Botryosphaeriaceae associated with *Terminalia catappa* in Cameroon, South Africa and Madagascar

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Abstract

Species in the Botryosphaeriaceae represent some of the most important fungal pathogens of woody plants. Although these fungi have been relatively well studied on economically important crops, hardly anything is known regarding their taxonomy or ecology on native or non-commercial tree species. The aim of this study was to compare the diversity and distribution of the Botryosphaeriaceae on *Terminalia catappa*, a tropical tree of Asian origin planted as an ornamental in Cameroon, Madagascar and South Africa. A total of 83 trees were sampled, yielding 79 Botryosphaeriaceae isolates. Isolates were initially grouped based on morphology of cultures and conidia. Representatives of the different morphological groups were then further characterised using sequence data for the ITS, tef 1-alpha, rpb2, BOTF15 and beta-tubulin gene regions. Five species of the Botryosphaeriaceae were identified, including *Neofusicoccum parvum*, *N. batangarum* sp. nov., *Lasiodiplodia pseudotheobromae*, *L. theobromae* and *L. mahajangana* sp. nov. *Lasiodiplodia pseudotheobromae* and *L. theobromae*, were the most commonly isolated species (62%), and were found at all the sites. *Neofusicoccum parvum* and *N. batangarum* were found in South Africa and Cameroon, respectively, whereas *L. mahajangana* was found only in Madagascar. Greenhouse inoculation trials performed on young *T. catappa* trees showed variation among isolates tested, with *L. pseudotheobromae* being the most pathogenic. The Botryosphaeriaceae infecting *T. catappa* appear to be dominated by generalist species that also occur on various other hosts in tropical and sub-tropical climates.

Introduction

The Botryosphaeriaceae is a diverse group of fungi that accommodates numerous species spread over many anamorph genera, the best known of which are *Diplodia*, *Lasiodiplodia*, *Neofusicoccum*, *Pseudofusicoccum*, *Dothiorella* and *Sphaeropsis* (Crous *et al*. 2006). Members of the Botryosphaeriaceae have a worldwide distribution and occur on a large variety of plant hosts including monocotyledons, dicotyledons, gymnosperms and angiosperms, on which they are found as saprophytes, parasites, and endophytes (Slippers and Wingfield 2007; von Arx 1987).

It has long been recognized that species of the Botryosphaeriaceae are important pathogens of several plants (von Arx 1987). Infected plants can exhibit a multiplicity of symptoms such as die-

back, canker, blight and rot on all above ground plant organs (Punithalingam 1980; Slippers *et al*. 2007). A particularly dangerous feature of these fungi is that they can live as endophytes in plant organs, in a latent phase, without producing clear symptoms, and diseases only emerge following the onset of unfavourable conditions to the tree (Smith *et al*. 1996). This implies that they can easily, and unobtrusively, be moved around the world with seeds, cuttings and even fruit.

Extensive studies have been conducted on diseases of economically important species of fruit (e.g. Lazzizera *et al*. 2008; Phillips 1998; Slippers *et al*. 2007; van Niekerk *et al*. 2004) and timber trees (e.g. Mohali *et al*. 2007; Sánchez *et al*. 2003) caused by fungi in the Botryosphaeriaceae. Much less is known about the Botryosphaeriaceae on plants with no large-scale international commercial value (Denman *et al*. 2003; Pavlic *et al*. 2008), such as *Terminalia catappa*, but which have social and environmental significance (Gure *et al*. 2005). Without knowledge of the Botryosphaeriaceae on hosts with limited or no commercial value, and hosts in their native environments, the impact and biology of the important pathogens in this group will never be fully understood.

Terminalia catappa, frequently referred to as “tropical almond”, belongs to the Combretaceae and originates from Southern India to coastal South-East Asia (Smith 1971). These trees are widely cultivated in tropical and subtropical coastal areas and utilised by local communities for a number of household uses. The multitude of non-wood products and services pertaining to this tree species make it an important component, especially for coastal communities. The tree is planted for shade and ornamental purposes in urban environments, the timber is converted into decorative tools, furniture and many other applications, leaves and bark are commonly used in traditional medicine, and its fruits contain edible kernels from which high energy oil is extracted and which can also be admixed into diesel fuel (Chen *et al*. 2000; Hayward 1990; Kinoshita *et al*. 2007).

The diversity and spatial distribution of the Botryosphaeriaceae, associated with a specific host, is important. Whether it accommodates similar or different fungal assemblages depending on the environment is useful in understanding the ecology and host–pathogen relationships of these fungi. This knowledge in turn can be applied where recommendations for disease management strategies are required. Several studies have compared assemblages of fungal endophytes in different geographic regions (Fisher *et al*. 1994; Gallery *et al*. 2007; Gilbert *et al*. 2007; Taylor *et al*. 1999). However, such studies dealing with a specific endophytic group of fungi are limited. Similarly, very few studies have compared the assemblages of Botryosphaeriaceae from a specific host at a regional level (Taylor *et al*. 2005; Úrbez-Torres *et al*. 2006).

Among all the species of *Terminalia* present on the African continent, *T. catappa* is one of the few species planted widely in West, Central, East and Southern Africa. As part of a larger project in which we explore diseases of *Terminalia* spp. in Africa, the broad distribution of this species over the continent made it an ideal candidate to characterise endophytic species of the Botryosphaeriaceae under variable geographic and climatic conditions. The aims of this study were, therefore, to investigate the diversity of the Botryosphaeriaceae occurring on introduced *T. catappa* and to analyse the patterns of their distribution in three African countries. Pathogenicity trials were also undertaken to assess the ecological significance of the Botryosphaeriaceae collected from *T. catappa*.

Materials and Methods

Isolates

Collections were made from *T. catappa* trees in Cameroon, Madagascar and South Africa. In Cameroon, samples were collected along the beach front of Kribi, a seaside town within the tropical forest and bordering the Atlantic Ocean (2°58.064′N, 9°54.904′E, 7 m asl). The climate in this area

is characterised by high humidity, precipitation up to 4,000 mm per annum and relatively high temperatures, averaging 26°C. In South Africa, sampling was done in Richards Bay (28°46.886′S, 32°03.816′E, 0 m asl), a harbour city on the Indian Ocean where *T. catappa* trees are planted to provide shade in open spaces and in parking areas. Climatic conditions in this area are typically subtropical to tropical. The average temperature in summer is 28°C and 22°C in the winter. The humidity levels tend to be very high in summer and the annual rainfall is ~1,200 mm. In Madagascar, samples were collected from the towns of Morondava (20°17.923′S, 44°17.926′E, 3 m asl) and Mahajanga (15°43.084′S, 46°19.073′E, 0 m asl), both located on the west coast of the country. In these areas, the climate is between semi-arid and tropical humid with mean annual temperatures of 23.5°C and average rainfall between 400 and 1,200 mm per annum.

Samples were collected from 83 *T. catappa* trees in all three countries in 2007. Forty trees were randomly sampled in Kribi, 15 in Richards Bay, and 20 and 8 in Morondava and Mahajanga, respectively. Except for the trees in Richards Bay, that were showing symptoms of die-back at the time of collection, those at all the other sites were healthy. One branch (~0.5–1 cm diameter) per tree was cut and all the samples placed in paper bags and taken to the laboratory where they were processed after 1 day.

From each branch, two segments (1 cm in length each) were cut and split vertically into four halves. Samples were surface sterilized by dipping the wood pieces in 96% ethanol for 1 min, followed by 1 min in undiluted 3.5% sodium hypochlorite and 1 min in 70% ethanol, before rinsing in sterile distilled water and allowing them to dry under sterile conditions. The four disinfected branch pieces from each tree were plated on 2% malt extract agar (MEA) (2% malt extract, 1.5% agar; Biolab, Midrand, Johannesburg, S.A.) supplemented with 1 mg ml^{-1} streptomycin (Sigma, St Louis, MO, USA) to suppress bacterial growth. The Petri dishes were sealed with Parafilm and incubated at 20°C under continuous near-Ultra Violet (UV) light. One week later, filamentous fungi growing out from the plant tissues and resembling the Botryosphaeriaceae were transferred to new Petri dishes containing fresh MEA.

All cultures are maintained in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. Representatives of all species have also been deposited at the Centaalbureau voor Schimmelcultures (CBS, Utrecht, Netherlands). Herbarium materials for previously underscribed species have been deposited at the National Fungal Collection (PREM), Pretoria, South Africa.

Morphology and cultural characteristics

Fungal isolates were grown on plates containing 1.5% water agar (Biolab) overlaid with three double-sterilized pine needles and incubated at 25°C under near UV-light for 2–6 weeks to induce the formation of fruiting bodies (pycnydia and/or pseudothecia). Morphological features of the resultant fruiting bodies were observed using a HRc Axiocam and accompanying Axiovision 3.1 camera (Carl Zeiss, München, Germany). For previously undescribed species, sections of fruiting bodies were made with a Leica CM1100 cryostat (Setpoint Technologies, Johannesburg, S.A.) and mounted on microscope slides in 85% lactic acid. For the undescribed species, 50 measurements of all relevant morphological characters were made for the isolate selected as the holotype and 30 measurements were made for the remaining isolates. These measurements are presented as the extremes in brackets and the range calculated as the mean of the overall measurements plus or minus the standard deviation.

The morphology of fungal colonies growing on 2% MEA at 25°C under near UV-light for2 weeks was described and colony colours (upper and reverse surfaces) of the isolates were recorded using the colour notations of Rayner (1970). Growth rates of cultures on 2% MEA in the dark was

determined at 5°C intervals from 10 to 35°C. For growth rates, evaluations of five plates were used for each isolate at each temperature. Two measurements, perpendicular to each other, were made after 3 days for each plate resulting in 10 measurements for each isolate at each temperature. The experiment was repeated once.

DNA extraction

Mycelium was scraped from 10-day-old cultures representing different morphological groups, using a sterile scalpel and transferred to 1.5 µl Eppendorf tubes for freeze-drying. The freeze-dried mycelium was mechanically ground to a fine powder by shaking for 2 min at 30.0 s−1 frequency in a Retsch cell disrupter (Retsch, Germany) using 2-mm-diameter metal beads. Total genomic DNA was extracted using the method described by Möller *et al*. (1992). The concentration of the resulting DNA was determined using a ND-1000 uv/Vis Spectrometer (NanoDrop Technologies, Wilmington, DE, USA) version 3.1.0.

