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Botryosphaeriaceae associated with *Pterocarpus angolensis* (kiaat) in South Africa

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Abstract: There have been several recent reports of *Pterocarpus angolensis* (kiaat) trees dying in South Africa, Zambia and Zimbabwe, where this tree is used in traditional medicine and is a valuable source of timber for woodcarving and furniture. A survey of material from diseased *P. angolensis* trees in South Africa yielded isolates of the Botryosphaeriaceae, an important fungal family known to cause a number of tree diseases. The aim of this study was to identify these Botryosphaeriaceae and to determine their pathogenicity to *P. angolensis* with branch inoculations. Seven species of the Botryosphaeriaceae were identified based on a combination of morphological characteristics and sequences from the ITS and EF-1α gene regions. Four of these represent undescribed taxa for which the names *Pseudofusicoccum violaceum*, *P. olivaceum*, *Diplodia alatafructa* and *Fusicoccum atrovirens* are provided. The remaining three species collected include *Lasiodiplodia theobromae*, *L. pseudotheobromae* and *L. crassispora*. Inoculation trials on tree branches showed that *L. pseudotheobromae* and one isolate of *D. alatafructa* differed significantly from control inoculations. The high levels of virulence and common occurrence of *L. pseudotheobromae* suggest that this species could play a role in tree dieback and death.

Key words: branch inoculation, *Diplodia*, endophyte, endophytic, *Fusicoccum*, *Lasiodiplodia*, *Pseudofusicoccum*

INTRODUCTION

Pterocarpus angolensis (kiaat) is a well known native southern African tree species prized for its use in traditional medicine (Coates Palgrave 1977) and as a source of timber in the woodcarving and furniture industries of several African countries (Lowore 1993). The heartwood of the tree is both durable and attractive, resulting in the species becoming a target for exploitation. This has raised concerns regarding the regeneration and health of the species (Caro et al. 2005).

In recent years there have been a number of reports of disease and death of *P. angolensis* trees in South Africa (Krynauw 1998, 2000), Zambia and Zimbabwe (van Wyk et al. 1993). In Zambia and Zimbabwe a disease known as mukwa, referring to the local name of the species in these areas, has been reported. Mukwa disease is characterized by defoliation, wilt, dieback, bark discoloration, vascular and phloem streaking and the production of epicormic shoots (Calvert 1972; Piearce 1979, 1986; van Wyk et al. 1993). In South Africa the disease has been characterized by branch dieback, heart rot and the death of mature trees (Krynauw 1998, 2000).

During a recent survey of diseased *P. angolensis* trees in the Mpumalanga Province of South Africa (Mehl unpubl) isolates resembling the Botryosphaeriaceae were collected from diseased trees. Species of this well known and widely distributed family of ascomycete plant pathogens (Schoch et al. 2009) occur endophytically in both gymnosperms and angiosperms and woody and herbaceous plants (von Arx and Müller 1954, Barr 1972). Diseases caused by species of the Botryosphaeriaceae include fruit rots, leaf spots, seedling damping-off and collar rot, cankers on stems and branches (including twigs) and roots, blight of shoots and seedlings, gummosis, blue-stain of the sapwood, dieback and tree death (Slippers et al. 2007, Slippers and Wingfield 2007). Stress due to drought and physical damage such as hail has been linked to disease expression (Slippers

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and Wingfield 2007), although a number of other predisposing factors might also favor the onset of disease.

The aim of this study was to identify species of the Botryosphaeriaceae associated with *P. angolensis* in South Africa. Species collected were characterized based on their morphology and comparisons of DNA sequence data. Pathogenicity of the species isolated was also evaluated by means of branch inoculations to assess their possible involvement with dieback of *P. angolensis*.

MATERIALS AND METHODS

Sample collection and isolation.—Branches and twigs showing symptoms of dieback as well as healthy specimens were collected from *P. angolensis* trees from five locations in Mpumalanga Province of South Africa. These locations were selected based on reports of tree mortality in the areas as well as to cover a broad range of sites representing the natural distribution of *P. angolensis*. Areas sampled included Mawewe Nature Reserve/Cattle Game Project, Buffelskloof Nature Reserve, Bushbuckridge settlement, the Sudwala Caves area and Pretoriuskop in Kruger National Park.

Branches, both symptomatic and asymptomatic, were collected from four trees at Pretoriuskop, 20 trees in the Sudwala Caves area, seven dying trees in Mawewe Nature Reserve, 14 trees alongside the road at Bushbuckridge Settlement and 20 trees in Buffelskloof Nature Reserve. Material was also collected from stem wounds on trees in the Sudwala Caves area and Mawewe Nature Reserve. Samples included those collected from dead and dying trees in Mawewe Nature Reserve and Buffelskloof Nature Reserve.

All samples were kept in a walk-in refrigerator and isolations were made after 2 wk and 4 wk. A pilot trial had been undertaken on the samples from Pretoriuskop to establish the optimal time for isolations to be made. In this case isolations were done after 1, 2, 4 and 8 wk. After 2 and 4 wk the largest number of isolates of Botryosphaeriaceae was recorded (results not shown). Isolations were made as outlined by Pavlic et al. (2004).

Culture characteristics and morphology.—Cultures were transferred to 2% water agar (Biolab, South Africa) overlaid with sterilized pine needles and small sections from branches of *P. angolensis*. Sporulation was induced following the methods of Mohali et al. (2006). Fruiting structures that emerged were sectioned by hand, and released conidia were mounted in 85% lactic acid on glass microscope slides. Digital microscopic photographs of conidia were taken with a HRc Axiocam digital camera and the accompanying Axiovision 3.1 software (Carl Zeiss Ltd., München, Germany). Thirty measurements were made of the lengths and widths of the conidia. Culture colors were determined using the color charts of Rayner (1970).

Single conidial cultures were made by spreading spores onto water agar with a sterile inoculation loop. Plates were incubated at room temperature 12–24 h and germinating conidia transferred to malt extract agar (1.5% malt extract, 2% agar) (Biolab, Midrand, South Africa) amended with 0.005 g streptomycin sulphate (MEA + S) (Sigma-Aldrich, Steinheim, Germany). For nonsporulating isolates single hyphal tips were transferred to MEA + S. Cultures were incubated at 25 C and maintained in the culture collection of Mike Wingfield (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria, Pretoria, South Africa. Dried cultures were deposited in the National Collection of Fungi (PREM) and representative strains with the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands. Culture morphology was captured with a mounted Sony digital still camera (DSC85 Cybershot 4.1 MP). Isolates were grouped based on culture and conidial morphology.

For new species of the Botryosphaeriaceae identified in this study 20 measurements were made of the conidiomata, 50 measurements were made of the locules (where present), 50 measurements were made of the conidiogenous cells (except one species where 40 measurements were made, as noted in the description) and 50 measurements were made of the conidia.

Growth studies.—Mycelial plugs (5 mm diam) of isolates representing new species of the Botryosphaeriaceae identified in this study were placed at the centers of 90 mm MEA plates with the plug orientated so that the mycelium faced the agar surface. For each isolate five plates each were incubated at 10–35 C at five-degree intervals and the experiment was repeated once.

DNA extraction and PCR amplification.—Three to four isolates of each morphological group were selected for DNA sequence comparisons. Isolates were also selected so that at least two different sampling sites were represented. DNA was extracted from cultures as described by van Wyk et al. (2006) except that the nucleic acid pellets were resuspended in 50 µL TE buffer (100 mM Tris-HCl, 10 mM EDTA, adjusted to pH 8.0) buffer and digested with 5 µL RNase A (1 mg/mL) at 60 C for 1 h. DNA concentrations were quantified with NanoDrop® ND-1000 and accompanying software (NanoDrop Technologies, DuPont Agricultural Genomics Laboratories, Delaware).

The ITS rDNA locus, including the ITS1, 5.8S gene and ITS2, were amplified with the primer pair ITS1 and ITS4 (White et al. 1990). A portion of the elongation factor 1α (EF1α) gene region was also amplified to verify the results from the ITS phylogeny with the primer pairs EF1-728F and EF1-986R (Carbone and Kohn 1999) and EF1F and EF2R (Jacobs et al. 2004). Both gene regions were sequenced for isolates representing all morphological groups. For isolates that did not produce fruiting structures on pine needles or branches and that consequently could not be grouped based on culture characteristics only the ITS rDNA gene region was sequenced. In addition the ITS rDNA locus was amplified and sequenced for isolates of the Botryosphaeriaceae with pigmented conidia that could not be distinguished based on morphology.

Polymerase chain reactions (PCRs) consisted of ~ 10 ng template DNA, 0.2 mM each primer, 2.5 mM each dNTP, 1.5 × PCR buffer, 25 mM $MgCl_2$, and 0.5 U *Taq* polymerase. Reaction volumes were adjusted to 25 µL by adding sterile

Sabax water (Adcock Ingram, Johannesburg, South Africa). Amplification reactions were performed on a Bio-Rad iCycler thermal cycler. Cycling conditions included an initial denaturation step of 96 C for 1 min followed by 35 cycles of denaturation at 94 C for 30 s, annealing at 54 C for 1 min and extension at 72 C for 90 s. A final extension at 72 C for 10 min was also performed. PCR products were separated on 2% agarose-ethidium bromide gels run on a TAE buffer system (Maniatis et al. 1982) and viewed under ultraviolet light. Product sizes were estimated with a Lambda DNA/*Eco*RI + *Hin*dIII marker 3 (Fermentas Life Sciences, USA).

DNA sequencing and phylogenetic analysis.—PCR products were purified with 6% Sephadex columns (Sigma-Aldrich, Steinheim, Germany) as per the manufacturer's instructions. Sequencing PCRs were made with the same primers as in the original PCR and also purified with Sephadex columns. PCR amplicons were sequenced in both directions with the ABI PRISM™ Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, California) following the manufacturer's instructions on an ABI PRISM 3100™ automated sequencer.

Sequences were analyzed and edited with MEGA4 (Tamura et al. 2007). Additional sequences for phylogenetic analysis were obtained from nucleotide BLAST comparisons (blastn) with sequences in GenBank. Sequences were aligned with MAFFT 6 (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/) (Katoh and Toh 2008) with a manual strategy based on the G-INS-i algorithm.

