et al. 2008). However, important differences in conidial sizes were observed for isolates from Cameroon (Table 3), raising the question as to whether they represent a different species. DNA sequence data for the ITS and *tef 1-α* gene regions, however, did not support the description of a discrete species for these isolates. Further sequences from additional gene regions (*β*-tubulin) not reported in this paper were found to be identical with those of original species of *L. parva* and, therefore, suggested that all these isolates represent the same species.

3.4 Pathogenicity

Pathogenicity trials conducted on *T. mantaly* using isolates of the Botryosphaeriaceae collected in this study revealed visible lesions within 6 weeks after inoculation (Fig. 4). Trees inoculated with sterile MEA also produced small lesions that represented only wound reactions as no Botryosphaeriaceae could be isolated from them. All the isolates of Botryosphaeriaceae were successfully re-isolated from the lesions emerging from inoculations. ANOVA showed that the mean lengths of lesions produced by all of the isolates on *T. mantaly* differed significantly (p < 0.0001) from the controls (Fig. 4). *L. pseudotheobromae* produced the longest lesions followed by *L. theobromae, L. parva* and *E. endophytica*.

On *T. catappa* trees, all isolates collected from *Terminalia* trees in Cameroon produced lesions significantly longer than those of the control inoculations (Fig. 5). Similar to the situation on *T. mantaly*, control inoculations showed only small lesions. However, re-isolations did not yield any Botryosphaeriaceae from the controls, whereas the original Botryosphaeriaceae were re-isolated from all the trees inoculated with fungal cultures. Analysis of variance indicated that lesion lengths produced on the cambium by all the isolates were significantly different (p < 0.0001) to those associated with the controls (Fig. 5). Isolates representing *L. pseudotheobromae* were most virulent and produced longer lesions than *L. theobromae* and *L. parva. E. endophytica* produced substantially smaller lesions than either *L. pseudotheobromae* or *L. theobromae*.

A positive correlation (r^2 = 77%) was found between inoculations on *T. mantaly* and *T. catappa.* On both tree species, *L. pseudotheobromae* was most virulent. In general, the lesions in *T. catappa* caused by *L. pseudotheobromae* and *L. theobromae* isolates were longer than those of *T. mantaly* . In contrast, the lengths of lesions produced by isolates of *L. parva* and *E. endophytica* on *T. catappa* were smaller than those observed on *T. mantaly.* However, this difference in susceptibility between *T. catappa* and *T. mantaly* was not statistically significant.

4 Discussion

This study represents the first attempt to identify the Botryosphaeriaceae on native *Terminalia* trees in Africa. Four species of the Botryosphaeriaceae were collected from *T. ivorensis* and *T. superba* and three species were found on samples from the non-native *T. mantaly*. A combination of morphological characteristics and DNA sequence comparisons was used to identify these species as *L. theobromae, L. pseudotheobromae, L. parva* and *E. endophytica.* These fungi are reported on these hosts for the first time. While *E. endophytica* was isolated only from *T. superba* and *T. ivorensis*, *L. pseudotheobromae, L. theobromae* and *L. parva* were collected from all the tree species sampled in this study.

The majority of isolates obtained in this study represented species of *Lasiodiplodia* of which isolates were identified as *L. theobromae, L. pseudotheobromae* and *L. parva* based on sequence data for the ITS and *tef 1-α* gene regions. Until recently, most *Lasiodiplodia* spp. from tropical trees were treated as *L. theobromae* (Punithalingam 1976). However, application of DNA sequence comparisons for the ITS and *tef 1-α* gene regions has resulted in the recognition of 10 new *Lasiodiplodia* spp. (Pavlic