PCR amplification

The oligonucleotide primers ITS1 (5′ TCCGTAGGTGAACCTGCGG 3′) and ITS4 (5′TCCT CCGCTTATTGATATGC 3′) (White *et al*. 1990), EF1 (5′ TGCGGTGGTATCGACAAGCG T 3′) and EF2 (5′ AGCATGTTGTCGCCGTTGAAG 3′) (Jacobs *et al*. 2004), BT2A (5′GGT AACCAAATCGGTGCTGCTTTC3′) and BT2B (5′ACCCTCAGTGTAGTGACCCTTGGC 3′) (Glass and Donaldson 1995), RPB2bot6F (5′GGTAGCGACGTCACTCCC3′) and RPB2bot7R (5′GGATGGATCTCGCAATGCG3′) (Sakaladis 2004), BOT15 (5′ CTGACTTGTGACGCCGGCTC3′) and BOT16 (5′ CAACCTGCTCAGCAAGCGAC3′) (Slippers *et al*. 2004c) were respectively used to amplify and sequence the internal transcribed spacer regions (ITS), including the complete 5.8 S gene, the translation elongation factor 1-α gene (tef 1-alpha), partial sequence of the β-tubulin gene (beta-tub), part of the second largest subunit of RNA polymerase II gene (rbp2) and an unknown locus (BotF15) containing microsatellite repeats. A "hot start" polymerase chain reaction (PCR) protocol was used on an Icycler thermal cycler (BIO-RAD, Hercules, CA, USA). The 25 µl PCR reaction mixtures for the ITS, BT and RPB2 regions contained 0.5 µl of each primer (10 mM) (Integrated DNA Technology, Leuven, Belgium), 2.5 µl DNTPs (10 mM), 4 µl of a 10 mM $MgCl_2$ (Roche Diagnostics, Mannheim, Germany), 2.5 µl of 10 mM reaction buffer (25 mM) (Roche Diagnostics), 1 U of Taq polymerase (Roche Diagnostics), between 60–100 ng/µl of DNA and 13.5 µl of sterile distilled water (SABAX water; Adcock Ingram, Bryanston, S.A.). The amplification conditions were as follows: an initial denaturation step at 96°C for 1 min, followed by 35 cycles of 30 s at 94°C, annealing for 1 min at 54°C, extension for 90 s at 72°C and a final elongation step of 10 min at 72°C. To amplify the tef 1-alpha gene region, the 25 µl PCR reaction mixture contained 0.5 µl of each primer (10 mM), 2.5 µl DNTPs (10 mM), 2.5 µl of 10 mM reaction buffer with $MgCl_2$ (25 mM) (Roche Diagnostics), 1 U of Taq polymerase, between 2–10 ng/µl of DNA and 17 µl of sterile SABAX water. The amplification conditions used were similar to those of Al-Subhi *et al*. (2006) and the conditions used to amplify the BotF15 locus were the same as those of Pavlic *et al*. (2009a, b). The PCR amplification products were separated by electrophoresis on 2% agarose gels stained with ethidium bromide in a 1× TAE buffer and visualised under UV light.

DNA sequencing

Amplified PCR fragments were cleaned using 6% Sephadex G-50 columns with 50–150 µm bead size (Sigma, Steinheim, Germany) following the manufacturer's instructions. Thereafter, 25 amplification cycles were carried out for each sample on an Icycler thermal cycler to generate sequences in both the forward and reverse directions using 10 µl mixtures. Each mixture contained 1 µl reaction buffer, 2 µl ready reaction buffer (Big dye), 1 µl primer (10 mM), 3 µl of the PCR

product and 3 µl Sabax water. The following PCR conditions were followed: one step at 96°C for denaturation of the double stranded DNA (10 s), followed by an annealing step at 50°C (5 s) and primer extension at 60°C (4 min). The BigDye Terminator v 3.1 Cycle sequencing Kit (PE Applied Biosystems) was used for sequencing reactions, following the manufacturer's protocols, on an ABI PRISM 3130xl genetic analyzer using Pop 7 polymer (Applied Biosystems, Foster City, California, USA).

DNA 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 PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Swofford 2001) where a final manual alignment was made. All the ambiguously aligned regions within each dataset were excluded from the analyses. Single gene phylogenetic analyses were run for the datasets representing the different gene regions and three combinations of analyses were also done: ITS and tef 1-alpha for all the isolates; ITS, tef 1-alpha, beta-tubulin, rbp2 and BOTF15 for *Neofusicoccum* and ITS, tef 1-alpha and beta-tubulin for *Lasiodiplodia* isolates. In the analyses, gaps were treated as fifth characters and all characters were unordered and of equal weight. The phylogenetic analyses for all the datasets were performed using the maximum parsimony (MP) option, with trees generated by heuristic searches with random stepwise addition in 1,000 replicates, tree bisection and reconnection (TBR) as branch swapping algorithms, and random taxon addition sequences for the construction of maximum parsimonious trees. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. MAXTREES was set to auto-increase in all analyses. *Guignardia philoprina* (Berk. & M.A. Curtis) Van der Aa was used as outgroup in analyses of ITS and tef 1-alpha datasets whereas no outgroup was inserted in additional analyses for the *Neofusicoccum* and *Lasiodiplodia* groups of isolates as the trees generated were unrooted. The support for branches of the most parsimonious trees was assessed with 1,000 bootstrap replications (Felsenstein 1985). Other measures used to assess the trees were tree length (TL), consistency index (CI), rescaled consistency index (RC), and the retention index (RI) (Hillis and Huelsenbeck 1992). A partition homogeneity test (Farris *et al*. 1995) was conducted in PAUP to assess the possibility of combining the ITS and tef 1-alpha datasets in analyses of all the isolates whereas Incongruence Length Difference (Farris *et al*. 1995) was used in combined analyses for the groups of *Neofusicoccum* and *Lasiodiplodia* isolates.

Bayesian analyses using the Markov Chain Monte Carlo (MCMC) method were performed to ascertain the topology of trees obtained with PAUP. Before launching the Bayesian analyses, the best nucleotide substitution models for each dataset were separately determined with MrModelTest version 2.2 (Nylander 2004) and included in each partition in MrBayes v3.1.2. (Huelsenbeck and Ronquist 2001). GTR+I+G and HKY+G were chosen as best-fitting models for the ITS and tef 1-alpha datasets, respectively, for the general analyses. In the following independent analyses, K80, HKY, GTR, and HKY+I, models were chosen for the ITS, tef 1-alpha, rbp2, beta-tubulin and BotF15 datasets, respectively, to analyse sequences of species in the *Neofusicoccum* group. In the second analyses for species in the *Lasiodiplodia* group, the following models were chosen: K80, HKY + I and HKY for the ITS, tef 1-alpha and beta-tubulin datasets, respectively. The MCMC analyses, with four chains, started from random tree topology and lasted 1,000,000 generations. Trees were saved every 100th generation. The burn-in number was graphically estimated from the likelihood scores and trees outside this point were discarded in the analyses. The consensus trees were constructed in MEGA version 4 and posterior probabilities were assigned to branches after 50% majority rule.

Pathogenicity tests

Two-year-old nursery-grown *T. catappa* plants with stems ranging from 50–100 cm in height and 1–1.5 cm in diameter, growing in peat moss soil in 20-L plastic bags were maintained in the greenhouse at 22°C and watered once a day for pathogenicity experiments. For inoculations, 15 isolates of Botryosphaeriaceae representing all the species identified in the study (Table 1) were grown on 2% MEA for 10 days prior to inoculation. To inoculate trees, wounds were made on the stems by removing the outer bark with a 7-mm-diameter cork-borer. A 7-mm-diameter plug of the test isolates was placed into each wound, with the mycelium facing the cambium, and covered with a strip of Parafilm to prevent desiccation of the wound and inoculum. Five trees, arranged in a completely randomised design, were used for each isolate and the trial was repeated once. For the controls, sterile MEA plugs were used instead of a fungal culture. After 6 weeks, the lengths of the bark and cambium lesions were measured to obtain an indication of the pathogenicity of the isolates tested. Small pieces of necrotic tissue from the edges of lesions were incubated on MEA to show that the inoculated fungi were associated with the lesions. The trial was repeated once. As no significant differences were noticed between the two repeats of the pathogenicity test, the data for all isolates of a particular species were pooled in a single dataset for analyses. Variations in the lengths of the lesions were assessed through a one-way analysis of variance (ANOVA) using SAS (SAS systems, version 8.2; SAS Institute).

Results

Isolates

In total, 79 isolates of Botryosphaeriaceae were obtained from 40 of the 83 *T. catappa* trees sampled in Cameroon, Madagascar and South Africa. Of these, 19 originated from branches on *T. catappa* in Kribi (Cameroon), 29 from Morondava, 8 from Mahajanga (Madagascar), and 25 from Richards Bay (South Africa). Only one isolate per tree was used for further morphological and molecular studies. The isolates obtained were grouped according to their colony and conidial morphology, and representative isolates of each group were selected for DNA sequence comparisons.

Morphologic characterization

All the isolates from *T. catappa* could group into two categories based on conidial morphology (Table 2). The isolates in the first category (Group A) produced hyaline, elongate and thin-walled, fusoid conidia. In the second category (Group B), isolates were characterised by hyaline or dark, thick-walled, aseptate or one-septate, ovoid conidia sometimes exhibiting longitudinal striations. Only anamorph structures were produced by the isolates collected from *T. catappa* when incubated on pine needles.

Based on colony morphology, only one group could be distinguished for all isolates collected in this study. All isolates on MEA grew fast, filling the Petri dishes within 5 days. The aerial mycelium was originally white, turning dark greenish-grey or greyish after 4–5 days at 25°C under near UV-light (Table 2). Based on a combination of colony morphology and morphology of conidia, it was possible to distinguish two groups of Botryosphaeriaceae from *T. catappa* with confidence and these were used in DNA sequence comparisons.

Table 1. Botryosphaeriaceae used for phylogenetic analyses in this study.