Sequence datasets were analyzed with PAUP 4.0b10 (Swofford 2002). Analyses were done with the heuristic search option with 100 random addition sequence replications on both gene datasets as well as the combined dataset. In all cases tree-bisection-reconnection (TBR) branch swapping was applied and MAXTREES was unlimited. Uninformative characters were excluded and the remaining gaps in the sequence alignment treated as a fifth base (Newstate). A partition homogeneity test (Swofford 2002) was done to determine whether the relationships generated from both gene regions were statistically congruent and that the two gene datasets could be combined. For the partition homogeneity test the heuristic search option was selected and 1000 replications were done. A bootstrap analysis (50% majority rule, 1000 replications) (Felsenstein 1995) was done on the individual datasets to determine the confidence levels of the tree branching points. Trees were rooted to two isolates of *Guignardia* sp. Viala & Ravaz (Pavlic et al. 2008) as outgroup taxa.

Bayesian analysis was done on the individual datasets to determine the posterior probability/stringency of the branch nodes with Monte Carlo Markov chain (MCMC) algorithms (Larget and Simon 1999). jModelTest 0.1.1 (Posada 2008) with the corrected Akaike information criterion (AIC) (Sugiura 1978) selected was used to determine the best nucleotide substitution model for the two individual datasets. MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001) was used for the Bayesian analysis.

Two independent runs were done, both of 5 000 000 generations. Four chains were used and trees were sampled every 100 generations, resulting in 50 000 trees. Burn-in was set at 15 000 generations (150 trees) after likelihood values had converged to stationary, providing 49 850 trees for sampling.

Pathogenicity tests.—Twenty trees in the Sudwala Caves area were selected for two branch inoculation trials in Mar and Sep 2007. Because each tree was genetically unique they were assigned labels for both inoculation trials when lesion lengths were measured. Branch diameter was measured for the second inoculation trial. Branches were inoculated with two representative isolates of each species. In the second inoculation trial CMW22674 and CMW22682 were included.

Branches were randomly selected on each tree for inoculation so that one branch per isolate and one branch per tree for the negative control (a sterile MEA plug) were inoculated. A cork borer (9 mm diam) was used to remove a disk of bark from the branches, exposing the cambium. The cork borer was sterilized between inoculations in 70% ethanol followed by flaming. Mycelial plugs (9 mm diam) taken from 7 d old cultures were placed in the wounds using a sterile scalpel with the mycelium orientated to face the cambium. Wounds were sealed with Parafilm to prevent desiccation. Lesion lengths were measured 6 wk after the branches were inoculated and re-isolations were done from all the inoculated branches on every fifth tree to ascertain that the lesions were associated with the isolate with which it was inoculated.

Data generated from the inoculation trials were subjected to a two-way analysis of variance (ANOVA) with the general linear model procedure from SAS, Type III sum of squares, F-test of SAS, and Fisher's pairwise test (SAS Institute 2004). For the data from the first trial the model included the tree, the species of fungus and the isolate nested within the species as predictors of lesion length where trees were considered as blocks. For the data from the second trial branch diameter was added as a covariable. When results were significantly different ($P < 0.05$), the least squares means were generated, and in conjunction with Fisher's pairwise test the differences among the means were evaluated for statistical significance.

RESULTS

Isolate collection and morphology.—Isolates produced anamorph structures, either on pine needles or small branches of host tissue or both concurrently. Structures yielded either dark *Diplodia*-like conidia (35 isolates) or hyaline *Fusicoccum*-like conidia (67 isolates) (total 102 isolates). The latter group with hyaline conidia could be broadly separated into two groups, based on conidial size and shape in combination with their culture morphology. The first of these groups produced conidia that were on average 15–40 × 5–15 µm (39 isolates) and the second group produced conidia that were 20–55 × 9–15 µm (28 isolates).

DNA sequencing and phylogenetic analyses.—PCR amplification of the ITS and EF-1α gene regions in

isolates yielded fragments of ~ 560 bp and 700–750 bp respectively. Sequences generated for the phylogenetic analysis in this study were deposited in GenBank (TABLE I). Identities of isolates together with culture and accession numbers, majority consensus bootstrap trees generated from the two datasets (TABLE II) as well as the trees resulting from the Bayesian analysis were deposited in TreeBASE (http://www.treebase.org/treebase/index.html) under accession number SN4677. Based on the results from jModelTest 0.1.1., three-parameter models were applied to both the EF-1α (TPM3uf) and ITS (TPM1uf) datasets. Gamma (G) and proportion of invariable site (I) parameters were applied to both models to accommodate variable rates across sites.

There were minor differences in the topologies of the trees emerging from analysis of sequences of the two gene regions, but these differences were observed only within genera among some species. For example some of the *Pseudofusicoccum* spp. could not be delineated with confidence based on the ITS sequences alone but were clearly resolved based on the EF-1α sequence data. Studies combining sequence data from multiple datasets, including the ITS and EF-1 gene regions, has provided the best resolution to distinguish among species (e.g. de Wet et al. 2003, Pérez et al. 2010, Begoude et al. 2011), and consequently those for this study were combined into a single dataset (TABLE II, FIG. 1).

The isolates from *P. angolensis* grouped in four genera representing *Pseudofusicoccum* Mohali, Slippers & M.J. Wingf. (FIG. 1, Clade 1), *Diplodia* Fr. (FIG. 1, Clade 2), *Lasiodiplodia* Ellis & Everh. (FIG. 1, Clade 3) and *Fusicoccum* Corda (FIG. 1, Clade 4, FIG. 2). Based on the phylogenetic analyses and spore measurements, seven species of the Botryosphaeriaceae could be resolved (FIG. 1). Three of these taxa represent known species, namely *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl., *L. crassispora* T.I. Burgess & Barber and *L. pseudotheobromae* A.J.L. Phillips, A. Alves & Crous. The remaining four taxa represented undescribed species that are described below.

TAXONOMY

Pseudofusicoccum olivaceum J.W.M. Mehl & B. Slippers sp. nov. FIG. 3

MycoBank MB512501

Conidiomata pycnidialia, subcuticula, unilocularis, atrobrunnea, pro parte maxima solitaria, applanata, mycelio tecta. *Ostiolum* centralis, rotundum et papillatum. Cellulae conidiogenae hyalinae, holoblasticae, glabrae, cylindricae, guttulatae, rotundae, percurrenter cum 1–2 proliferationibus obscuris prolificentes, vel in plano eodem periclinaliter incrassatae, paraphyses visae. Conidia hyalina, parietibus tenuis, unicellularia, eseptata, interdum contento granulari, guttulata, circumdata strato mucido persistente, apicibus basibusque obtusis ad rotunda late, bacilliforma.

Conidiomata on both pine needles and host material pycnidial, subcuticular, unilocular, dark brown, mostly solitary, applanate, covered with hyphae/mycelium, wall composed of three layers: an outer thick-walled dark to light brown textura angularis; a middle layer of thin-walled light brown cells; and an inner layer of thin-walled hyaline cells, (480.4–)531.9–646.2(–688.3) µm diam (average of 50 conidiomata 589.0 µm). Ostiole central, circular and papillate. Conidiogenous cells hyaline, holoblastic, smooth, cylindrical, guttulate, circular, proliferating percurrently to form one or two indistinct annellations, or proliferating at same level giving rise to periclinal thickenings, paraphyses present, (2.8–)4.7–8.6(–12.7) × (1.7–)2.9–4.5(–6.3) µm (average of 50 conidiogenous cells 6.6 × 3.7 µm). Conidia hyaline, thin-walled, unicellular, aseptate, occasionally granular, guttulate, surrounded by a persistent mucoid sheath, both apex and base blunt to broadly rounded, bacilliform, (17.9–)19.9–25.7(–30.4) × (5.9–)6.3–7.7(–8.9) µm (average of 50 conidia 22.8 × 7.0 µm).

Cultures. Mycelium fluffy, initially white to amber (21′b) on the edges and olivaceous (23k) on the edges, becoming white to olivaceous with age. Optimum temperature for growth 25 C.

Etymology. Name refers to the olivaceous color formed in culture.

Teleomorph. Not observed but expected to be *Botryosphaeria*-like based on phylogenetic inference.

Habitat. Asymptomatic branches and twigs of *Pterocarpus angolensis*.

Known distribution. South Africa.

Specimens examined. SOUTH AFRICA. MPUMALANGA PROVINCE: Kruger National Park: Pretoriuskop, from an asymptomatic branch of *P. angolensis*, Sep 2005, *J. Roux* (CMW20881/ CBS124939) in Herb. PREM60328 (HOLOTYPE). SOUTH AFRICA. MPUMALANGA PROVINCE: Sudwala Caves area, from an asymptomatic branch of *P. angolensis*, Dec 2005, *J.W.M. Mehl & J. Roux* (CMW22637/ CBS124940) in Herb. PREM60329 (PARATYPE). SOUTH AFRICA. MPUMALANGA PROVINCE: Sudwala Caves area, from an asymptomatic branch of *P. angolensis*, Dec 2005, *J.W.M. Mehl & J. Roux* (CMW22643/CBS124941) in Herb. PREM60331 (PARATYPE). SOUTH AFRICA. MPUMALANGA PROVINCE: Kruger National Park, Pretoriuskop, from an asymptomatic branch of *P. angolensis*, Sep 2005, *J. Roux* (CMW20442) in Herb. PREM60332 (PARATYPE). SOUTH AFRICA. MPUMALANGA PROVINCE: Sudwala Caves area, from an asymptomatic branch of *P. angolensis*, Dec 2005, *J.W.M. Mehl & J. Roux* (CMW22639) in Herb. PREM60330 (PARATYPE).