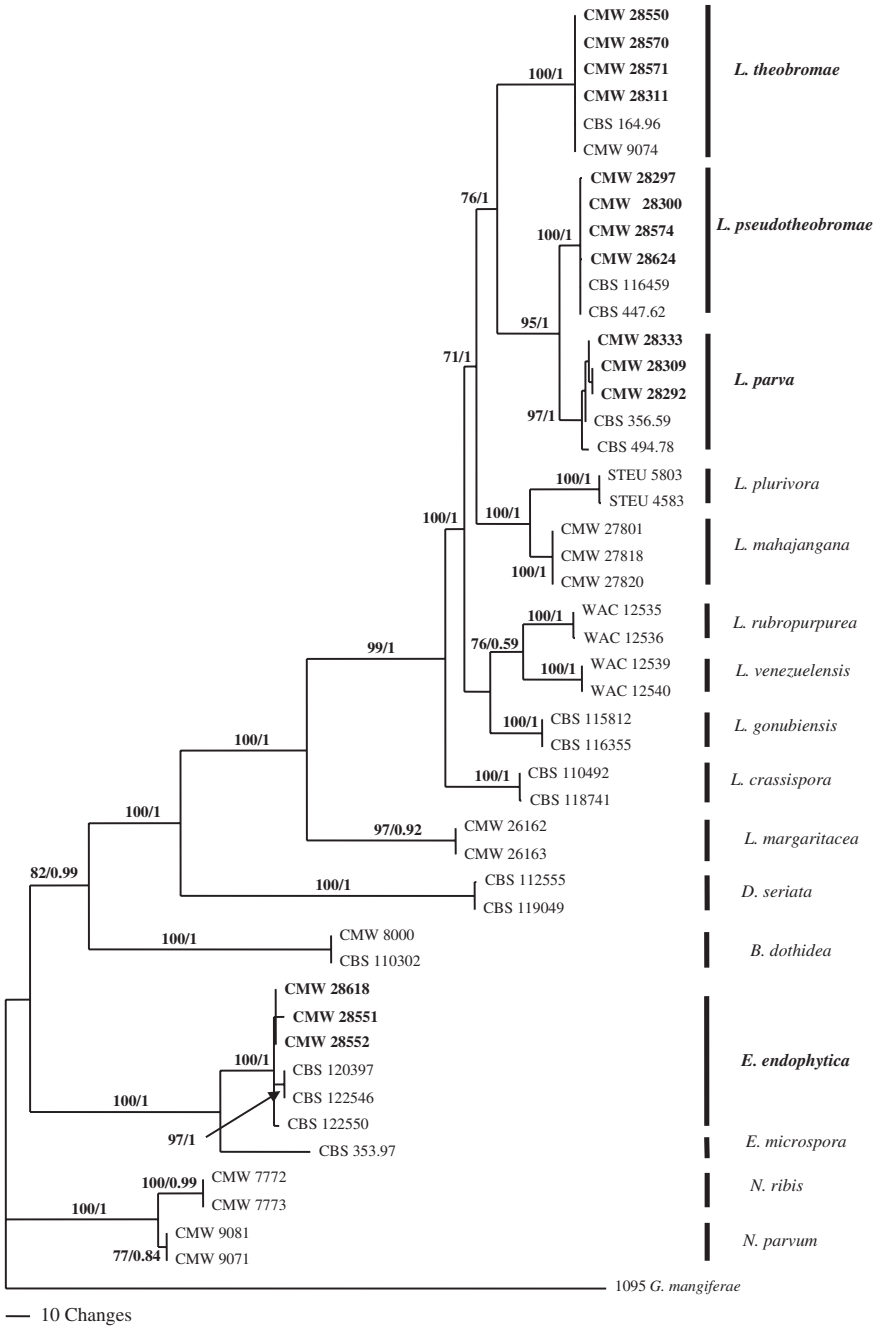

Fig. 3. One of the most parsimonious trees obtained from analyses of the combined ITS and tef 1-α sequence data of the Botryosphaeriaceae from *Terminalia spp.* Bootstrap support (%) followed by Posterior probabilities from 1000 replications are given on the branches (BS/PP). Isolates marked in bold represent those obtained from *Terminalia spp.* in Cameroon.

et al. 2004; Burgess et al. 2006; Damm et al. 2007; Alves et al. 2008; Pavlic et al. 2008; Begoude et al. 2010). These species share similar morphological characteristics, such as slowly maturing conidia with thick walls that turn dark with age and develop longitudinal striations.

Lasiodiplodia theobromae has a wide geographic distribution and it has been found on more than 500 forest and agricultural plant species in tropical and subtropical areas (Punithalingam 1980). It is well known as an endophyte on healthy tropical trees (Suryanarayanan et al. 2002; Begoude et al. 2010). Furthermore, *L. theobromae* can act as a latent pathogen causing disease symptoms after onset of conditions unfavourable for the host (Schoeneweiss 1981; Mullen et al. 1991; Slippers and Wingfield 2007). *L. theobromae* has previously been reported as an endophyte in the inner bark and twigs of healthy *T. arjuna* (Tejesvi et al. 2005), leaves of *T. tomentosa* and *T. bellerica* (Suryanarayanan et al. 2002) and the twigs and bark of healthy *T. catappa* (Begoude et al. 2010) in the tropics. On *Terminalia* spp., *L. theobromae* has mostly been recorded as the causal agent

Table 3. Conidial dimensions of the Botryosphaeriaceae from *Terminalia* spp. in Cameroon and comparison with those reported in previous studies.