Species	Culture number[a]	Origin[b]	Host	Collectors	Genbank Accession No.				
					ITS	*tef 1-alpha*	*rbp2*	*beta-tub*	BotF15
Botryosphaeria dothidea	CMW 7999	Switzerland	*Ostrya* sp.	B. Slippers	AY236948	AY236897			
	CMW 8000	Switzerland	*Prunus* sp.	B. Slippers	AY236949	AY236898			
Dichomera eucalypti	CMW 15952	Australia	*Eucalyptus diversicolor*	T. Burgess/K.I.Goei	DQ093194	DQ093215			
	CMW 15953	Australia	*E. diversicolor*	T. Burgess/K.I.Goei	DQ093194	DQ093216			
Diplodia mutila	CBS 112553	Portugal	*Vitis vinifera*	A.J.L. Phillips	AY259093	AY573219			
	CBS 230.30	USA	*P. dactylifera*	L.L. Huillier	DQ458886	DQ458869			
D. seriata	CMW 7774	USA	*Ribes* sp.	B.Slippers/G.Hudler	EF445343	EF445382			
	CMW 7775	USA	*Ribes* sp	B.Slippers/G.Hudler	EF445344	EF445383			
Guignardia philoprina	CMW 7063	Netherlands	*T. baccata*	H.A. van der Aa	AY236956	AY236905			
Lasiodiplodia crassispora	WAC 12533	Venezuela	*E. urophylla*	S. Mohali	DQ103552	DQ103556			
	WAC 12534	Australia	*Santalum album*	T.I. Burgess/B. Dell	DQ103550	DQ103557			
	WAC 12535	Australia	*S. album*	T.I. Burgess/B. Dell	DQ103551	DQ103558			
L. gonubiensis	CBS 115812	South Africa	*Syzigium cordatum*	D. Pavlic	DQ458892	DQ458877			
	CBS 116355	South Africa	*S. cordatum*	D. Pavlic	AY639594	DQ103567			
L. mahajangana	CMW 27801	Madagascar	*Terminalia catappa*	J. Roux	FJ900595	FJ900641		FJ900630	
	CMW 27818	Madagascar	*T. catappa*	J. Roux	FJ900596	FJ900642		FJ900631	
	CMW 27820	Madagascar	*T. catappa*	J. Roux	FJ900597	FJ900643		FJ900632	
L. margaritacea	CMW 26162	Australia	*Adansonia gibbosa*	D. Pavlic	EU144050	EU144065			
	CMW 26163	Australia	*A. gibbosa*	D. Pavlic	EU144051	EU144066			
L. parva	CBS 356.59	Sri Lanka	*Theobroma cacao*	A. Riggenbach	EF622082	EF622062		EU673113	
	CBS 494.78	Colombia	Cassava-field soil	O. Rangel	EF622084	EF622064		EU673114	
L. plurivora	STEU-5803	South Africa	*Prunus salicina*	U.Damm F.	EF445362	EF445395			
	STEU-4583	South Africa	*V. vinifera*	Halleen	AY343482	EF445396			
L. pseudotheobromae	CMW 26721	South Africa	*T. catappa*	D. Begoude/J. Roux	FJ900598	FJ900644			
	CMW 26716	South Africa	*T. catappa*	D. Begoude/J. Roux	FJ900599	FJ900645			
	CMW 27802	Madagascar	*T. catappa*	J. Roux	FJ900600	FJ900646			
	CMW 27817	Madagascar	*T. atappa*	J. Roux	FJ900601	FJ900647			
	CBS 116459	Costa Rica	*Gmelinea arborea*	J.Carranza/Velásquez	EF622077	EF622057		EU673111	
	CBS 447.62	Suriname	*Citrus aurantium*	C. Smulders	EF622081	EF622060		EU673112	
L. rubropapurea	WAC 12535	Australia	*E. grandis*	T.I. Burgess/G.Pegg	DQ103553	DQ103571			
	WAC 12536	Australia	*E. grandis*	T.I. Burgess/G.Pegg	DQ103554	DQ103572			
L. theobromae	CMW 28317	Cameroon	*T. catappa*	D. Begoude/ J.Roux	FJ900602	FJ900648			
	CMW 28319	Cameroon	*T. catappa*	D. Begoude/J. Roux	FJ900603	FJ900649			
	CMW 26715	South Africa	*T. catappa*	D. Begoude/J. Roux	FJ900604	FJ900650			
	CMW 27810	Madagascar	*T. catappa*	D. Begoude/J. Roux	FJ900605	FJ900651			
	CMW 9074	Mexico	*Pinus* sp.	T. Burgess	EF622074	EF622054		AY236930	
	CBS 164.96	New Guinea	Fruit along coral reef coast	Unknown	AY640255	AY640258		EU673110	
L. venezuelensis	WAC 12539	Venezuela	*Acacia mangium*	S. Mohali	DQ103547	DQ103568			
	WAC 12540	Venezuela	*A. mangium*	S. Mohali	DQ103548	DQ103569			
Neofusicoccum batangarum	CMW 28315	Cameroon	*T. catappa*	D. Begoude/ J.Roux	FJ900606	FJ900652	FJ900614	FJ900633	FJ900622
	CMW 28363	Cameroon	*T. catappa*	D. Begoude/ J. Roux	FJ900607	FJ900653	FJ900615	FJ900634	FJ900623
	CMW 28320	Cameroon	*T. catappa*	D. Begoude/ J.Roux	FJ900608	FJ900654	FJ900616	FJ900635	FJ900624
	CMW 28637	Cameroon	*T. catappa*	D. Begoude/ J. Roux	FJ900609	FJ900655	FJ900617	FJ900636	FJ900625
N. cordaticola	CMW 13992	South Africa	*S. cordatum*	D. Pavlic	EU821898	EU821868	EU821928	EU821838	EU821802
	CMW 14056	South Africa	*S. cordatum*	D. Pavlic	EU821903	EU821873	EU821933	EU821843	EU821807
	CMW 14054	South Africa	*S. cordatum*	D. Pavlic	EU821906	EU821876	EU821936	EU821846	EU821810
N. kwambonambiense	CMW 14023	South Africa	*S. cordatum*	D. Pavlic	EU821900	EU821870	EU821930	EU821840	EU821804
	CMW 14025	South Africa	*S. cordatum*	D. Pavlic	EU821901	EU821871	EU821931	EU821841	EU821805
	CMW 14123	South Africa	*S. cordatum*	D. Pavlic	EU821924	EU821894	EU821954	EU821864	EU821828
N. parvum	CMW 9081	New Zealand	*P. nigra*	G.J. Samuels	AY236943	AY236888	EU821963	AY236917	EU821837
	CMW 9079	New Zealand	*A. deliciosa*	S.R. Pennicook	AY236940	AY236885	EU821961	AY236915	EU821835
	CMW 26714	South Africa	*T. catappa*	D. Begoude/ J. Roux	FJ900610	FJ900656	FJ900618	FJ900637	FJ900626
	CMW 26717	South Africa	*T. catappa*	D. Begoude/ J. Roux	FJ900611	FJ900657	FJ900619	FJ900638	FJ900627
	CMW 26718	South Africa	*T. catappa*	D. Begoude/ J. Roux	FJ900612	FJ900658	FJ900620	FJ900639	FJ900628
	CMW 26720	South Africa	*T. catappa*	D. Begoude/ J. Roux	FJ900713	FJ900659	FJ900621	FJ900640	FJ900629
N. ribis	CMW 7772	USA	*Ribes* sp.	B. Slippers/G.Hudler	AY236935	AY236877	EU821958	AY236906	EU821832
	CMW 7773	USA	*Ribes* sp.	B. Slippers/G Hudler	AY236936	AY236878	EU821959	AY236907	EU821833
N. umdonicola	CMW 14106	South Africa	*S. cordatum*	D. Pavlic	EU821899	EU821869	EU821929	EU821839	EU821803
	CMW 14058	South Africa	*S. cordatum*	D. Pavlic	EU821904	EU821874	EU821934	EU821844	EU821808
	CMW 14060	South Africa	*S. cordatum*	D. Pavlic	EU821905	EU821875	EU821935	EU821845	EU821809

Table 2. Conidial dimensions of *Neofusicoccum* spp. and *Lasiodiplodia* spp. from *Terminalia catappa* and comparison with those reported in previous studies.

Species	Conidial size (μm)		Source of data
	This study	Previous studies	
N. parvum	(10.5–)14 – 19(–20.5) × (4–)5.5 – 6.5(–7.5)	(12–)15 – 19(–24) × 4 – 6	Slippers et al. 2004b
N. batangarum	(12–)14 – 17.5(–20) × (4–)4.5 – 6(–6.5)	(22.5–)23.5 – 32(–33)× (13.3–)14 – 18(–20)	This study
L. pseudotheobromae	(21.5–)24.5 – 29.5(–31) × (13.5–)14 – 16.5(–18)		Alves et al. 2008.
L. theobromae	(20.5–)22.5 – 26(–30.5) × (11.5–)12.5 – 15(–17)	(19–)21 – 31(–32.5)× (12–)13 – 15.5(–18.5)	Alves et al. 2008.
L. mahajangana	(13.5–)15.5 – 19(–21.5) × (10–)11.5 – 13(–14)		This study

DNA extraction and PCR amplification

A total of 40 isolates, each originating from a separate *T. catappa* tree, were selected for ITS sequence comparisons to obtain a broad indication of their identities and to select isolates for the datasets used in the final analyses. These comprised 12 from Group A and 28 from Group B. Of these, 19 isolates were selected for tef 1-alpha sequence comparisons and were considered in the final analyses. Sequences from the beta-tubulin, rbp2 and BotF15 gene regions were used to clarify the relationships between isolates that could not be clearly resolved with ITS and tef 1-alpha sequences. DNA extraction and PCR was conducted successfully for all gene regions selected. PCR fragments for the ITS were ~580 bp in size, while those for tef 1-alpha, beta-tubulin, rbp2 and BOTF15 were 710 bp, 440 bp, 615 bp and ~350 bp, respectively.

DNA sequence analyses

ITS analyses

The ITS dataset comprised 82 sequences of which 40 originated from *T. catappa* and 42 sequences were retrieved from GenBank. Of the 543 characters present in the ITS sequence dataset, 24% were parsimony informative. The MP analyses generated 11 trees with identical topology (TL=401, CI= 0.840, RI=0.977, RC=0.821). Isolates from *T. catappa* grouped into five well-separated clades, representing *Neofusicoccum* [bootstrap support (BS)=74% and Bayesian posterior probabilities (BPP)=1] and *Lasiodiplodia* (BS=51%, BPP=0.61), which also corresponded with the two groups defined based on isolate morphology.

Within the *Neofusicoccum* clade, isolates from *T. catappa* were divided into two groups. The first comprised only isolates from Cameroon, grouping in a single clade (with no bootstrap value) close to the recently described *N. umdonicola* Pavlic, Slippers, & M.J. Wingf. The second clade accommodated isolates from *T. catappa* in South Africa, together with isolates of *N. parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips., with no sequence variation among them. Bayesian analyses supported the separation of the isolates in the *Neofusicoccum* group as observed with MP analyses.

Isolates from *T. catappa* formed three clades within *Lasiodiplodia* based on ITS sequence data. Isolates in the first two clades grouped with *L. theobromae* (Pat.) Griff. & Maubl. and *L. pseudotheobromae* A.J.L. Phillips, A. Alves & Crous., and included isolates from all three countries. Three isolates (CMW27801, CMW27818, CMW27820) from *T. catappa* in Madagascar constituted a clade (BS=63% and BPP=0.91), which did not group with any known *Lasiodiplodia* sp., suggesting a possibly undescribed species, most closely related to *L. parva* A.J.L. Phillips, A. Alves & Crous. The topology of the consensus tree generated with Bayesian analyses was similar in overall topology to the one obtained with MP analyses.

ITS and tef 1-alpha analyses

The partition homogeneity test for the ITS and tef 1-alpha datasets showed that they could be combined (P =0.303) as no conflict was found between the gene genealogies. The combined dataset consisted of 59 isolates and contained 804 characters of which 38% were parsimony informative. Gaps were treated as a fifth character. After heuristic searches, eight most parsimonious trees were obtained (TL=884; CI=0.782, RI=0.960, RC=0.750, TreeBase No: SN4517). The topology of the tree generated from the combined analyses with MP, as well as with the 50% majority rule consensus tree (Fig. 1), was congruent with the trees obtained with the individual analyses of ITS and tef 1-alpha, presenting the same clades. However, the Cameroonian isolates with hyaline, thin-walled conidia formed a single sub-group (BS=56% and BPP=0.94) close to *N. umdonicola* within the larger clade containing species close to *N. ribis*, similar to that observed on the tree obtained for the ITS analyses. The clade (BS=100% and BPP=1) accommodating the apparently undescribed *Lasiodiplodia* sp. from Madagascar (CMW27801, CMW27818, CMW27820) was basal to *L. plurivora* as observed on the tree obtained using tef 1-alpha analyses.

Analyses of both ITS and tef 1-alpha separately, as well as combined, identified the same groups amongst the isolates collected from *T. catappa*. These included *N. parvum*, *L. theobromae*, *L. pseudotheobromae* and two previously unidentified groups. Some uncertainty was, however, present regarding the Cameroonian isolates with hyaline, thin-walled conidia. Although these isolates consistently grouped in a unique clade within the *N. ribis*/*N. parvum* complex, only low statistical support was observed in all analyses. These results raised uncertainties regarding their relationship with other closely related species and prompted analyses using additional gene regions in an attempt to clarify their identity.

Additional analyses using five loci

To resolve uncertainties in the relationships among the Cameroonian isolates (CMW28315, CMW28363, CMW28320, CMW28637) grouping close to *N. ribis*, additional independent multilocus analyses were used for taxa included in the genus. Twenty-one isolates taken from the *N. ribis* and *N. parvum* complex were included in the ITS, tef 1-alpha, BOTF15, rbp2 and beta-tubulin datasets (Table 3). For each dataset, trees obtained from both MP and Bayesian analyses showed identical topologies. Isolates from *T. catappa* in Cameroon grouping in the *N. ribis*/*N. parvum* complex formed a distinct clade in four of the five individual partitions (Fig. 2a, b, d, e). These isolates were more closely related to *N. ribis* and *N. umdonicola* than to any other species of the complex. The clade accommodating the Cameroonian *Neofusicoccum* isolates was characterised by Bootstrap and Bayesian posterior probabilities values between 60 and 97%. The ITS and BOTF15 gene regions contained one unique fixed polymorphism each, while nucleotide sequences of tef 1-alpha and beta-tubulin showed a number of base variations (one deletion and two substitutions for tef 1-alpha and one substitution for beta-tub) from those representing *N. ribis* but no fixed polymorphism was observed (Table 4). No differences were observed in rbp2 sequences between Cameroonian isolates and those representing N. ribis (Table 4) (Fig. 2c). However, analysis of the data from the rbp2 locus remained informative and could not cancel out the lineage sorting of the Cameroonian isolates. To provide a better resolution in the relationship of these isolates, we combined the data from each partition into one phylogenetic analysis.