Notes. BLAST results for the ITS sequences revealed an identity of 98% with sequences of *Ps. kimberleyense*

TABLE I. Isolates used in the phylogenetic analysis. Culture numbers in bold indicate ex-type cultures. Accession numbers in italics were obtained from GenBank

Culture number	Other collection number	Identity	Host	Location[a]	Collector	GenBank accession number	
						ITS	EF-1α
CBS119047		*Botryosphaeria corticis*	*Vaccinium corymbosum*	Hammonton, New Jersey, USA	PV Oudemans	*DQ299245*	*EU017539*
ATCC22927		*B. corticis*	*Vaccinium* sp.	North Carolina, USA	RD Millholland	*DQ299247*	*EF614931*
CMW8000	CBS115476	*B. dothidea*	*Prunus* sp.	Crocifisso, Ticino, Switzerland	B Slippers	*AY236949*	*AY236898*
CMW7780	BOT1636	*B. dothidea*	*Fraxinus excelsior*	Molinizza, Ticino, Switzerland	B Slippers	*AY236947*	*AY236896*
CMW13425	CBS117445	*B. mamane*	*Acacia mangium*	Portuguesa State, Venezuela	S Mohali	*EF118046*	GU134939
CMW13429	CBS117446	*B. mamane*	*Eucalyptus* hybrid	Cojedes State, Venezuela	S Mohali	*EF118048*	GU134940
CBS418.64		*"Botryosphaeria" tsugae*	*Tsuga heterophylla*	British Columbia, Lake Cowichan, Canada	A Funk	*DQ458888*	*DQ458873*
CMW15198	WAC12398	*Dichomera eucalypti*	*E. diversicolor*	Warne, WA	TI Burgess	*AY744371*	*DQ093214*
CMW15953	BOT10	*Dic. eucalypti*	*E. diversicolor*	Denmark, WA	TI Burgess	*DQ093195*	*DQ093216*
CBS120835	STE-U5908	*Diplodia africana*	*Prunus persica*	Paarl, Western Cape, S. Africa	U Damm	*EF445343*	*EF445382*
CBS121104	STE-U5946	*D. africana*	*Pr. persica*	Paarl, Western Cape, S. Africa	U Damm	*EF445344*	*EF445383*
CMW22627	CBS124931	*D. alatafructa*	*Pterocarpus angolensis*	Road servitude, Sudwala Caves area, S. Africa	J Mehl & J Roux	FJ888460	FJ888444
CMW22635	CBS124932	*D. alatafructa*	*Pt. angolensis*	Road servitude, Sudwala Caves area, S. Africa	J Mehl & J Roux	FJ888461	FJ888445
CMW22721	CBS124933	*D. alatafructa*	*Pt. angolensis*	Buffelskloof Nature Reserve, S. Africa	J Mehl & J Roux	FJ888478	FJ888446
CBS168.87		*D. cupressi*	*Cupressus sempervirens*	Bet Dagan, Israel	Z Solel	*DQ458893*	*DQ458861*
CBS261.85		*D. cupressi*	*C. sempervirens*	Bet Dagan, Israel	Z Solel	*DQ458894*	*DQ458862*
CMW7060	CBS431.82	*D. mutila*	*Fraxinus excelsior*	Kleine Plas, Maarseveen, Netherlands	HA van der Aa	*AY236955*	*AY236904*
CBS112553		*D. mutila* ("*B. stevensii*")	*Vitis vinifera*	Montemor-o-Novo, Portugal	AJL Phillips	*AY259093*	*AY573219*
CBS121887		*D. olivarum*	*Olea europaea*	Puglia, Italy	S Frisullo	*EU392302*	*EU392279*
CBS121886		*D. olivarum*	*O. europaea*	Puglia, Italy	S Frisullo	*EU392301*	*EU392278*
CMW1185	CBS109727	*D. pinea* A morphotype	*Pinus. radiata*	Jonkershoek, Stellenbosch, S. Africa	WJ Swart	*DQ458897*	*DQ458882*
CMW4881	CBS109725	*D. pinea* C morphotype	*Pi. patula*	Plantation 3, Habinsaran, Sumatra, Indonesia	MJ Wingfield	*DQ458896*	*DQ458881*
CBS116470	Pr3	*D. rosulata*	*Pr. africana*	Gambo, Ethiopia	A Gure	*EU430265*	*EU430267*
CBS116472	Pr5	*D. rosulata*	*Pr. africana*	Gambo, Ethiopia	A Gure	*EU430266*	*EU430268*
CMW189	CBS118110	*D. scrobiculata*	*Pi. resinosa*	USA	MA Palmer	*AY253292*	*AY624253*
CMW7775	BOT1642	*D. seriata* ("*B. obtusa*")	*Ribes* sp.	New York, USA	B Slippers	*AY236954*	*AY236903*
CBS112555		*D. seriata*	*Vitis vinifera*	Portugal, Montemor-o-Novo	AJL Phillips	*AY259094*	*AY573220*
CBS115035		*Dothiorella iberica*	*Quercus ilex*	Monzón, Aragón, Spain	N Ibarra	*AY573213*	*AY573228*

TABLE I. Continued

Culture number	Other collection number	Identity	Host	Location[a]	Collector	GenBank accession number	
						ITS	EF-1α
CBS115041		*Do. iberica*	*Q. ilex*	Aragón, Spain,	AJL Phillips	*AY573202*	*AY573222*
IMI 63581b		*Do. sarmentorum*	*Ulmus sp.*	Warwickshire, England	EA Ellis	*AY573212*	*AY573235*
CBS115038		*Do. sarmentorum*	*Malus pumila*	Delft, Netherlands	AJL Phillips	*AY573206*	*AY573223*
CMW22674	CBS124934	*Fusicoccum atrovirens*	*Pt. angolensis*	Mawewe Nature Reserve, S. Africa	J Mehl & J Roux	FJ888473	FJ888456
CMW22682	CBS124935	*F. atrovirens*	*Pt. angolensis*	Mawewe Nature Reserve, S. Africa	J Mehl & J Roux	FJ888476	FJ888457
CMW26167	CBS122069	*F. ramosum*	*E. camaldulensis*	Bell Gorge, WA	TI Burgess	*EU144055*	*EU144070*
MUCC684		*Guignardia* sp.	*Agonis flexuosa*	Yalgorup, WA	TI Burgess	*EU675682*	*EU686573*
MUCC685		*Guignardia* sp.	*Ag. flexuosa*	Yalgorup, WA	TI Burgess	*EU675681*	*EU686572*
CMW14691	WAC12533	*Lasiodiplodia crassispora*	*Santalum album*	Ord River, Kununurra, WA	TI Burgess	*DQ103550*	*DQ103557*
CMW14688	WAC12534	*L. crassispora*	*S. album*	Ord River, Kununurra, WA	TI Burgess	*DQ103551*	*DQ103558*
CMW22653		*L. crassispora*	*Pt. angolensis*	Mawewe Nature Reserve, S. Africa	J Mehl & J Roux	FJ888465	FJ888452
CMW22654		*L. crassispora*	*Pt. angolensis*	Mawewe Nature Reserve, S. Africa	J Mehl & J Roux	FJ888466	FJ888453
CMW22655		*L. crassispora*	*Pt. angolensis*	Mawewe Nature Reserve, S. Africa	J Mehl & J Roux	FJ888467	FJ888454
CMW22697		*L. crassispora*	*Pt. angolensis*	Mawewe Nature Reserve, S. Africa	J Mehl & J Roux	FJ888477	FJ888455
CMW14077	CBS115812	*L. gonubiensis*	*Syzygium cordatum*	Gonubie, Eastern Cape, S. Africa	D Pavlic	*AY639595*	*DQ103566*
CMW14078	CBS116355	*L. gonubiensis*	*S. cordatum*	Gonubie, Eastern Cape, S. Africa	D Pavlic	*AY639594*	*DQ103567*
CMW26162	CBS122519	*L. margaritacea*	*Adansonia gibbosa*	Tunnel Creek Gorge, WA	TI Burgess	*EU144050*	*EU144065*
CMW26163	CBS122065	*L. margaritacea*	*Ad. gibbosa*	Tunnel Creek Gorge, WA	TI Burgess	*EU144051*	*EU144066*
CBS456.78		*L. parva*	Cassava-field soil	Dep. Meta, Vilavicencio, Colombia	O Rangel	*EF622083*	*EF622063*
CBS356.59		*L. parva*	*Theobroma cacao*	Agalawatta, Sri Lanka	A Riggenbach	*EF622082*	*EF622062*
CBS120832	STE-U5803	*L. plurivora*	*Pr. salicina*	Stellenbosch, S. Africa	U Damm	*EF445362*	*EF445395*
CBS121103	STE-U4583	*L. plurivora*	*V. vinifera*	S. Africa	F Halleen	*AY343482*	*EF445396*
CBS116459		*L. pseudotheobromae*	*Gmelina arborea*	San Carlos, Costa Rica	J Carranza-Velásquez	*EF622077*	*EF622057*
CBS116460		*L. pseudotheobromae*	*Ac. mangium*	San Carlos, Costa Rica	J Carranza-Velásquez	*EF622078*	*EF622058*
CMW22650		*L. pseudotheobromae*	*Pt. angolensis*	Mawewe Nature Reserve, S. Africa	J Mehl & J Roux	FJ888464	FJ888447
CMW22666		*L. pseudotheobromae*	*Pt. angolensis*	Mawewe Nature Reserve, S. Africa	J Mehl & J Roux	FJ888470	FJ888448

TABLE I. Continued

Culture number	Other collection number	Identity	Host	Location[a]	Collector	GenBank accession number	
						ITS	EF-1α
CMW22667		*L. pseudotheobromae*	*Pt. angolensis*	Mawewe Nature Reserve, S. Africa	J Mehl & J Roux	FJ888471	FJ888449
CBS111530		*L. theobromae*	Unknown	Unknown	Unknown	*EF622074*	*EF622054*
CMW18420	BOT979	*L. theobromae*	*Casuarina cunninghamii*	Mbale, Uganda	J Roux	*DQ103534*	*DQ103564*
CMW22663		*L. theobromae*	*Pt. angolensis*	Mawewe Nature Reserve, S. Africa	J Mehl & J Roux	FJ888468	FJ888450
CMW22664		*L. theobromae*	*Pt. angolensis*	Mawewe Nature Reserve, S. Africa	J Mehl & J Roux	FJ888469	FJ888451
CMW13455	CBS117453	*Neofusicoccum andinum*	*Eucalyptus* sp.	Mountain Range, Mérida state, Venezuela	S Mohali	*AY693976*	*AY693977*
CMW13446	CBS117452	*N. andinum*	*Eucalyptus* sp.	Mountain Range, Mérida state, Venezuela	S Mohali	*DQ306263*	*DQ306264*
CBS110299		*N. luteum*	*V. vinifera*	Quinta do Marquês, Oeiras, Portugal	AJL Phillips	*AY259091*	*AY573217*
CMW9076	BOT2482	*N. luteum*	*Malus* × *domestica*	Kemeu, New Zealand	SR Pennycook	*AY236946*	*AY236893*
CMW9080	BOT2486	*N. parvum*	*Populus nigra*	TePuke/BP, New Zealand	GJ Samuels	*AY236942*	*AY236887*
CMW15950		*N. parvum*	*E. globulus*	Western Australia	TI Burgess	*DQ093193*	*DQ093213*
CMW26147	CBS122055	*Pseudofusicoccum adansoniae*	*Adansonia gibbosa*	Derby, WA	TI Burgess	*EF585523*	*EF585571*
CMW26148	CBS122056	*P. adansoniae*	*Ficus opposita*	Tunnel Creek National Park, Derby, WA	TI Burgess	*EF585524*	*EF295489*
CMW26159	CBS122062	*P. ardesiacum*	*Ad. gibbosa*	Mount Hardman, Great North Highway, WA	TI Burgess	*EU144060*	*EU144075*
CMW26155	CBS122063	*P. ardesiacum*	*Ad. gibbosa*	Derby, WA	TI Burgess	*EU144061*	*EU144076*
CMW26156	CBS122058	*P. kimberleyense*	*Ac. synchronica*	Tunnel Creek National Park, Derby, WA	TI Burgess	*EU144057*	*EU144072*
CMW26158	CBS122060	*P. kimberleyense*	*Ad. gibbosa*	Tunnel Creek National Park, Derby, WA	TI Burgess	*EU144058*	*EU144073*
CMW20881	CBS124939	*P. olivaceum*	*Pt. angolensis*	Pretoriuskop, Kruger National Park, S. Africa	J Roux	FJ888459	FJ888437
CMW22637	CBS124940	*P. olivaceum*	*Pt. angolensis*	Road servitude, Sudwala Caves area, S. Africa	J Mehl & J Roux	FJ888462	FJ888438
CMW22639		*P. olivaceum*	*Pt. angolensis*	Road servitude, Sudwala Caves area, S. Africa	J Mehl & J Roux	FJ888463	FJ888439
CMW13434	CBS117448	*P. stromaticum*	*Eucalyptus* hybrid	San Carlos/Cojedes state, Venezuela	S Mohali	*AY693974*	*AY693975*