Species	Conidial size (μm)		Source of data
	This study	Previous studies	
L. pseudotheobromae	(20.5–)23.5–27.5(–31.5) × (10.5–)12–14(–16.5)	(22.5–)23.5–32(–33) × (13.3–)14–18(–20)	Alves et al. (2008)
L. theobromae	(17.5–)21.5–27.5(–31) × (10.5–)12–14(–16.5)	(19–)21–31(–32.5) × (12–)13–15.5(–18.5)	Alves et al. (2008)
L. parva	(24.5–)26.5–29.5(–33.5) × (11–)12–14.5(–17.5)	(15.5–)16–23.5(–24.5) × (10–)10.5–13(–14.5)	Alves et al. (2008)
E. endophytica	(5.5–)6–7.5(–8) × (3–)3.5–4(–4.5)	(4.7–)5.5–7.5(–10.0) × (3.0–)3.5–4.5(–6.2)	Rojas et al. (2008)

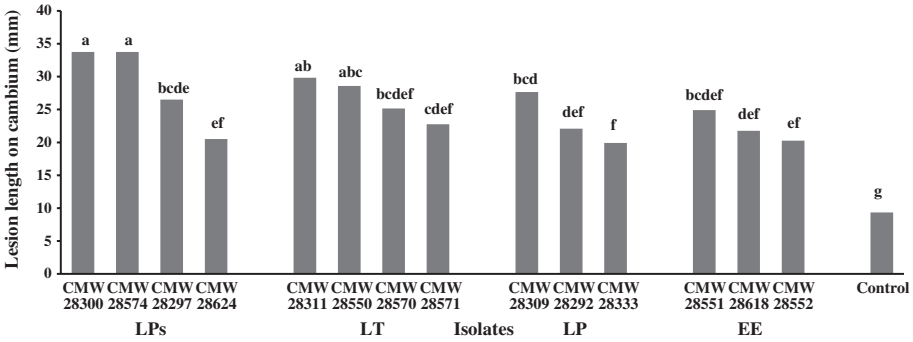

Fig. 4. Mean lesion lengths (mm) on cambium of *T. mantaly* 6 weeks after inoculation with isolates of *L. pseudotheobromae* (LPs), *L. theobromae* (LT), *L. parva* (LP), *E. endophytica* (EE), Control. Lesion lengths caused by isolates marked with the same letter are not significantly different ($p < 0.0001$).

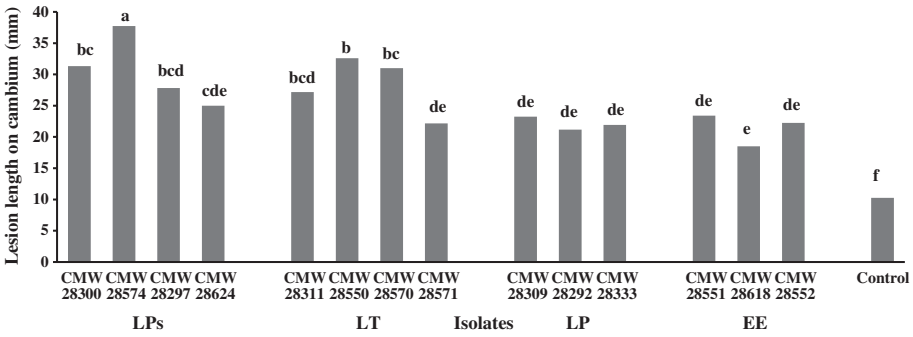

Fig. 5. Mean lesion lengths (mm) on cambium of *T. catappa* 6 weeks after inoculation with isolates of *L. pseudotheobromae* (LPs), *L. theobromae* (LT), *L. parva* (LP), *E. endophytica* (EE), Control. Lesion lengths caused by isolates marked with the same letter are not significantly different ($p < 0.0001$).

of blue stain of logs, soon after felling (Lamb and Ntima 1971; Groulez and Wood 1985; Apetorgbor et al. 2004). However, in Cameroon, *L. theobromae* is best known as the cause of die-back of cacao (*Theobromae cacao*) (Mbenoun et al. 2008). In the current study, *L. theobromae* was the second most abundant species identified on *Terminalia* spp. All isolates collected were from healthy trees, but pathogenicity trials on young *T. catappa* and *T. mantaly* showed that it is highly pathogenic to these trees. Pathogenicity tests on *T. ivorensis* and *T. superba* should, however, be conducted to determine whether it can cause disease on these important native trees.