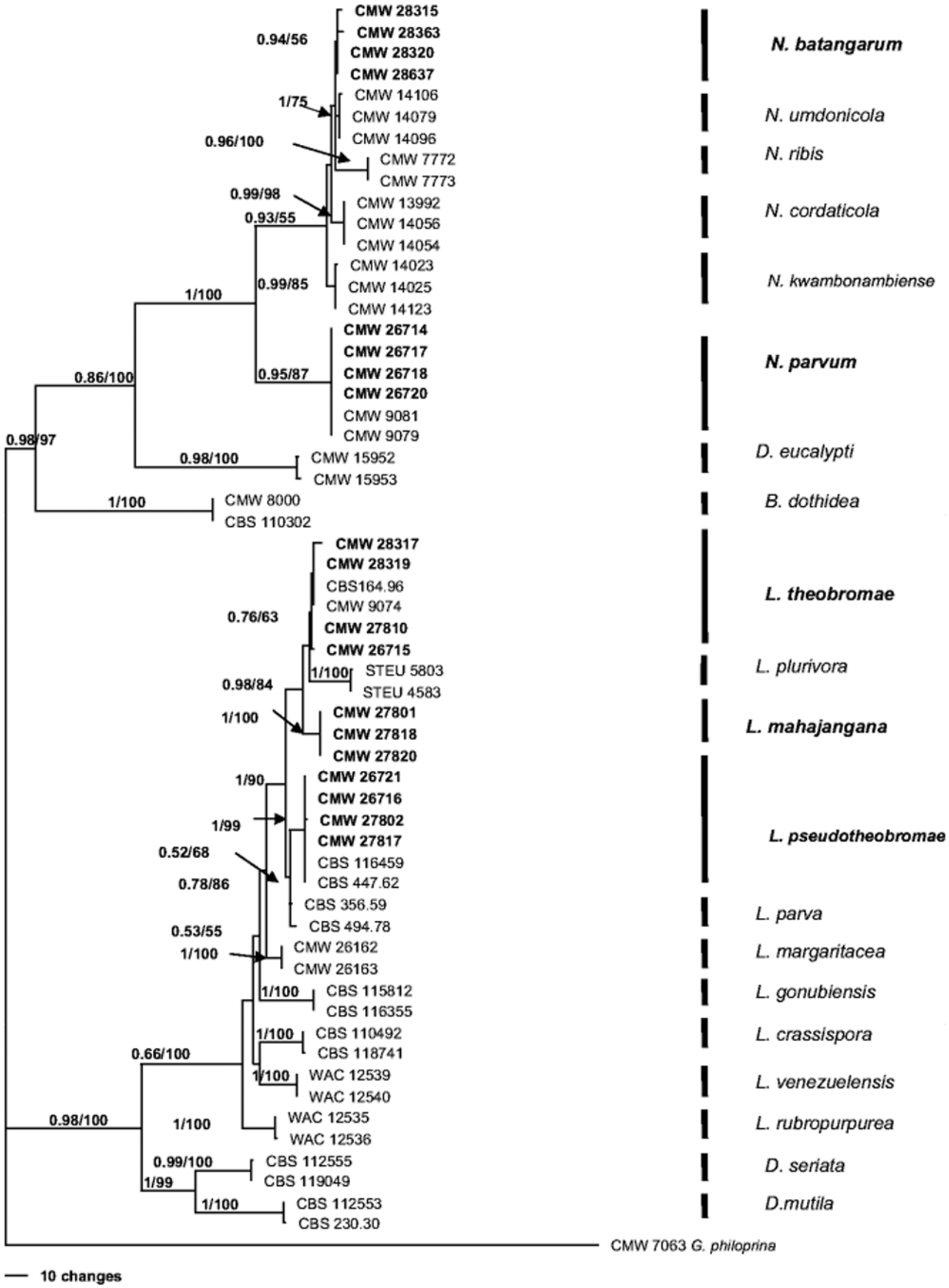

Fig. 1. One of the most parsimonious trees obtained from Maximum Parsimony analyses of the combined ITS and tef 1-alpha sequence data of the representative taxa of the Botryosphaeriaceae. Posterior probabilities followed by bootstrap support (%) from 1,000 replications are given on the branches (PP/BS). Isolates marked in bold represent those obtained from *T. catappa*.

Table 3. Sequence dataset characteristics and phylogenetic information for ITS, tef 1-alpha, rbp2, beta-tubulin and BotF15 and combined datasets of *Neofusicoccum* spp.

Dataset	Sequence range (bp)	No. variable sites	No. informative sites	No. most parsimonious trees	Tree length	Consistency index	Retention index	Monophyletic taxa
ITS	502	15	10	1	15	1	1	*N. kwambonambiense*, *N. cordaticola*, *N. batangarum*, *N. umdonicola*, *N. ribis*, *N. parvum*
tef 1-alpha	263	24	23	6	25	0.960	0.977	*N. kwambonambiense*, *N. cordaticola*, *N. batangarum*, *N. umdonicola*, *N. ribis*, *N. parvum*
rpb2	566	19	16	1	19	1	1	*N. kwambonambiense*, *N. cordaticola*, *N. umdonicola*, *N. parvum*
beta-tub	420	12	12	2	13	0.923	0.974	*N. kwambonambiense*, *N. cordaticola*, *N. batangarum*, *N. umdonicola*, *N. ribis*, *N. parvum*
BotF15	364	26	25	1	26	1	1	*N. kwambonambiense*, *N. cordaticola*, *N. batangarum*, *N. parvum*
Combined data	2115	96	86	1	98	0.980	0.992	*N. kwambonambiense*, *N. cordaticola*, *N. batangarum*, *N. umdonicola*, *N. ribis*, *N. parvum*

Table 4. Polymorphic nucleotides from sequence data of the ITS, tef 1-alpha, rbp2, beta-tubulin and BOTF15gene regions for isolates in the *Neofusicoccum ribis*, *N. kwabonambiense*, *N. umdonicola*, *N. cordicola* and *N. parvum* clade introduced from outgroup comparisons.

Identity	Culture number	ITS	*tef1-alpha*	*rbp2*	*beta-tub*	BOTF15
		51 115 141 163 168 173 372 389 416	43 44 66 67 77 81 153 211 227 257	10 22 49 97 100 112 205 265 280 343 382 397 409 421 475 526	42 50 93 106 125 167 185 245 261 326 389 407	97 98 102 105 166 215 252 301 311
N. ribis	CMW 7772	A G T C T A A A T	G I T G T C C A - G	T T C T G C T G C C G T T G C T	C G C C G T C G G C T T	G A 0 C 0 C G C G
	CMW 7773	. . . . .	. I . . . . A . .	. . . . .	. . . T	. . . . .
N. batangarum	CMW 28315	. . . . . G .	. 0 . . . . G . .	. . . . .	. . . . C	. . . . . A . .
	CMW 28320	. . . . . G .	. 0 . . . . G . .	. . . . .	. . . . C	. . . . . A . .
	CMW 28363	. . . . . G .	. 0 . . . . G . .	. . . . .	. . . . C	. . . . . A . .
	CMW 28637	. . . . . G .	. 0 . . . . G . .	. . . . .	. . . . C	. . . . . A . .
N. kwabonambiense	CMW 14023	. . T . G . .	. 0 . . . . G . .	. C G C . . C . . A C . A . C	. . . . A C T A A . C C	. T I T I . . T .
	CMW 14025	. . T . G . .	. 0 . . . . G . .	. C G C . . C . . A C . A . C	. . . . A C T A A . C C	. T I T I . . T .
	CMW 14123	. . T . G . .	. 0 . . . . G . .	. C G C . . C . . A C . A . C	. . . . A C T A A . C C	. T I T I . . T .
N. umdonicola	CMW 14106	. . . . C . .	. 0 . . . T . G C .	. . . . . T	A . . . C	. . . . .
	CMW 14079	. . . . C . .	. 0 . . . T . G C .	. . . . . T	A . . . C	. . . . .
	CMW 14096	. . . . C . .	. 0 . . . T . G C .	. . . . . T	A . . . C	. . . . .
N. cordaticola	CMW 13992	. . . . . G . C	. 0 . . C . . G . .	. C . C A T C A . . . C C . .	T . . T . C . . . G C C	T C . T . . . T .
	CMW 14056	. . . . . G . C	. 0 . . C . . G . .	. C . C A T C A . . . C C . .	T . . T . C . . . G C C	T C . T . . . T .
	CMW 14054	. . . . . G . C	. 0 . . C . . G . .	. C . C A T C A . . . C C . .	T . . T . C . . . G C C	T C . T . . . T .
N. parvum	CMW 9081	T	. 0 C A . . T G . A	C C . C . . C . . T . C . . T	. . T A C	. . . T . . T . T A
	CMW 9079	T	. 0 C A . . T G . A	C C . C . . C . . T . C . . T	. . T A C	. . . T . . T . T A

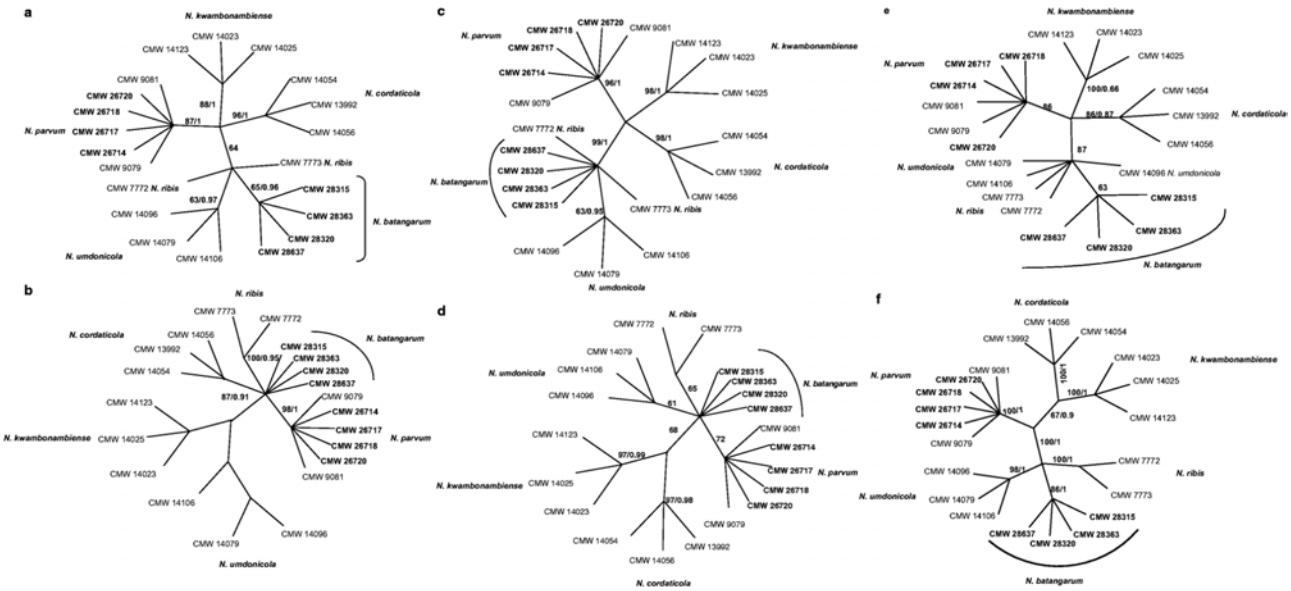

Fig. 2. One of the most parsimonious unrooted trees inferred from independent analyses of each dataset (**a** ITS, **b** tef 1-alpha, **c** rbp2, **d** beta-tubulin, **e** BOTF15, **f** combination of sequences of the

five loci) in the *Neofusicoccum* spp. group of the Botryosphaeriaceae from *T. catappa*. Bootstrap support (%) from 1,000 replications followed by Posterior probabilities are given on the branches (BS/PP). Isolates marked in bold represent those obtained from *T. catappa*.