TABLE I. Continued

Culture number	Other collection number	Identity	Host	Location[a]	Collector	GenBank accession number	
						ITS	EF-1α
CMW13435	CBS117449	*P. stromaticum*	*Eucalyptus* hybrid	San Carlos/Cojedes state, Venezuela	S Mohali	*DQ436935*	*DQ436936*
CMW22679	CBS124936	*P. violaceum*	*Pt. angolensis*	Mawewe Nature Reserve, S. Africa	J Mehl & J Roux	FJ888474	FJ888442
CMW20436	CBS124937	*P. violaceum*	*Pt. angolensis*	Pretoriuskop, Kruger National Park, S. Africa	J Roux	FJ888458	FJ888440
CMW22671	CBS124938	*P. violaceum*	*Pt. angolensis*	Mawewe Nature Reserve, S. Africa	J Mehl & J Roux	FJ888472	FJ888441
CMW22681		*P. violaceum*	*Pt. angolensis*	Mawewe Nature Reserve, S. Africa	J Mehl & J Roux	FJ888475	FJ888443

[a] Abbreviations: KNP = Kruger National Park, S. Africa = South Africa, WA = Western Australia, USA = United States of America.

(GenBank accession EU144059; 505 of 512 bases), *Ps. ardesiacum* (GenBank accession EU144062; 504 of 512 bases), *Ps. adansoniae* (GenBank accession EF585532; 502 of 512 bases) and *Ps. stromaticum* (GenBank accession DQ436935; 502 of 512 bases), while BLAST results for the elongation factor 1α sequences revealed an identity of 97% with sequences of *Ps. stromaticum* (GenBank accession DQ436936; 292 of 298 bases), *Ps. ardesiacum* (GenBank accession EU144077; 291 of 298 bases) and *Ps. kimberleyense* (GenBank accession EU144074; 291 of 298 bases).

Pseudofusicoccum violaceum J.W.M. Mehl & B. Slippers sp. nov. FIG. 4

MycoBank MB513500

Conidiomata pycnidialia, superficialia, unilocularis, atrobrunnea, pro parte maxima solitaria, fere globosa, mycelio tecta. *Ostiolum* centralis, rotundum et papillatum. Cellulae conidiogenae hyalinae, holoblasticae, glabrae, cylindricae, percurrenter cum 1–2 proliferationibus distinctis prolificentes, vel in plano eodem periclinaliter incrassatae, paraphyses non visae. Conidia hyalina, parietibus tenuis, unicellularia, eseptata, contento granulari, guttulata, circumdata strato mucido persistente, apicibus basibusque obtusis ad rotunda late, bacilliforma.

Conidiomata on both pine needles and host material pycnidial, superficial, unilocular, dark brown, mostly solitary, more or less globose/circular, covered with hyphae/mycelium, wall composed of three layers: an outer thick-walled dark to light brown textura angularis; a middle layer of thin-walled light brown cells; and an inner layer of thin-walled hyaline cells, (470.4–)498.2–615.9(–659.4) μm diam (average of 50 cells 557.1 μm). Ostiole central, circular and papillate. *Conidiogenous cells* hyaline, holoblastic, smooth, cylindrical, proliferating percurrently to form one or two distinct annellations, or proliferating at same level giving rise to periclinal thickenings, paraphyses not observed, (5.9–)6.1–11.2(–17.0) × (2.7–)3.6–5.0(–6.3) μm (average of 50 conidiogenous cells 8.6 × 4.3 μm). Conidia hyaline, thin-walled, unicellular, aseptate, granular, guttulate, surrounded by a persistent mucoid sheath, both apex and base blunt to broadly rounded, bacilliform, (26.5–)29.8–36.1(–39.6) × (8.0–)8.7–10.3(–11.6) μm (average of 50 conidia 33.0 × 9.5 μm).

Cultures. Mycelium fluffy, initially white to amber (21′b) in the center and violet (59′i) on the edges, turning olivaceous (23k) to greenish black (31′′′′′k) in the center and becoming olivaceous to greenish black with age. Optimum temperature for growth 30 C.

Etymology. Name refers to the distinct violet/purple often formed in culture.

Teleomorph. Not observed but expected to be *Botryosphaeria*-like based on phylogenetic inference.

Habitat. Asymptomatic branches and twigs of *Pterocarpus angolensis*.

Known distribution. South Africa.

Specimens examined. SOUTH AFRICA. MPUMALANGA PROVINCE: Mawewe Nature Reserve, from an asymptomatic branch of *P. angolensis*, Dec 2005, *J.W.M. Mehl & J. Roux* (CMW22679/CBS124936) in Herb. PREM60333 (HOLOTYPE). SOUTH AFRICA. MPUMALANGA PROVINCE: Mawewe Nature Reserve, from an asymptomatic branch of *P. angolensis*, Dec 2005, *J.W.M. Mehl & J. Roux* (CMW22671/CBS124938) in Herb. PREM60334 (PARATYPE). SOUTH AFRICA. MPUMALANGA PROVINCE: Mawewe Nature Reserve, from an asymptomatic branch of *P. angolensis*, Dec 2005, *J.W.M. Mehl* (CMW22675) in Herb. PREM60335 (PARATYPE). SOUTH AFRICA. MPUMALANGA PROVINCE: Mawewe Nature Reserve, from an asymptomatic branch of *P. angolensis*, Dec 2005, *J.W.M. Mehl* (CMW22683) in Herb. PREM60336 (PARATYPE).

Notes. BLAST results for the ITS sequences revealed an identity of 99% with sequences of *Ps. kimberleyense* (GenBank accession EU144059; 511 of 513 bases) and *Ps. ardesiacum* (GenBank accession EU144062; 510 of 513 bases), while BLAST results for the elongation factor 1α sequences revealed an identity of 98% with sequences of *Ps. ardesiacum* (GenBank accession EU144077; 292 of 297 bases) and *Ps. kimberleyense* (GenBank accession EU144074; 292 of 297 bases).

The violet/purple formed in culture is distinctive of *Ps. violaceum* and has not been observed in any other *Pseudofusicoccum* species. Conidiomata of *Ps. violaceum* on both pine needles and branches of *P. angolensis* are superficial as are conidiomata of *Ps. stromaticum* (Mohali et al. 2006), while conidiomata of *Ps. olivaceum* are subcuticular and conidiomata of *Ps. adansoniae*, *Ps. ardesiacum* and *Ps. kimberleyense* semi-immersed (Pavlic et al. 2008). Conidia of *Ps. violaceum* are larger than those of *Ps. stromaticum*, *Ps. adansoniae* and *Ps. ardesiacum*, only slightly larger than *Ps. kimberleyense*, and remain aseptate post-germination (Mohali et al. 2006, Pavlic et al. 2008) (TABLE III). Conidia of *Ps. olivaceum* are smaller than those of *Ps. kimberleyense* and wider than those of *Ps. adansoniae* (Pavlic et al. 2008) (TABLE III). Conidiogenous cells of *Ps. violaceum* and *Ps. olivaceum* are wider than those of other *Pseudofusicoccum* species, while conidiogenous cells of *Ps. olivaceum* are wider than only *Ps. stromaticum* (Mohali et al. 2006, Pavlic et al. 2008). Both conidia and coniodogenous cells are larger in *Ps. violaceum* than those of *Ps. Olivaceum*, and this feature helps to distinguish between the two species.

Diplodia alatafructa J.W.M. Mehl & B. Slippers sp. nov. FIG. 5

MB513498

Conidiomata pycnidialia, superficialia, unilocularis, atrobrunnea vel nigra, pro parte maxima solitaria, fere globosa, mycelio tecta. Ostiolum centrale et cylindrice, histogene. Cellulae conidiogenae holoblasticae, hyalinae, discretae, cylindricae, percurrenter cum 2–3 proliferationibus distinctis prolificentes, vel in plano eodem periclinaliter incrassatae. Conidia primo hyalina, cum maturitate pigmenta et atrobrunnea, unicellularia, raro uniseptata aut biseptata, raro striata, ellipsoidea vel obovoidea, parietibus crassis, contento granulari, apice rotundata, sine guttulis, glabris.

Conidiomata on both pine needles and host material pycnidial, superficial, unilocular, dark brown to black, mostly solitary, more or less globose/circular, covered with mycelium/hyphae, wall composed of three layers: an outer thick-walled dark brown textura angularis; a middle layer of light brown to reddish brown thin-walled cells; and an inner layer of hyaline thin-walled cells, (114.0–)129.0–154.0(–160.0) μm diam (average of 50 conidiomata 141.4 μm). Ostiole central and circular. Conidiophores absent. Conidiogenous cells holoblastic, hyaline, discrete, spherical to cylindrical, proliferating percurrently to form two or three distinct annellations, or proliferating at same level giving rise to periclinal thickenings, (10.0–)12.6–18.2(–23.2) × (8.1–)10.8–14.2(–15.6) μm (average of 40 conidiogenous cells 15.4 × 12.5 μm). Conidia initially hyaline becoming pigmented and dark brown with age, unicellular, rarely septate or biseptate, rarely striate, ellipsoid to obovoid, thick-walled, granular, rounded at apices, eguttulate, smooth, (22.4–)24.6–29.2(–32.9) × (9.3–)11.0–13.8(–15.8) μm (average of 50 conidia 26.9 × 12.4 μm).

Cultures. Mycelium fluffy, initially white to amber (21′b) in the center turning dark amber within 7 d and becoming white to dark amber (19′b), almost olivaceous (23k) with age. Submerged mycelia (reverse) same except becoming white to dark amber, almost olivaceous, on the edges, and olivaceous in the center with age. Optimum temperature for growth 25 C.