Lasiodiplodia pseudotheobromae was the most commonly collected species of Botryosphaeriaceae, collected from all the species of *Terminalia* sampled in this study. This fungus was originally described from *Rosa* spp. in the Netherlands, *Gmelina arborea* and *Acacia mangium* in Costa Rica, *Coffea* spp. in Democratic Republic of Congo and *Citrus aurantium* in Suriname (Alves et al. 2008). In a recent study investigating the Botryosphaeriaceae on *T. catappa* in Cameroon, South Africa and Madagascar (Begoude et al. 2010), *L. pseudotheobromae* was also the most abundant species found in all the sampled areas.

The information generated in the current study, which is supported by a previous one on *T. catappa*, suggests that *L. pseudotheobromae* has a worldwide distribution. In pathogenicity trials *L. pseudotheobromae* was found to be the most virulent species. This was also the case in a study of *T. catappa* (Begoude et al. 2010). *L. pseudotheobromae* is, therefore, the most likely species of Botryosphaeriaceae to cause health problems on *Terminalia* trees in Africa where they are subjected to stressful conditions.

Lasiodiplodia parva was only recently described and was previously treated as *L. theobromae*, together with *L. pseudotheobromae* (Alves et al. 2008). Isolates collected from *Terminalia* spp. in this study, however, differed in their conidial sizes from descriptions for the type specimen. The conidia of isolates from Cameroon were larger than those previously described for *L. parva*. DNA sequence data for both ITS and *tef 1-α, β*-tubulin, however, confirmed that isolates from Cameroon represent *L. parva*, despite minor differences for two nucleotides in ITS sequences and a single nucleotide in *tef 1-α* sequences. Our results thus show that some isolates of *L. parva* can produce conidia as large as those produced by other closely related species, such as *L. pseudotheobromae* and *L. theobromae*. This emphasizes the importance of considering multiple criteria for species identification when treating species of the Botryosphaeriaceae.

Prior to this study, *L. parva* was known only to occur in agricultural field soil and crops in Latin America (Alves et al. 2008). Although *L. parva* was the least abundant *Lasiodiplodia* spp. isolated from *Terminalia* spp., its occurrence on these trees in Cameroon has substantially broadened its host range and geographic distribution. Previously, the only plant host from which *L. parva* was known was *Theobroma cacao* in Colombia (Alves et al. 2008) and no information concerning its pathogenicity to this tree is available. In the current study, assessment of its pathogenicity on *T. mantaly* and *T. catappa* trees showed that *L. parva* consistently produced lesions on both hosts. However, in comparison to *L. theobromae* and *L. pseudotheobromae*, *L. parva* was only mildly pathogenic, suggesting that this fungus is unlikely to emerge as an important pathogen on these trees.

Endomelanconiopsis endophytica is a recently described species found as an endophyte in leaves of *T. cacao* and associated native woody hosts in the same environment (Rojas et al. 2008). Isolates of *E. endophytica* found in the present study were shown to group with the South American isolates of the fungus. The Cameroonian isolates were obtained from *T. ivorensis* and *T. superba*. These tree species are commonly used in cacao farms to establish a 'taungya' agri-sylvicultural system in which they provide shade or improve soil fertility (Norgrove and Hauser 2002). It would not, therefore, be surprising to obtain further isolates of this fungus on hosts such as cocoa trees in Cameroon. The collection of *E. endophytica* from native *Terminalia* spp. in Cameroon adds to previous records of the fungus from plants in South America (Rojas et al. 2008). Even though very few isolates representing *E. endophytica* were found in this study, its presence on tropical species of *Terminalia* is particularly interesting as this could indicate a possible tropical origin of the fungus.

Two distinct genera of Botryosphaeriaceae, *Lasiodiplodia* and *Endomelanconiopsis*, were found associated with species of *Terminalia* in Cameroon. Although little information related to the ecology of the genus *Endomelanconiopsis* is available, both *Lasiodiplodia* and *Endomelanconiopsis* appear to be tropical species. Apart from *E. endophytica*, which was isolated only from *T. superba* and *T. ivorensis*, no evidence of host specialization was observed for species of *Lasiodiplodia* identified in this study. This is characteristic of many species of Botryosphaeriaceae (Slippers and Wingfield 2007) and contributes to their potential to cause diseases on trees. Although this study focussed exclusively on healthy tree tissue, the common occurrence of generalist species such as *L. theobromae* and *L. pseudotheobromae*, which are reputed virulent pathogens on a wide range of hosts (Punithalingam 1980; Slippers and Wingfield 2007; Begoude et al. 2010) suggests that they could be pathogens if unfavourable conditions for the host occur.

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