The Incongruence Length Difference calculated for all the datasets related to all the isolates included in the *N. ribis* and *N. parvum* complex (I =0) (Table 3) indicated that the gene phylogenies were congruent. This was illustrated by strong statistical (BS=86%; BPP=1) support observed on the consensus tree obtained from both MP and Bayesian analyses of the combined dataset (Fig. 2f). Analyses of the combined datasets confirmed the grouping of the isolates from Cameroon into an undescribed and unique lineage (Fig. 2f).

Additional analyses for the *Lasiodiplodia* clade, including nine isolates representing *L. theobromae*, *L. pseudotheobromae*, *L. parva* and isolates of the apparently undescribed species from Madagascar were conducted using part of the ITS, tef 1-alpha and beta-tubulin gene regions (Table 5). From the MP tree topologies, which were identical to those obtained in Bayesian analyses, for each partition, as well as the combined analysis, it was clear that isolates from Madagascar consistently formed a clade distinct from *L. theobromae*, *L. pseudotheobromae* and *L. parva* (Fig. 3a–d). There was considerable sequence variation across the three gene regions among isolates representing the undescribed species and those of *L. theobromae*, *L. pseudotheobromae* and *L. parva* (Table 6). The resolution in the relationship of these isolates was improved by combining individual datasets in one phylogenetic analysis. The Incongruence Length Difference calculated for all the datasets for this group of isolates (I =2) (Table 5) indicated a congruence in the phylogenies of all three genes. Strong statistical (BS=100%; BPP=1) support was observed for the consensus tree obtained for both MP and Bayesian analyses (Fig. 3d) of the combined dataset. Analyses of the combined datasets confirmed the monophyly of isolates (CMW27801, CMW27818, CMW27820) from Madagascar in the *Lasiodiplodia* clade (Fig. 3d).

Table 5. Sequence dataset characteristics and phylogenetic information for ITS, tef 1-alpha and beta-tubulin and combined datasets for *Lasiodiplodia* spp.

Dataset	Sequence range	No. of variable sites	No. of informative sites	No. of most parsimonious trees	Tree length	Consistency index	Retention index	Monophyletic taxa
ITS	461	5	5	1	5	1	1	*L. theobromae, L. pseudotheobromae, L. parva, L. mahajangana*
tef 1-alpha	276	51	47	1	55	0.982	0.990	*L. theobromae, L. pseudotheobromae, L. parva, L. mahajangana*
beta-tub	422	9	8	1	9	1	1	*L. theobromae, L. pseudotheobromae, L. parva, L. mahajangana*
Combined data	1,159	65	60	1	71	0.958	0.976	*L. theobromae, L. pseudotheobromae, L. parva, L. mahajangana*

Table 6. Polymorphic nucleotides from sequence data of the ITS, tef 1-alpha and beta-tubulin gene regions for isolates in the *Lasiodiplodia theobromae*, *L. parva*, *L. pseudotheobromae* and *L. mahajangana* clade introduced from outgroup comparisons.

Identity	Culture number	ITS	*tef 1-alpha*	*beta-tub*
		42 46 98 116 439	20 22 23 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 50 53 55 58 59 61 62 68 82 99 232 267 282	85 124 143 157 177 189 230 244
L. theobromae	CBS 164.96	A C C T C	G C C C A C G C A T G T C G T T T T T T A T C A T C - - 1 C C A A A	C C G T T A C C
	CMW 9074	. . . . .	. . . . . - - 1	. . . . .
L. parva	CBS 356.59	. T . . .	- G A A . G A T T . T C - - C C - - - C . G T G C T C A 0 . T . . G	T . C C C G A .
	CBS 494.78	. T . . .	- G A A . G A T T . T C - - C C - - - C . G T G C T C A 0 . T . . G	T . C C C G A .
L. pseudotheobromae	CBS 447.62	G T . C T	- T . A T T T . C C C C - - C C . C C C . G T G C T T A 0 . . G G G	T . . C C . . .
	CBS 116459	G T . C T	- T . A T T T . C C C C - - C C . C C C . G T G C T T A 0 . . G G G	T . . C C . . .
L. mahajangana	CMW 27801	. T T . .	. . . . . G . . . . . T . . . . . - - 0 T T . . .	T T C C C G . A
	CMW 27818	. T T . .	. . . . . G . . . . . T . . . . . - - 0 T T . . .	T T C C C G . A
	CMW 27820	. T T . .	. . . . . G . . . . . T . . . . . - - 0 T T . . .	T T C C C G . A

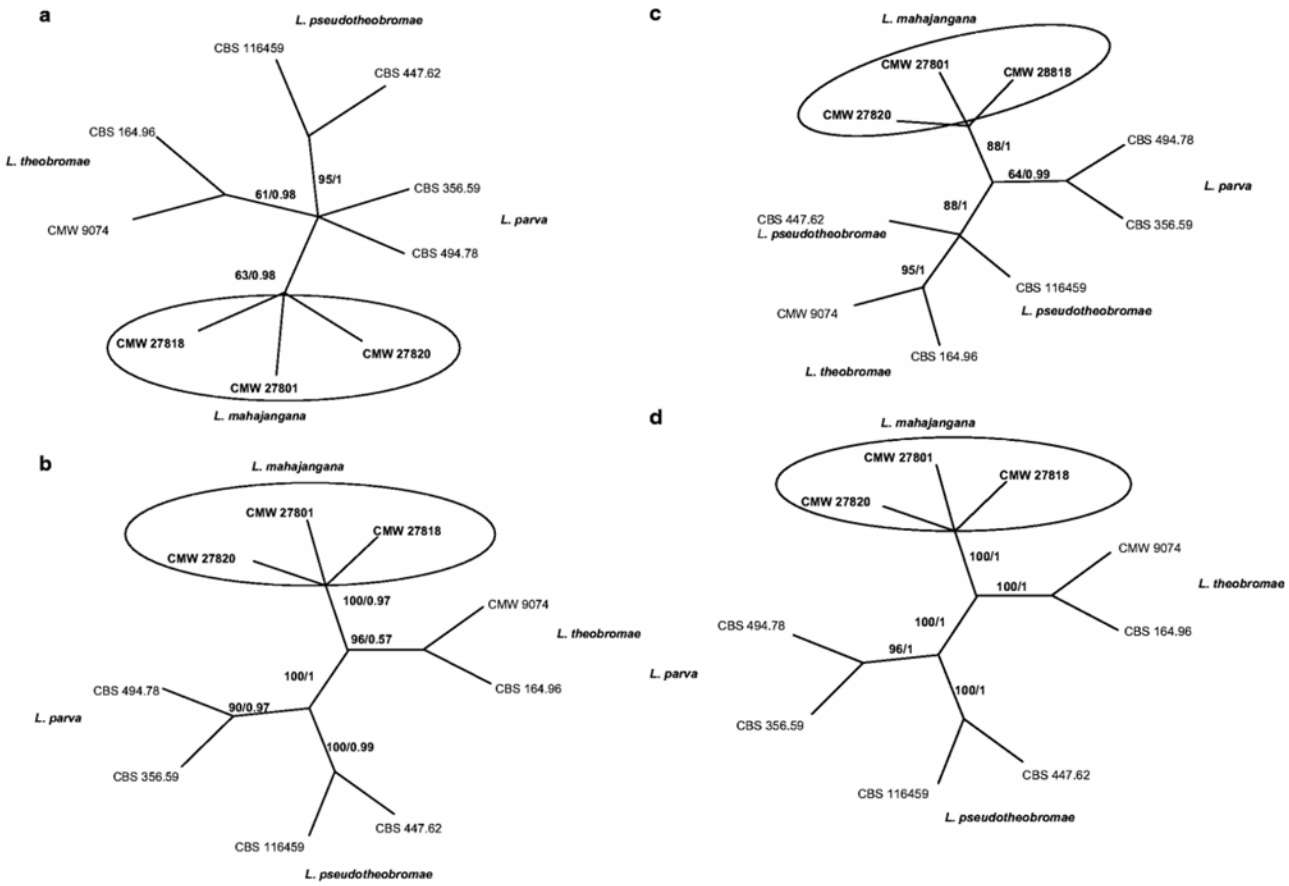

Fig. 3. Most-parsimonious unrooted trees inferred from independent analyses of each dataset (**a** ITS, **b** tef 1-alpha, **c** beta-tubulin, **d** combination of sequences of the three loci) of the *Lasiodiplodia* spp. from *T. catappa* and related species. Bootstrap support (%) from 1,000 replications followed by Posterior probabilities are given on the branch (BS/PP). Isolates marked in bold represent those obtained from *T. catappa*.

Taxonomy

DNA sequence data for the ITS, tef 1-alpha, BOTF15, rbp2 and beta-tubulin gene regions revealed the presence of two previously undescribed species of Botryosphaeriaceae amongst the isolates collected from *T. catappa* in this study. A study of the morphology of these isolates confirmed that they are distinct from previously described species and they are consequently described as new here:

Lasiodiplodia mahajangana Begoude, Jol. Roux, Slippers, sp. nov. MB514012 Fig. 4.

Etymology: The name refers to the locality where this fungus was collected for the first time.

Description: Conidia pycnidialia usque ad 300 µm lata, in foliis Pini in MEA in 14 diebus facta, solitaria mycelio tecta, superficialia conica. Conidiophorae ad cellulas conidiogenas reductae. Cellulae conidiogenae holoblasticae discretae hyalinae cylindricae. Conidia primo non septata, hyalinae ellipsoideae vel ovoideae parietibus crassis <2.5 µm, contentis granularibus, demum semel septataet liberata colorata, matura verticaliter striata 17.5×11.5 µm.