Etymology. Name refers to the Latinized form of the genus of the host from which it was isolated; *Pterocarpus* (L.) Jacq. is Greek for ''winged fruit'', hence the equivalent Latin ''alatafructa''.

Teleomorph. Not observed but expected to be *Botryosphaeria*-like based on phylogenetic inference.

Habitat. Stem wounds and diseased tissue of *Pterocarpus angolensis*.

Known distribution. South Africa.

Specimens examined. SOUTH AFRICA. MPUMALANGA PROVINCE: Sudwala Caves area, from a stem wound on *P. angolensis*, Dec 2005, *J.W.M. Mehl & J. Roux* (CMW22627/CBS124931) in Herb. PREM60337 (HOLOTYPE). SOUTH AFRICA. MPUMALANGA PROVINCE: Sudwala Caves area, from a stem wound on *P. angolensis*, Dec 2005, *J.W.M. Mehl & J. Roux* (CMW22635/CBS124932) in Herb. PREM60338 (PARATYPE). SOUTH AFRICA. MPUMALANGA PROVINCE: Buffelskloof Nature Reserve, from diseased tissue of *P. angolensis*, Dec 2005, *J.W.M. Mehl & J. Roux*

TABLE II. Identity, location and source material of isolates collected from *P. angolensis*

Site	Species	Number of isolates	Source(s) of isolates collected
Buffelskloof Nature Reserve	*Diplodia alatafructa*	2	Branch and trunk of two dead trees
	Lasiodiplodia theobromae	1	Base of trunk of dead tree
Bushbuckridge Settlement	*Pseudofusicoccum olivaceum*	21	Endophytic from branch pith
	P. violaceum	4	Endophytic from branch pith
Mawewe Nature Reserve	*Fusicoccum atrovirens*	2	Endophytic from branch pith
	L. crassispora	4	Trunk wounds
	L. pseudotheobromae	16	Trunk wounds and lesion (n = 14) and endophytic from branch pith (n = 2)
	L. theobromae	4	Trunk lesion and wounds
	P. violaceum	15	Endophytic from branch pith
Pretoriuskop, Kruger National Park	*P. olivaceum*	9	Endophytic from branch pith
	P. violaceum	6	Endophytic from branch pith
Road Servitude, Sudwala Caves area	*D. alatafructa*	2	Trunk wounds
	L. pseudotheobromae	7	Trunk wounds
	P. olivaceum	10	Endophytic from branch pith

(CMW22721/CBS124933) in Herb. PREM60339 (PARATYPE). SOUTH AFRICA. MPUMALANGA PROVINCE: Buffelskloof Nature Reserve, from diseased tissue of *P. angolensis*, Dec 2005, *J.W.M. Mehl & J. Roux* (CMW22703) in Herb. PREM60340 (PARATYPE).

Notes. BLAST results for the ITS sequences revealed an identity of 99% with sequences of *D. seriata* (GenBank accession EU080933; 501 of 503 bases), while BLAST results for the elongation factor 1α sequences revealed an identity of 95% with sequences of both *D. seriata* (GenBank accession EU392282; 250 of 259 bases) and *D. pinea* (GenBank accession EU392263; 249 of 259 bases).

Although BLAST results indicated close homology to *D. seriata*, *D. alatafructa* is a distinct species based on both morphology and phylogenetic inference. Morphologically *D. seriata* has conidiogenous cells shorter (5.5 μm) than those of *D. alatafructa* (~ 10.0–23.0 μm). In the description of this species it was noted that *D. seriata* has immersed conidiomata while *D. alatafructa* produces superficial conidiomata, but further studies are needed to determine whether this is a consistent character.

Fusicoccum atrovirens J.W.M. Mehl & B. Slippers sp. nov. FIG. 6

MB513499

Conidiomata pycnidialia superficialia, multilocularis, atrobrunnea vel nigra, eustromatica, multipleces, effusa, globosa, mycelio tecta. Loculus defixus sine ostiolis visis, histogenis. Cellulae conidiogenae hyalinae, holoblasticae, glabrae, discretae, cylindricae, percurrenter cum 1–2 proliferationibus distinctis prolificentes, vel in plano eodem periclinaliter incrassatae. Conidia hyalina, parietibus tenuis, unicellularia, eseptata, contento granulari, ellipsoidea vel obovoidea.

Conidiomata on both pine needles and host material pycnidial, superficial, multilocular, dark brown to black, eustromatic, complex, effuse, globose, covered with hyphae/mycelium, wall composed of three layers: an outer thick-walled dark to light brown textura angularis; a middle layer of thin-walled light brown cells; and an inner layer of thin-walled hyaline cells, (179.8–)212.3–273.4(–285.8) μm diam (average of 50 cells 242.8 μm). Locule embedded without visible ostioles, (36.9–)42.8–60.4(–68.2) μm diam (average of 50 locules 51.6 μm). *Coniophores* absent. Conidiogenous cells hyaline, holoblastic, smooth, discrete, cylindrical, proliferating percurrently to form one or two distinct annellations, or proliferating at same level giving rise to periclinal thickenings, paraphyses present, (10.5–)13.7–19(–21.9) × (2.1–)3.3–4.4(–5.4) μm (average of 50 coniodogenous cells 16.3 × 3.8 μm). Conidia hyaline, thin-walled, unicellular, aseptate, rarely becoming septate on germination, granular, ellipsoid to obovoid, (27.1–)30.9–36.0(–40.3) × (5.7–)7.1–9.9(–11.8) μm (average of 50 conidia 33.5 × 8.5 μm).

Cultures. Mycelium fluffy, initially white to olivaceous (23k) in the center, edges becoming olivaceous to greenish black (31′′′′′k) with age. Submerged mycelia (reverse) initially white to dark amber (19′b) on the edges to olivaceous in the center, becoming olivaceous to greenish black with age. Optimum temperature for growth 30 C.

Etymology. Name refers to the dark green color formed by the fungus in culture.

Teleomorph. Not observed but expected to be *Botryosphaeria*-like based on phylogenetic inference.

Habitat. Asymptomatic branches and twigs of *Pterocarpus angolensis*.

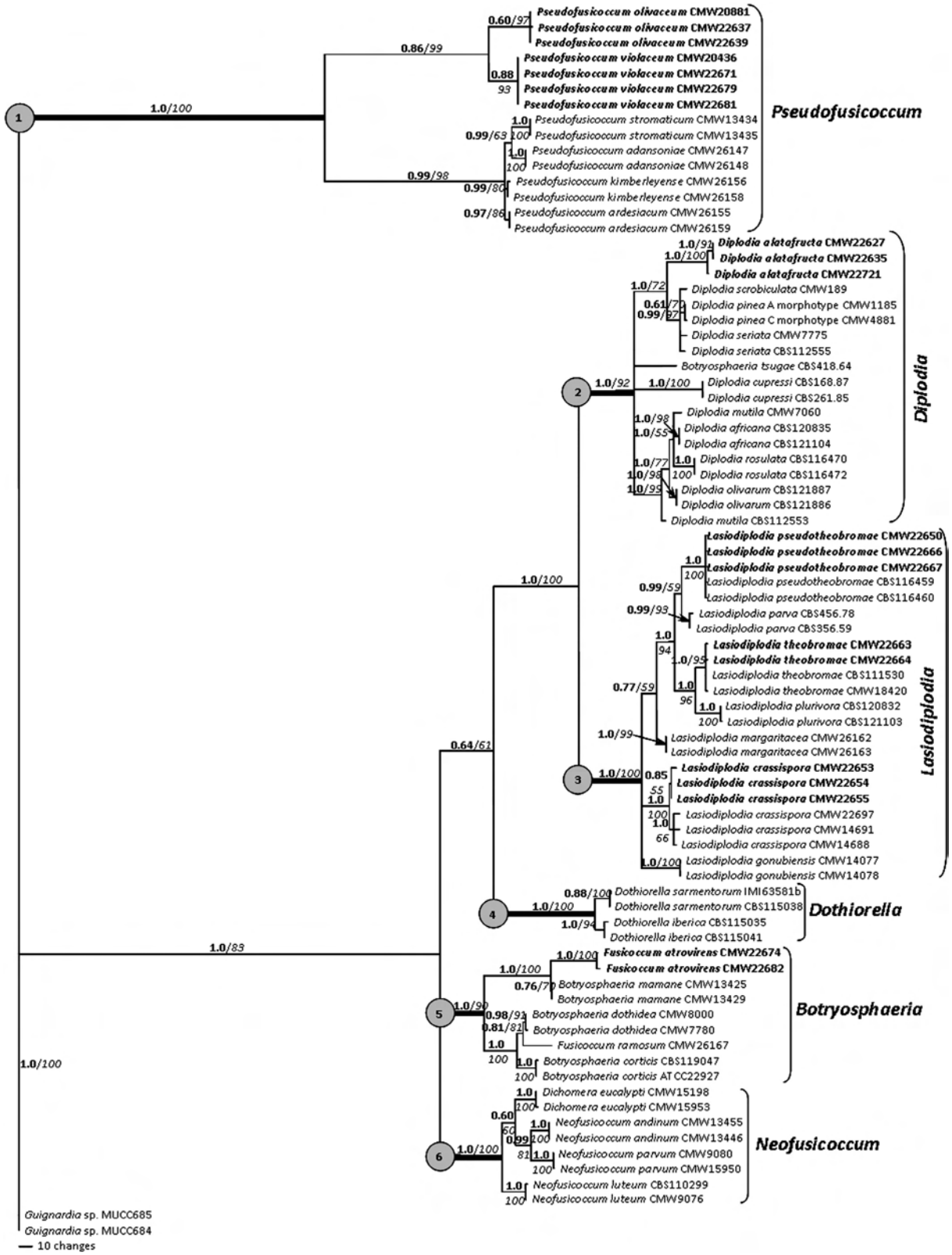

FIG. 1. One of 416 most parsimonious trees of 1602 steps obtained for the combined dataset of the ITS and EF-1α gene regions, after exclusion of uninformative characters. The tree was rooted to two isolates of *Guignardia* sp. Posterior probability values from the Bayesian analysis are provided in bold above the branches, followed by Bootstrap values (1000 replicates, values lower than 50% not shown) in italics either below or above branches. Genera defined by Crous et al. (2006) and Phillips et al.

Known distribution. South Africa.