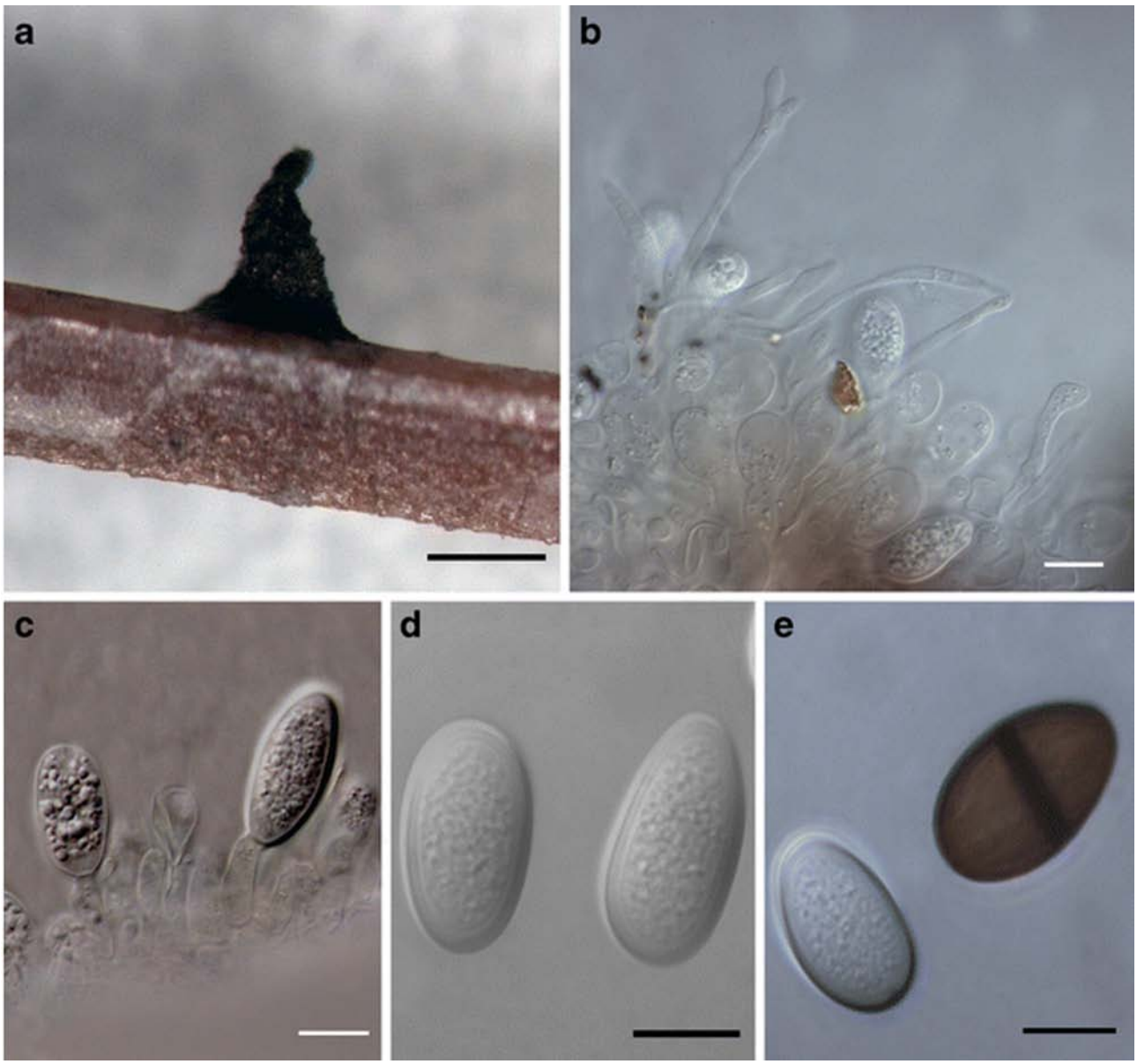

Fig. 4. *Lasiodiplodia mahajangana*. **a** Pycnidium formed on pine needle in culture. **b** Paraphyses. **c** Conidiogenous cells with developing conidia. **d** conidia. **e** mature conidium showing septum. Bars **a** 500 µm, **b**–**e** 10 µm.

Conidiomata: pycnidial (up to 300 µm wide), produced on pine needles on MEA within 14 days, solitary and covered by mycelium, superficial, conical, unilocular, with long necks (up to 200 µm) and single ostioles at the tips, locule walls thick, consisting of two layers: an outer dark brown textura angularis, lined with inner thin-walled, hyaline cells. Paraphyses: rare, cylindrical, hyaline, aseptate 1-celled (27.5)33.5 – 52.5(66) × (2)2.5 – 3.5(5) µm, (average 50 paraphyses 43 × 3 µm), rounded at the tips, unbranched. Conidiophores: reduced to conidiogenous cells. Conidiogenous cells: holoblastic, discrete, hyaline, cylindrical, proliferating percurrently to form a periclinal thickening (10)10.5 – 18(26) × (3)3.5 – 5.5(6) µm (average 50 conidiogenous cells 14.5 × 4.5 µm, l/w 3.2). Conidia: initially aseptate, hyaline, ellipsoid to ovoid, thick-walled (<2.5 µm), granular content, becoming one-septate and pigmented after release, vertical striations observed at maturity, (13.5)15.5 – 19(21.5) × (10)11.5 – 13(14) µm (average 50 conidia 17.5 × 11.5 µm, l/w 1.4). Cultural characteristics: white fluffy and abundant aerial mycelium, becoming pale olivaceous grey (23‴″f) after 4 days, with the reverse sides of the colonies olivaceous grey (23‴″b). Optimum temperature for growth: 25–30°C, covering a 90-mm-diameter Petri dish after 3 days on MEA in the dark, no growth observed at 10°C.

Teleomorph: Not observed.

Host: *Terminalia catappa*.

Distribution: Mahajanga, Madagascar.

Specimen examined: Madagascar, Mahajanga, 15°43′.084N, 46°19′.073E, 0 m asl: isolated from healthy branches of *Terminalia catappa*, Oct 2007, J. Roux, holotype (PREM 60288), a dry culture on pine needles CMW27801 = CBS 124925; ex-type culture CMW27820 = CBS 124927.

Additional specimens: Madagascar, Mahajanga, 15°43′.084N, 46°19′.073E, 0 m asl: isolated from healthy branches of *Terminalia catappa*, Oct 2007, J. Roux, ex-paratype (PREM 60289) CMW27818 = CBS 124926.

Neofusicoccum batangarum Begoude, Jol. Roux, Slippers, sp. nov. MB514013 Fig. 5.

Etymology: Name refers to the Batanga people who live in the area where the type specimen was collected.

Description: Conidia pycnidialia in foliis Pini in 14 diebus facta, solitaria mycelio tecta, primo immersa, matura 3/4 per foliis emergentia, obpyriformia vel ampulliformia. Conidiophorae ad cellulas conidiogenas reductae. Cellulae conidiogenae holoblasticae hyalinae cylindricae. Conidia non septata, hyalinae fusoideae vel ovoideae parietibus tenuis, 15.5 × 5.5 µm.

Conidiomata: pycnidial produced on pine needles within 14 days, solitary and covered by mycelium, initially embedded, 3/4 erumpant through the pine needles at maturity, obpyriform to ampulliform with a central and circular ostiole at the neck, unilocular, locule wall thick consisting of two layers: an outer layer of dark brown textura angularis, lined with an inner layer of thin-walled, hyaline cells. Conidiophores: reduced to conidiogenous cells. Conidiogenous cells: holoblastic, hyaline, cylindrical, proliferating percurrently, sometimes forming a periclinal thickening, smooth producing a single conidium, (11)12.5 – 19(27) × (2)2.5 – 3(3.5) µm (average of 50 conidiogenous cells 15.5 × 2.5 µm, l/w 6). Conidia: aseptate, hyaline, smooth, fusoid to ovoid, thin-walled, (12)14 – 17.5(20) × (4)4.5 – 6(6.5) µm (average 50 conidia 15.5 × 5.5 µm, l/w 2.9). Cultural characteristics: colonies forming concentric rings on MEA, mycelium white and immersed at the leading edge, becoming smokey grey (21′′′′d) to grey olivaceous (21′′′′b) from the old ring after 5 days on MEA. Optimum temperature for growth: 25°C, covering the 90-mm-diameter Petri plate after 4 days on MEA in the dark, little growth observed at 10 and 35°C (Fig. 6).

Teleomorph: Not observed.

Host: *Terminalia catappa*.

Distribution: Kribi, Cameroon.

Specimen examined: Cameroon, Kribi, Beach, 2°58′.064N, 9°54′.904E, 7 m asl, isolated from healthy branches of *Terminalia catappa*, Dec 2007, D. Begoude and J. Roux, ex-paratype (PREM 60285), a dry culture on pine needles CMW28315 = CBS 124922; ex-type culture (PREM 60286) CMW28363 = CBS 124924.

Additional specimens: Cameroon, Kribi, Beach, 2°58′.064N, 9°54′.904E, 7 m asl, isolated from healthy branches of *Terminalia catappa*, Dec 2007, D. Begoude and J. Roux ex-paratype (PREM 60284) CMW28320 = CBS 124923; (PREM 60287) CMW28637.

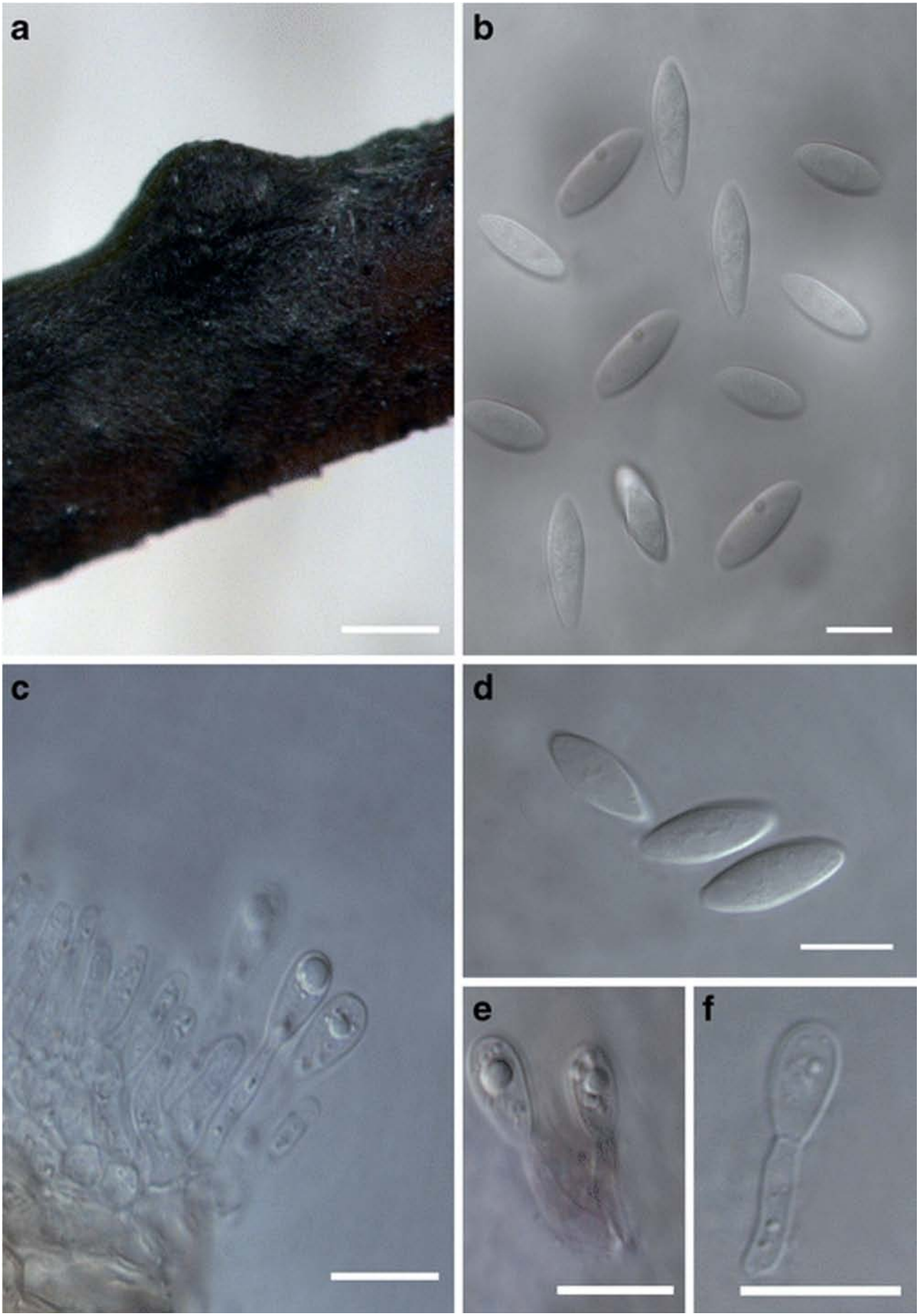

Fig. 5. *Neofusicoccum batangarum*. **a** Pycnidium formed on pine needle in culture. **b**,**d** Conidia. (**c**,**e**,**f**) Conidiogenous cells with developing conidia. Bars **a** 500 μm, **b**–**e** 10 μm