Specimens examined. SOUTH AFRICA. MPUMALANGA PROVINCE: Mawewe Nature Reserve, from an asymptomatic branch of *P. angolensis*, Dec 2005, *J.W.M. Mehl & J. Roux* (CMW22674/CBS124934) in Herb. PREM60341 (HOLOTYPE). SOUTH AFRICA. MPUMALANGA PROVINCE: Mawewe Nature Reserve, from an asymptomatic branch of *P. angolensis*, Dec 2005, *J.W.M. Mehl & J. Roux* (CMW22682/CBS124935) in Herb. PREM60342 (PARATYPE).

Notes. BLAST results for the ITS sequences revealed an identity of 91% with sequences of *B. mamane* (GenBank accession EF118052; 455 of 501 bases) while BLAST results for the elongation factor 1α sequences revealed an identity of 87% with sequences of *B. corticis* (GenBank accession EU673291; 231 of 265 bases) and 100% homology with sequences of *B. mamane* generated in this study. However the highly divergent ITS phylogeny (19 steps with 100% Bootstrap support) and several morphological characters (Gardner 1997) supported the delineation of *F. atrovirens* as a distinct species from *B. mamane*. These morphological characters include ostiolate locules 100–200 μm diam in *B. mamane*, whereas locules in *F. atrovirens* are smaller (35–70 μm diam) and seemingly do not possess obvious ostioles. Furthermore microconidia have been observed in *B. mamane* but not in *F. atrovirens*, and paraphyses have been reported only in *F. atrovirens*. Conidia can be one- or two-sepatate in *B. mamane* (Mohali et al. 2007) but remain aseptate until germination in *F. atrovirens*.

Phylogenetically and morphologically *F. atrovirens* is distinct from the remaining species in the genus. The conidiomata on both pine needles and *P. angolensis* branch tissue are superficial, in contrast to *B. dothidea*, *B. corticis* and *F. ramosum* where conidiomata are semi-immersed/embedded in host tissue and both conidiogenous cells and conidia are larger than those of *F. ramosum*. Conidia are also larger than those of *B. dothidea* and *B. corticis* (TABLE III).

Species distribution.—The most common species isolated in this study were the two *Pseudofusicoccum* spp., *Ps. olivaceum* (38 isolates, 37.26%) and *Ps. violaceum* (28 isolates, 27.45%). Both were isolated from asymptomatic branches, suggesting that they are endophytes of *P. angolensis*. *Lasiodiplodia pseudotheobromae* was the third most common species isolated (22 isolates, 21.57%), mostly from stem wounds. Only five or fewer isolates were obtained for the remaining species identified in this study (TABLE IV, FIG. 7). In terms of locations sampled 35 isolates (34.31%) representing five of the species obtained in this study originated from Mawewe Nature Reserve, 25 isolates (24.51%) representing two species were obtained from Bushbuckridge Settlement, 19 isolates (18.63%) representing three species were obtained from the Sudwala Caves area, 15 isolates representing two species were obtained from Pretoriuskop and three isolates representing two species were obtained from Buffelskloof Nature Reserve.

Pathogenicity tests.—Inoculations resulted in lesions (FIG. 8) within 6 wk for both inoculation trials for all isolates and for some of the control inoculations (FIG. 9). In the first trial isolate ($F = 3.01$, $P = 0.0046$), fungal species ($F = 4.96$, $P < 0.0001$) and tree genotype ($F = 1.77$, $P = 0.0261$) were significant predictors of lesion length. Isolates of *L. pseudotheobromae* (CMW22629, $P = 0.006$, and CMW22656, $P = 0.0001$), *L. crassispora* (CMW22653, $P = 0.0209$) and *D. alatafructa* (CMW22703, $P = 0.0004$) differed significantly from those associated with the control inoculations. In the second trial only fungal species ($F = 15.24$, $P < 0.0001$) was a significant predictor of lesion length. Neither isolate ($F = 1.44$, $P = 0.1776$) nor branch diameter ($F = 3.05$, $P = 0.0819$) were significant predictors of lesion length. Isolates of *L. pseudotheobromae* (CMW22629, $P < 0.0001$; CMW22656, $P < 0.0001$) and *D. alatafructa* (CMW22703, $P = 0.0002$) differed significantly from those associated with the control inoculations. In all cases the inoculated fungi were re-isolated from the branches and no Botryosphaeriaceae were isolated from the branches inoculated as controls.

DISCUSSION

In this study seven species of the Botryosphaeriaceae were isolated from *P. angolensis* trees in South Africa. Of these fungi four represent new species described here as *Pseudofusicoccum olivaceum*, *Ps. violaceum*, *Diplodia alatafructa* and *Fusicoccum atrovirens*. The remaining species represent the known taxa, *Lasiodiplodia theobromae*, *L. crassispora* and *L. pseudotheobromae*. Before this study only an unknown species of *Sphaeropsis* Sacc., a genus taxonomically related to species in the Botryosphaeriaceae (Denman et al. 2000, Phillips et al. 2008), had been isolated from the tree (Vermeulen 1990).

←

(2008) are indicated by circled numbers (1 = *Pseudofusicoccum*, 2 = *Diplodia*, 3 = *Lasiodiplodia*, 4 = *Dothiorella*, 5 = *Botryosphaeria*, 6 = *Neofusicoccum*).

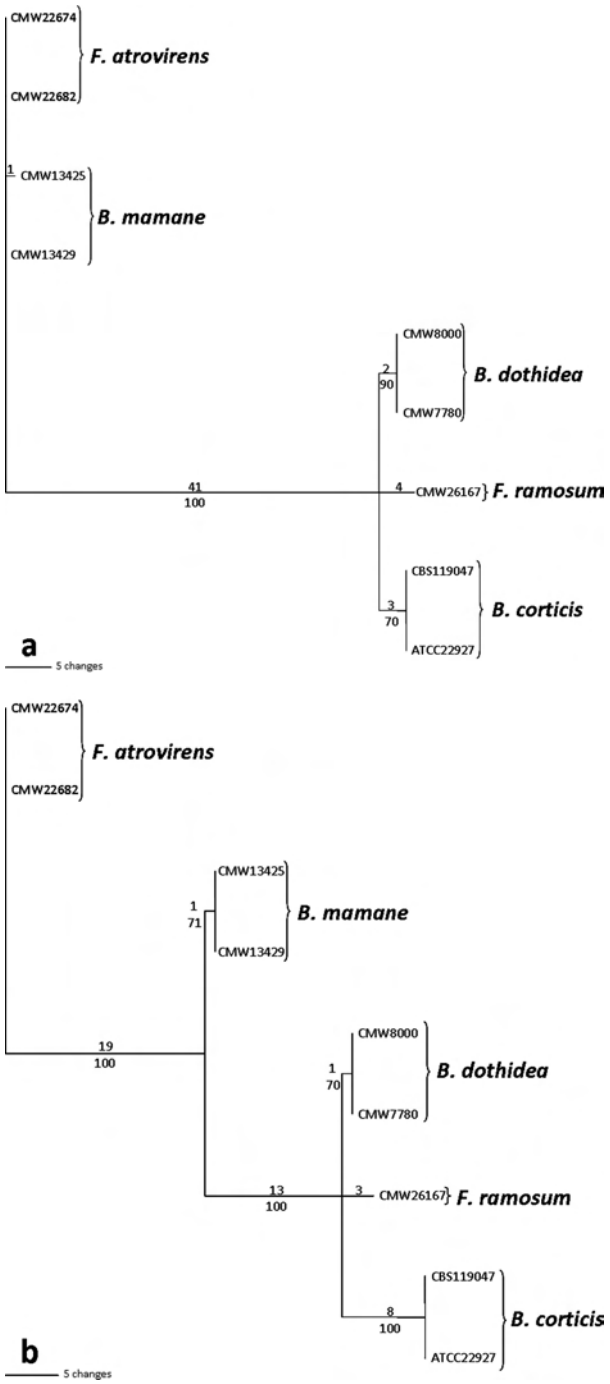

FIG. 2. Unrooted majority consensus phylograms of the a. EF-1α and b. ITS gene regions for species in the *Fusicoccum* clade, after exclusion of uninformative characters. Bootstrap values (1000 replicates, values lower than 50% not shown) are indicated below the branches and branch lengths above.

The description of *Ps. olivaceum* and *Ps. violaceum* from *P. angolensis* expands the host and geographic range of *Pseudofusicoccum* spp. This recently described genus accommodates species of the Botryosphaeriaceae producing *Fusicoccum*-like conidia with

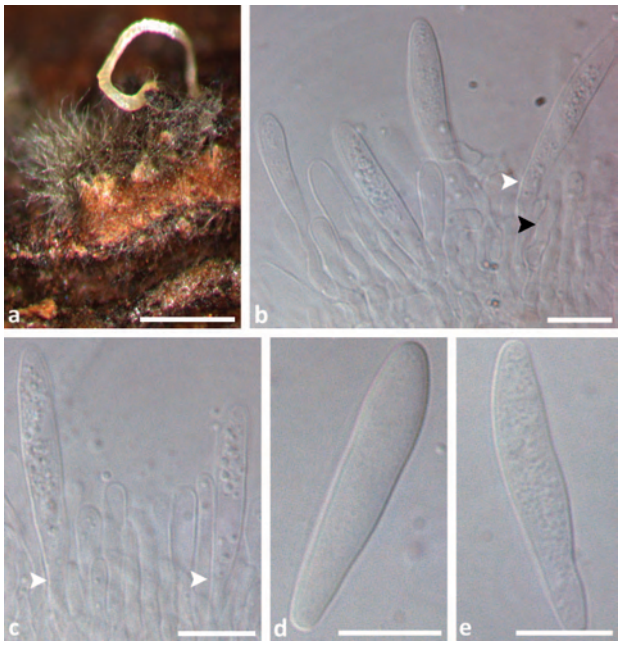

FIG. 3. *Pseudofusicoccum olivaceum*. a. Pycnidia sporulating on a branch of the host (*Pterocarpus angolensis*). b, c. Conidiogenous cells with immature, developing conidia. d, e. Mature conidia. White arrows indicate annellations while black arrows indicate paraphyses. Bars: a = 500 μm, b–e = 10 μm.

persistent mucoid sheaths (Crous et al. 2006). At the time of its description *Pseudofusicoccum* was monotypic for *Ps. stromaticum* isolated from hybrid *Eucalyptus* (Myrtaceae) trees in Cojedes state (Mohali et al. 2006) and *Acacia mangium* in Portuguesa state, both in Venezuela (Mohali et al. 2007). Pavlic et al. (2008) identified three new species, *Ps. adansoniae*, *Ps. kimberleyense* and *Ps. Ardesiacum*, from several native Australian trees including a *Eucalyptus* sp., *Ficus opposita* (Moraceae), *Acacia synchronica* (Fabaceae: Mimosidae) and *Adansonia gibbosa* (Bombacaceae) in northern Western Australia. Each species was isolated from at least two of these tree species. These results suggested that species of *Pseudofusicoccum* occur mostly on native trees, have a broad host range and are tropical in distribution. All species have optimal growth temperatures of 25–30 C and all but one (*Ps. stromaticum* isolated ~ 10° north of the equator) are found in the southern hemisphere. Begoude (2009) isolated *Ps. olivaceum* from native *Terminalia sericea* trees, also in the Sudwala Caves area, strengthening the view that this fungus is a common endophyte on other native tree species in South Africa and that species in the genus are plurivorous instead of host-specific.

Both isolates of *F. atrovirens* obtained in this study were from a single tree in Mawewe Nature Reserve. Its limited occurrence, despite extensive sampling in this

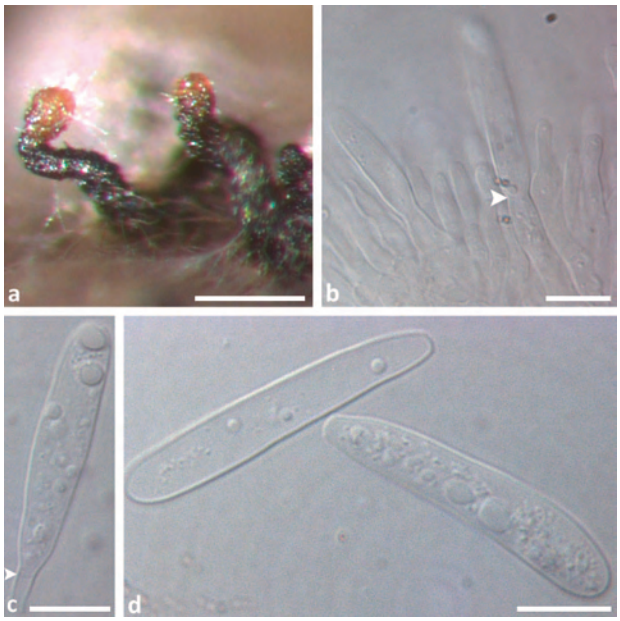

FIG. 4. *Pseudofusicoccum violaceum*. a. Pycnidia sporulating on a branch of the host (*P. angolensis*). b, c. Conidiogenous cells and immature, developing conidia. d. Mature conidia. White arrows indicate annellations. Bars: a = 200 µm, b–d = 10 µm.

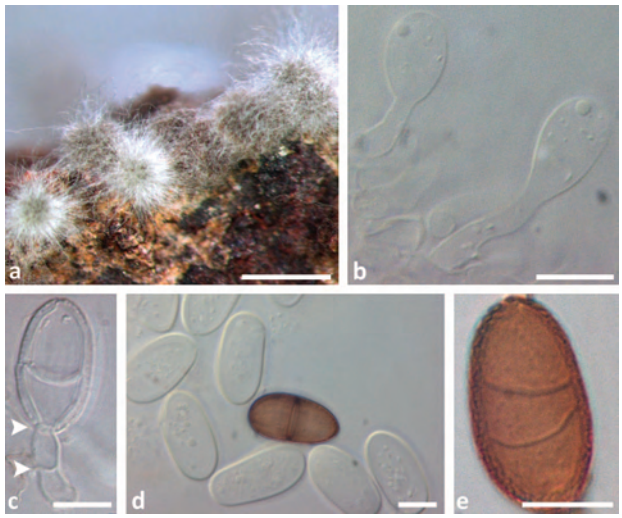

FIG. 5. *Diplodia alatafructa*. a. Pycnidia sporulating on a branch of the host (*P. angolensis*). b, c. Conidiogenous cells and immature, developing conidia. d, e. Mature conidia. White arrows indicate annellations. Bars: a = 1000 µm, b–e = 10 µm.

and the other areas, suggests that the species possibly originated from another plant host in the area. The remaining species in the genus *Botryosphaeria* (anamorph: *Fusicoccum*) (*B. dothidea*, *B. corticis*, *B. mamane* and *F. ramosum*) have also been reported on multiple plant hosts, with the exception of *F. ramosum* that was isolated as an endophyte of *E. camaldulensis* in Western Australia (Pavlic et al. 2008). *Botryosphaeria dothidea*, the type species of the genus, has a cosmopolitan distribution and a broad host range, including angiosperms and gymnosperms (de Wet et al. 2007). The fungus has been isolated from asymptomatic *Eucalyptus* hybrids (Mohali et al. 2007) and is the cause of shoot blight and canker formation on pistachio in USA (Ma and Michailides 2002). *Botryosphaeria corticis* has only been reported from *Vaccinium* spp. in USA where it was associated with stem cankers (Phillips et al. 2006).

Fusicoccum atrovirens is most closely related to *B. mamane*. *Botryosphaeria mamane* was reported recently from hybrid *E. urophylla* × *E. grandis* clones and *A. mangium* in Venezuela, associated with dieback symptoms, but also isolated as an endophyte (Mohali et al. 2007). Before the fungus was reported associated with witches' broom on *Sophora chrysophylla* in Hawaii (Gardner 1997) but there were no authentic cultures and attempts to re-isolate the species were

TABLE III. Statistics for sequence datasets generated and information on maximum parsimony trees for each set of analyses

	Gene region				
	All			*Fusicoccum*	
Clade considered	EF-1α	ITS	Combined	EF-1α	ITS
Total characters in dataset	826	701	1527	742	599
Variable characters	592	473	386	699	540
Parsimony informative	234	228	1141	43	59
Excluded characters	624	504	945	699	559
Analyzed characters	202	197	582	43	40
Number of most parsimonious trees	Unlimited	Unlimited	416	4	2
Tree length	620	497	1602	49	45
Consistency Index (CI)	0.5855	0.5775	0.6398	0.9592	0.9556
Retention Index (RI)	0.9186	0.9141	0.9333	0.9836	0.9688
Rescaled Consistency Index (RC)	0.5378	0.5278	0.5972	0.9435	0.9257
Figure			1	2a	2b

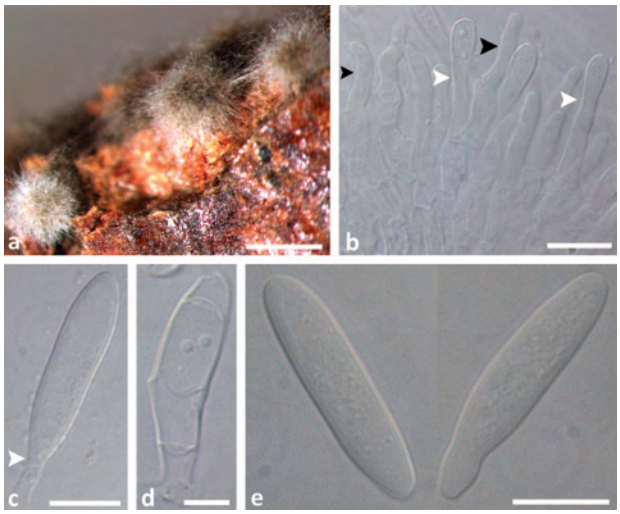

FIG. 6. *Fusicoccum atrovirens*. a. Pycnidia sporulating on a branch of the host (*P. angolensis*). b, c. Conidiogenous cells and immature, developing conidia. d. Mature germinating conidium. e. Mature conidia. White arrows indicate annellations, while black arrows indicate paraphyses. Bars: a = 1000 μm; b, c, e = 10 μm, d = 5 μm.

unsuccessful (Crous et al. 2006). The distinction between the species rests on several SNPs (single nucleotide polymorphisms) and three indels in the 18S rDNA of the small ribosomal subunit and ITS1 regions of *F. atrovirens*. They are also distinct morphologically (as noted in the species description) and occur on different continents on divergent hosts, including in isolated native environments. Despite these distinguishing data, *B. mamane* and *F. atrovirens* have identical sequences in the EF-1α gene region. The reason for this is unclear and further information is needed to clarify.

The four isolates of *D. alatafructa* obtained in this study originated from two locations and were isolated both as endophytes from healthy as well as diseased plant tissue. It is likely that the species originated from other plant hosts in these areas, especially considering that Begoude (2009) reported it from *T. sericea* trees in the Sudwala Caves area, where it was collected together with *Ps. olivaceum*. Despite the diversity of species in the genus, only six species of *Diplodia*, confirmed based on sequence data, are known to occur in the southern hemisphere, including *D. pinea* A morphotype, *D. seriata*, *D. cupressi*, *D. africana*, *D. rosulata* and *D. porosum* (de Wet et al. 2000, van Niekerk et al. 2004, Gure et al. 2005, Alves et al. 2006, Damm et al. 2007, Phillips et al. 2007). Of these six species *D. pinea* and *D. cupressi* have been reported only from gymnosperms, *D. seriata* has been reported from both gymnosperms and angiosperms, and *D. africana*, *D. rosulata* and *D. porosum*, like *D. alatafructa*, are known only from angiosperm hosts (de Wet et al. 2007).

Three *Lasiodiplodia* spp., *L. crassispora*, *L. pseudotheobromae* and *L. theobromae*, were isolated from *P. angolensis* in this study. This was not surprising because *Lasiodiplodia* spp. are known to occur in tropical and subtropical regions where *P. angolensis*

TABLE IV. Comparison of conidial dimensions of new (in boldface) species described in this study with previously described species included in the phylogenetic analyses