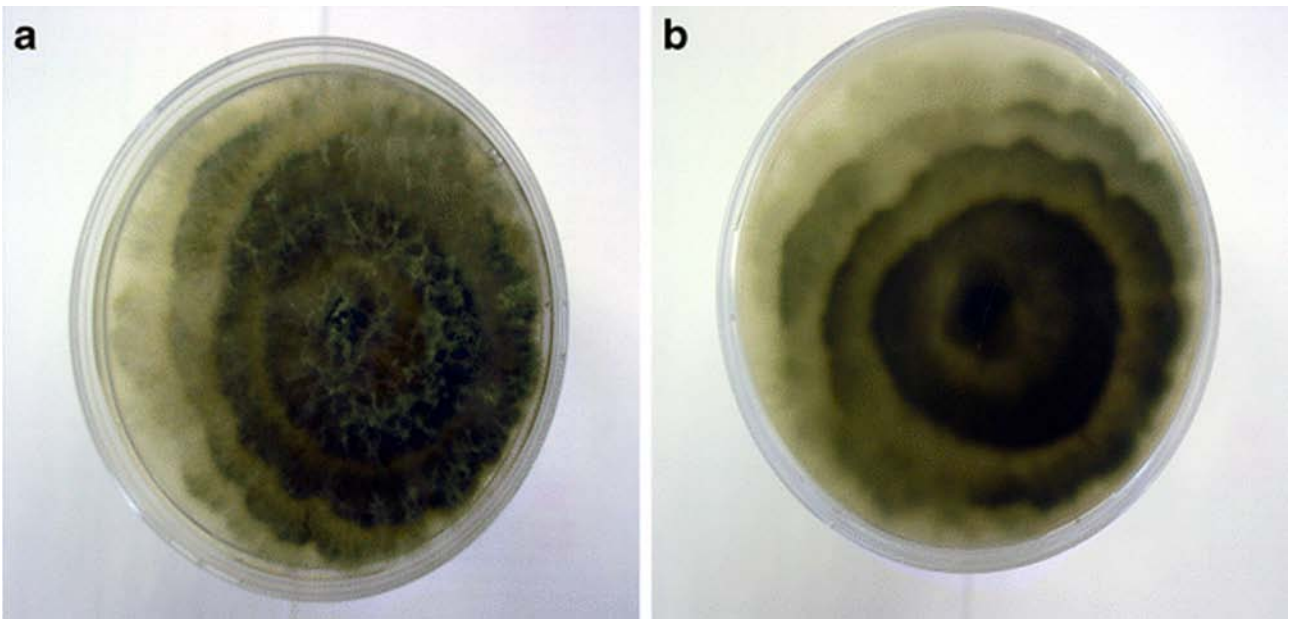

Fig. 6. *Neofusicoccum batangarum* culture on MEA. (a) Front plate. (b) Reverse plate

Distribution of the Botryosphaeriaceae

In total, five species of Botryosphaeriaceae were isolated from *T. catappa* in South Africa, Madagascar and Cameroon. Two cosmopolitan species, *L. pseudotheobromae*, the most commonly isolated species which represented 42% of the isolates collected, and *L. theobromae* were collected from trees in all three countries. The other three species, *N. parvum*, *N. batangarum* and *L. mahajangana*, were each isolated only in South Africa, Cameroon and Madagascar, respectively (Fig. 7).

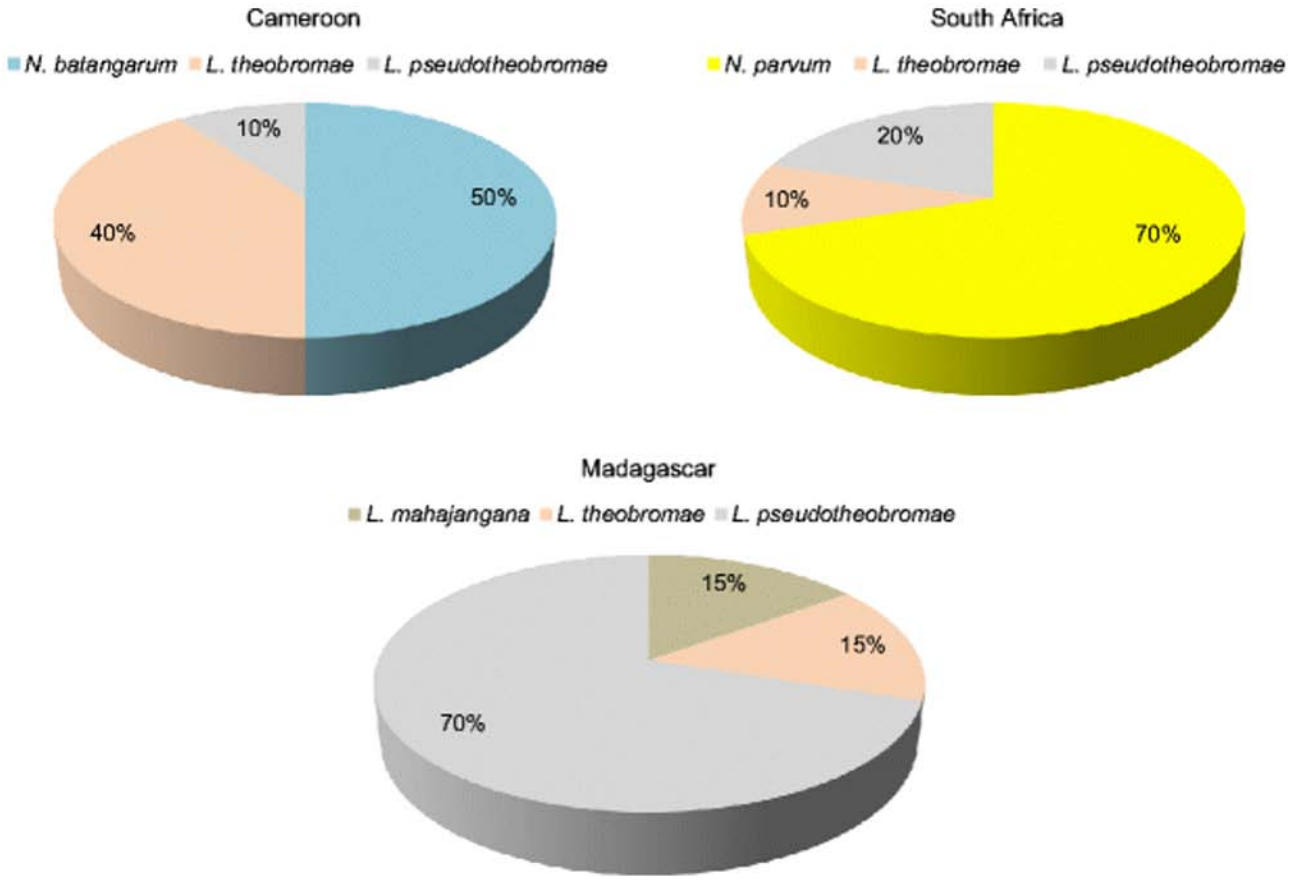

Fig. 7. Distribution of Botryosphaeriaceae collected from *T. catappa* per locality.

Pathogenicity

All inoculations with isolates of Botryosphaeriaceae collected in this study resulted in visible lesions on the bark and cambium of T. catappa trees after six weeks. Analysis of variance showed that there were significant differences in the pathogenicity among species ($P<0.0001$). Overall, L. pseudotheobromae, L. theobromae, N. parvum and N. batangarum produced the longest lesions on both bark and cambium, whereas L. mahajangana produced the smallest lesions (Figs. 8 and 9). Considerable variation in levels of pathogencity was also observed among isolates of the same species. There was a positive correlation ($R^2=75\%$) between lesions produced on the bark and those on the cambium. Re-isolations from lesions on the inoculated trees resulted in the recovery of the inoculated fungi.

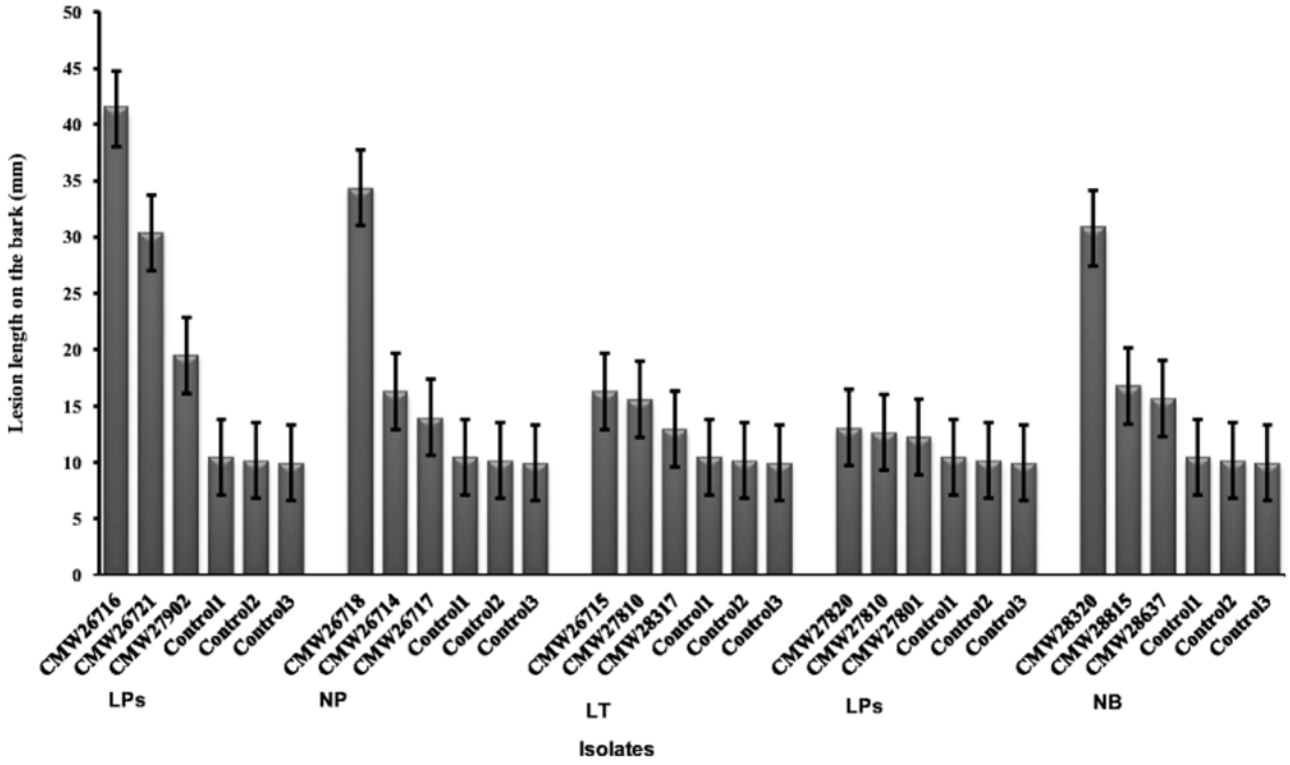

Fig. 8. Mean bark lesion lengths (mm) for each Botryosphaeriaceae isolate 6 weeks after inoculation on *T. catappa* ($P<0.0001$). *L. pseudotheobromae* (LPs), *N. parvum* (NP), *L. theobromae* (LT), *L. mahajangana* (LM), *N. batangarum* (NB), Control.

Discussion

This study presents the first consideration of the possible fungal pathogens of *T. catappa*. It is also the first study of the Botryosphaeriaceae on these popular ornamental trees. In total, five species of the Botryosphaeriaceae were identified and two of these were new taxa that were described and provided with the names *N. batangarum* and *L. mahajangana*.

Slippers *et al*. (2004a), in comparing the assemblage of Botryosphaeriaceae on native and introduced *Eucalyptus* trees in Australia and South Africa, emphasised the importance of individually identifying species affecting a specific host in every country or environment where it occurs. This was because they found more pathogenic fungal species on *Eucalyptus* spp. outside their native environment (South Africa) than in the area (Australia) where these trees were native. Although the assemblage of the Botryosphaeriaceae found in the current study varied from one country to another, colonization patterns on *T. catappa* in the three areas showed similar trends. In each country, three species of Botryosphaeriaceae were found, one of which was specific to that

country and two species occurring in all three countries. These patterns might be explained by climatic differences as has been shown for the distribution of Botryosphaeriaceae in California (Úrbez-Torres *et al*. 2006).

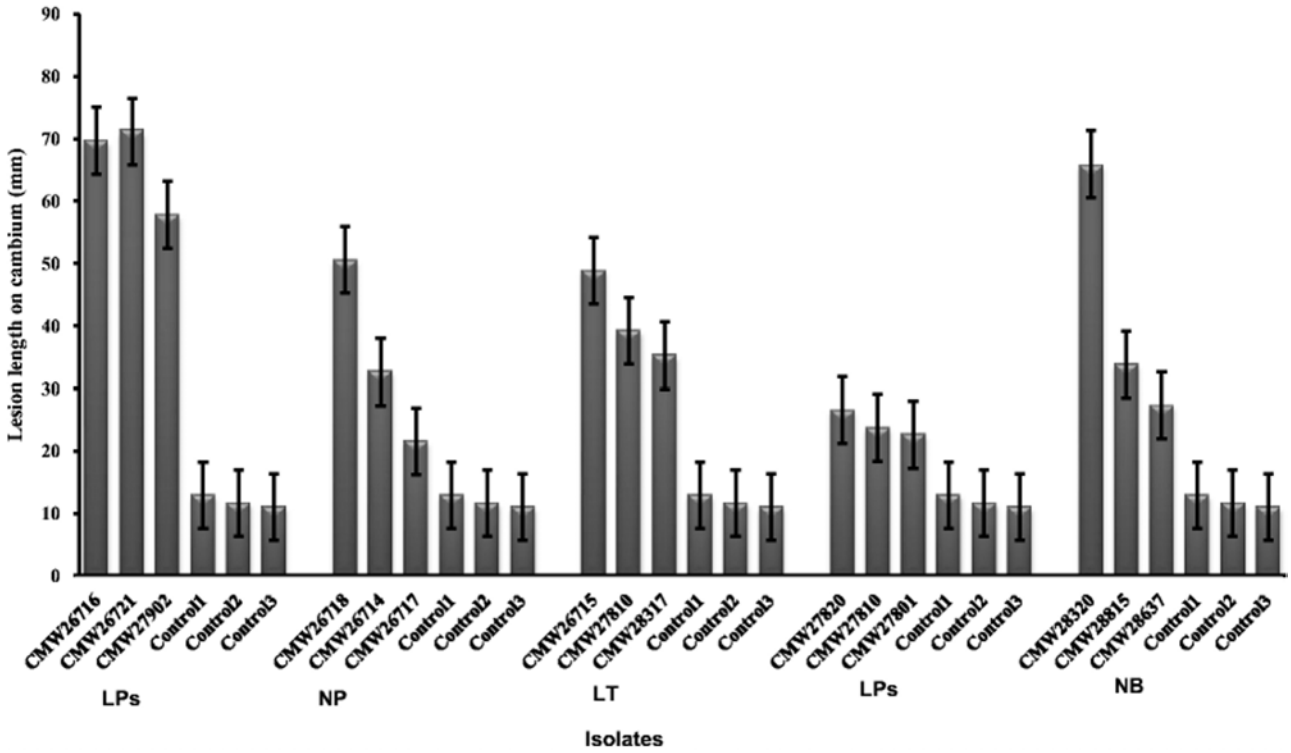

Fig. 9. Mean cambial lesion lengths (mm) for each Botryosphaeriaceae isolate six weeks after inoculation on *T. catappa* ($P<0.0001$). *L. pseudotheobromae* (LPs), *N. parvum* (NP), *L. theobromae* (LT), *L. mahajangana* (LM), *N. batangarum* (NB), Control.

Phylogenetic relationships for the Botryosphaeriaceae from *T. catappa* and other known members of this fungal family were determined using combined sequence datasets of the ITS and tef 1-alpha gene regions. However, the resulting phylogenies did not clearly separate all the species. This was especially true for isolates from Cameroon grouping in the *N. ribis*/*N. parvum* complex. Within the Botryosphaeriaceae, species in the *N. ribis*/*N. parvum* complex have been difficult to distinguish based on phylogenies of single gene regions (Pavlic *et al*. 2007; Slippers *et al*. 2004b). A recent study by Pavlic *et al*. (2009a, b) thus made use of the Genealogical Concordance Phylogenetic Species recognition (GCPSR) approach (Taylor *et al*. 2000) to resolve species boundaries in the complex. These authors were able to identify three cryptic species in the *N. ribis*/*N. parvum* complex. The same approach was used in the present study, to confirm the unique nature of *N. batangarum*. Isolates of *N. batangarum* were distinct from *N. ribis* based only on four fixed unique single nucleotide polymorphisms (SNPs) out of 86 informative characters across four gene regions. The gene genealogies across the five different loci were not different, as illustrated by the similarity in the sums of the length of the gene trees for the observed and resampled data. Under these conditions, a recent clonal mutation, most likely due to geographical and host isolation (Geiser *et al*. 1998), provides the best explanation for these results.

Even though DNA sequence data provided the most important basis used to discriminate *N. batangarum* from other species in the *N. ribis*/*N. parvum* complex, some morphologically informative characters were also found. The most obvious of these were the fact that colonies of *N. batangarum* formed concentric rings on MEA (Fig. 6), a characteristic that was not observed in any other species of the complex.

Neofusicoccum batangarum was found as an endophyte on healthy twigs of *T. catappa* in

Cameroon. Although no more information regarding its ecology is available, *N. batangarum* was able to produce lesions on young *T. catappa* in pathogenicity trials. This suggests that *N. batangarum* lives in a latent phase in plant organs and is able to convert to being a virulent pathogen when environmental conditions become unfavorable for the tree host.

The second previously undescribed species, *L. mahajangana*, was found in samples from Madagascar, a country where very few studies of microfungi have been conducted. *L. mahajangana* is phylogenetically most closely related to *L. theobromae* and *L. parva*. However, 6 and 14 SNPs amongst 60 informative characters across ITS, tef 1-alpha and beta-tubulin gene regions distinguish *L. mahajangana* from *L. theobromae* and *L. parva*, respectively. Moreover, *L. mahajangana* can also be distinguished from these fungi based on conidial size, its paraphyses and growth characteristics. Conidia of *L. mahajangana* are smaller than those of its closest relatives, *L. theobromae* and *L. pseudotheobromae*, but larger than those of *L. parva*. The paraphyses in this species are aseptate while those of *L. theobromae* and *L. parva* are septate. Moreover, *L. mahajangana* exhibited growth at temperatures as high as 35°C.

Isolates of *L. mahajangana* were obtained from healthy plant material where they occurred as endophytes. Besides this particular feature, there are no data relating to its ecology, distribution and host range. Our consideration of its pathogenicity on *T. catappa* trees showed that *L. mahajangana* was less pathogenic than the other Botryosphaeriaceae found on this host. Lesions produced by *L. mahajangana*, although smaller than those produced by the other species collected from T. catappa in this study, were also significantly different from the control inoculations. The relatively small lesions produced by *L. mahajagana*, together with the fact that it was isolated only from healthy material, provides an indication that it is not a primary pathogen of these trees.

Lasiodiplodia theobromae is considered to be a pantropical pathogen that occurs on numerous hosts worldwide (Punithalingam 1980). Thus, it was not surprising to isolate it from the tropical *T. catappa*. The relatively common occurrence of *L. theobromae* in Cameroon, compared to the other regions sampled in this study, could also reflect a climatic influence. *Lasiodiplodia theobromae* appears to occur most commonly in consistently warm areas (Taylor *et al*. 2005; Urbez-Torrez *et al*. 2008) and the climatic conditions in the localities where samples were collected in this study apparently support the findings.

Neofusicoccum parvum was the most common species collected form *T. catappa* in South Africa and produced lesions on young trees of *T. catappa* in pathogenicity trials. *N. parvum* is a well-known pathogen of forest and fruit trees (Davidson and Tay 1983; Mohali *et al*. 2007; Slippers *et al*. 2004a; van Niekerk *et al*. 2004). In the current study, isolates of this species were obtained from branches of *T. catappa* showing symptoms of die-back. This might indicate that it is the pathogen responsible for branch die-back and death of *T. catappa* in South Africa. In previous studies conducted in South Africa, *N. parvum* was common on non-native *Eucalyptus* trees and on native *Syzygium cordatum*, where it has been shown to be pathogenic to these hosts (Pavlic *et al*. 2007; Slippers *et al*. 2004a). The common occurrence and wide host range of *N. parvum* in South Africa suggests that this fungus might be native to this area.

Lasiodiplodia pseudotheobromae emerged from a recent separation of cryptic species originally identified as *L. theobromae* (Alves *et al*. 2008). It is known from Africa, Europe and Latin America, where it occurs on forest and fruit trees. However, no information concerning its pathogenicity to these trees is available. *Lasiodiplodia pseudotheobromae* was the most abundant species isolated from *T. catappa* and it occurred in all the sampled areas. The known host range of *L. pseudotheobromae* is very limited, with single isolates obtained from *Rosa* sp. in the Netherlands, *Gmelina arborea* and *Acacia mangium* in Costa Rica, *Coffea* sp. in Democratic Republic of Congo and *Citrus aurantium* in Suriname (Alves *et al*. 2008). Results of this study have substantially

increased the geographic areas from which the fungus is known, and they suggest that *L. pseudotheobromae*, like *L. theobromae*, has a worldwide distribution and a very wide host range.

The inoculation trials conducted in this study have shown that *L. pseudotheobromae* was the most pathogenic of all the species tested. *Lasiodiplodia theobromae* and *N. parvum* have previously been shown to be pathogens of several hosts (Davidson and Tay 1983; Mohali *et al*. 2007; Pavlic *et al*. 2007; Slippers *et al*. 2004a). It was, therefore, not surprising that they caused lesions on *T. catappa* in this study. However, this study has provided the first data for the pathogenicity of *L. pseudotheobromae*, which suggests that its importance has been overlooked in the past, most likely because it was considered collectively with *L. theobromae*. It will now be important to determine its host range and distribution in order to understand the threat that it might pose as a pathogen, as well as to guide possible quarantine and other control measures.

The origins of the species of Botryosphaeriaceae collected from *T. catappa* in this study are unknown. However, its common occurrence on both introduced and native plants has led to suggestions that N. parvum might be part of the indigenous fungal flora of South Africa (Pavlic *et al*. 2007, 2008, 2009a, b). In contrast, *L. theobromae*, which has a wide host range and has been reported on native and introduced hosts on many continents, may have been introduced to Africa. Population genetic studies on this fungus will likely provide answers to the questions related to its origin and movements. As limited information is available regarding the recently described *L. pseudotheobromae*, the origin of this species cannot be considered here. In this study, the close relationship between *N. batangarum* and *N. ribis* suggests that *N. batangarum*, which was commonly isolated form *T. catappa* in Cameroon, could be derived from a clonal mutation possibly arising from geographical and host isolation of *N. ribis*, a fungus that has been reported with certainty only from the United States of America on *Ribes* sp. (Slippers *et al*. 2004b). More sampling, both in other areas and hosts, is clearly needed to address the question of its origin.

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