Species	Conidial dimensions (μm)	Reference
Pseudofusiccum olivaceum	(17.9–)19.9–25.7(–30.4) × (5.9–)6.3–7.7(–8.9) [Avg. 22.8 × 7.0]	This study
P. violaceum	(26.5–)29.8–36.1(–39.6) × (8.0–)8.7–10.3(–11.6) [Avg. 33.0 × 9.5]	This study
P. adansoniae	(19–)21–24(–26) × (3.5–)4.5–6(–6.5) [Avg. 22.5 × 5.2]	Pavlic et al. 2008
P. ardesiacum	(17.5–)21–29(–32) × (6.3–)7–8(–9) [Avg. 25 × 7.5]	Pavlic et al. 2008
P. kimberleyense	(24–)28–33(–34) × (6.5–)7–8(–8.5) [Avg. 30.7 × 7.4]	Pavlic et al. 2008
P. stromaticum	(19–)20–23(–24) × (4–)5–6 [Avg. 21.5 × 5.5]	Mohali et al. 2006
Diplodia alatafructa	(22.4–)24.6–29.2(–32.9) × (9.3–)11.0–13.8(–15.8) [Avg. 26.9 × 12.4]	This study
D. africana	(17–)25.5–33(–34) × (10–)12–14(–15) [Avg. 29.2 × 13]	Damm et al. 2007
D. cupressi	(21.5–)23.5–28.5(–30.5) × (12.0–)13.5–15.0(–16.0) [Avg. 24.9 × 14.2]	Alves et al. 2006
D. mutila	(23.5–)25.1–25.7(–27.4) × (12.4–)13.2–13.5(–14.3) [Avg. 25.4 × 13.4]	Alves. 2004
D. olivarum	(21.5–)22–27.5(–28.5) × (10–)11–13.5(–14.5) [Avg. 24.4 × 12.4]	Lazzizera et al. 2008
D. pinea	30–45 × 10–16	Punithalingam and Waterston 1970
D. rosulata	(21–)25–32(–36) × (10–)11–17.5(–19.5) [Avg. 28 × 14.5]	Gure et al. 2005
D. scrobiculata	(37.5–)39.5(–41.5) × (13–)14(–15.5)	de Wet et al. 2003
D. seriata	(21.5–)22–27(–28) × (11–)11.5–14.5(–15.5) [Avg. 24.9 × 12.9]	Phillips et al. 2007
Fusicoccum atrovirens	(27.1–)30.9–36.0(–40.3) × (5.7–)7.1–9.9(–11.8) [Avg. 33.5 × 8.5]	This study
Botryosphaeria corticis	(20.5–)23.5–32.5(–34.5) × (5–)5.5–7(–7.5) [Avg. 28.9 × 6.4]	Phillips et al. 2006
B. dothidea	(17–)18–20(–22) × 4–5 [Avg. 19.6 × 4.8]	Slippers et al. 2004
B. mamane	(21–)28–43(–52) × (4–)5–7(–8) [Avg. 35.5 × 6.1]	Mohali et al. 2007
F. ramosum	(11–)12–15(–16) × (4.7–)5–6(–7) [Avg. 13.4 × 5.7]	Pavlic et al. 2008

FIG. 7. Lesions produced on inoculated *P. angolensis* branches in the field inoculations. From left to right: control inoculation, *Pseudofusicoccum violaceum*, *P. olivaceum*, *Diplodia alatafructa*, *Lasiodiplodia pseudotheobromae*, *L. theobromae* and *L. crassispora*. Incisions in the branch mark the edge of visible lesions and are indicated by arrows.

also occurs. All three species are known to be plurivorous with *L. theobromae* reported from both gymnosperms and angiosperms and the remaining species known only from angiosperm hosts. Both *L. pseudotheobromae* and *L. theobromae* have a cosmopolitan distribution and have been reported from the tropics in both the northern and southern hemispheres (Alves et al. 2008). However *L. crassispora* appears restricted to the southern hemisphere (Burgess et al. 2006, Begoude 2009) and results of this study add weight to that view. It is thus likely that many if not most woody plant species in tropical regions are hosts of these common and often pathogenic *Lasiodiplodia* spp.

In the pathogenicity trials both isolates of *L. pseudotheobromae* and one isolate of *D. alatafructa* differed significantly from the control inoculations. Although some of the control inoculations resulted in lesions, only saprophytes that were absent from experimental inoculations could be isolated from these lesions. Differences between the means of both trials in the controls are thought to be due to seasonal variation; the first trial was conducted toward the end of summer and the beginning of fall and the second toward the beginning of spring. In the isolations *L. pseudotheobromae* was the only species for which isolates were obtained from both external stem wounds as well as from internal asymptomatic tissue. The other species obtained were either all isolated externally from stem wounds (*L. theobromae*, *L. crassispora*) and diseased tissue (*D. alatafructa*) or internally from asymptomatic branches (*F. atrovirens*, *P. olivaceum*, *P. violaceum*). The virulence of the isolates of *L. pseudotheobromae* along with results of other similar studies (Begoude 2009, Begoude et al. 2009) suggests that the species can exist as both a latent pathogen within *P. angolensis* and could play a role in increasing the likelihood of tree dieback and death when trees are stressed. The single isolate of *D. alatafructa* with a high level of virulence in both trials was obtained from diseased tissue, specifically from the stem of a dying tree, while the other isolate used in the trials was from a stem wound. The isolates of *D. alatafructa* were obtained from geographically distinct areas, and genotypic differences were observed in the sequences obtained, indicating that the two are different strains with different levels of aggressiveness. This is not surprising because it is well known that different isolates of Botryosphaeriaceae can differ in their aggressiveness (Old et al. 1986, Pavlic et al. 2007, Stanosz et al. 2007, Mohali et al. 2009).

The results of this study are similar to those of Taylor et al. (2009) in species diversity and their pathogenicity in a native system. They found seven species of which four were new. Of these only isolates of *Neofusicoccum australe* were significantly different from the control inoculations while the rest produced no significant lesions compared to the controls. Similarly in this study seven species were found of which four are newly described. Of these only isolates of *D. alatafructa* and *L. pseudotheobromae* produced significant lesions while the rest were insignificant when compared to the control. From this comparison, as well as other similar studies (Pavlic et al. 2007; Begoude et al. 2010a, b; Pérez et al. 2010), we conclude that native environments are likely to contain large numbers of species of the Botryosphaeriaceae of which only one or two will be pathogenic. The role of the nonpathogenic species as endophytes remains unclear.

This study was the first to consider the role of the Botryosphaeriaceae in the decline and dieback of *P. angolensis* trees. Some of the seven species of the Botryosphaeriaceae identified in this study are clearly endophytes in this tree. These include species with both hyaline (*F. atrovirens*, *P. olivaceum*, *P. violaceum*) and pigmented (*L. pseudotheobromae*) conidia. Isolates of only two species were pathogenic in field trials. Nevertheless isolation of the species identified in this study from dead and dying trees and from stem wounds suggested that they could contribute to the overall decline of *P. angolensis* trees in South Africa but they are probably not primary agents of disease. It is likely, given that the Botryosphaeriaceae are associated with trees under stress (Slippers and Wingfield 2007), that the decline of trees observed in South Africa is the result of environmental stresses. The diversity of the Botryosphaeriaceae associated

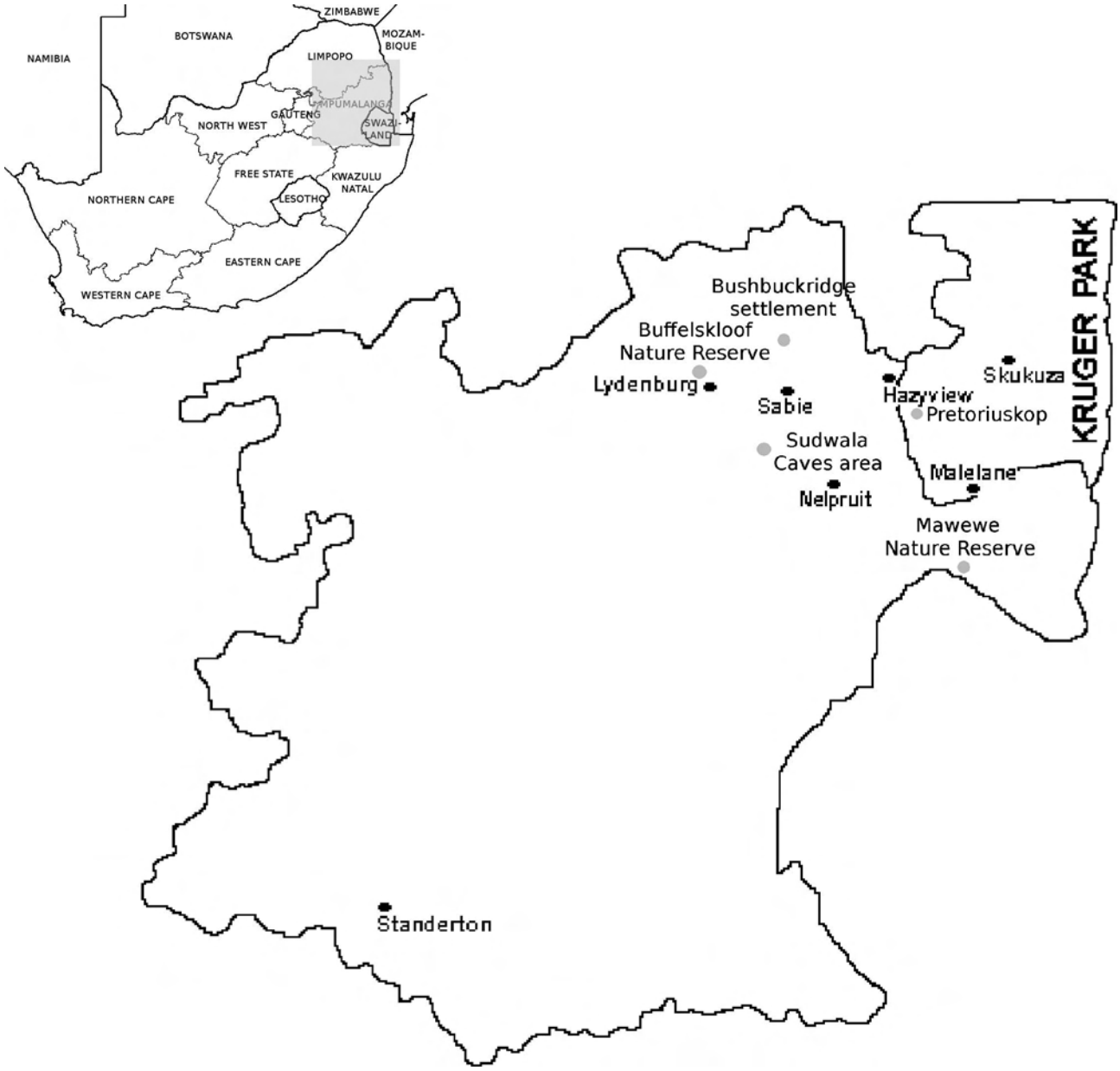

FIG. 8. Sites sampled. The gray area on the South African map indicates Mpumalanga Province where samples were collected. Sampling sites on the larger map of Mpumalanga Province are denoted by a gray dot with sample sizes (n), fungal species and distribution fungal distributions obtained indicated (South Africa map source: http://upload.wikimedia.org/wikipedia/commons/thumb/b/bc/Map_of_South_Africa_with_Mpumalanga_highlighted.svg/763px-Map_of_South_Africa_with_Mpumalanga_highlighted.svg.png, accessed 27/7/2009, Mpumalanga Province map source: http://www.stayinsa.co.za/southafrica/mpumalanga_hotels.html, accessed 27/7/2009).

with *P. angolensis* trees in neighboring countries and the association of this group of fungi with Mukwa disease in Zambia and Zimbabwe merit further study.

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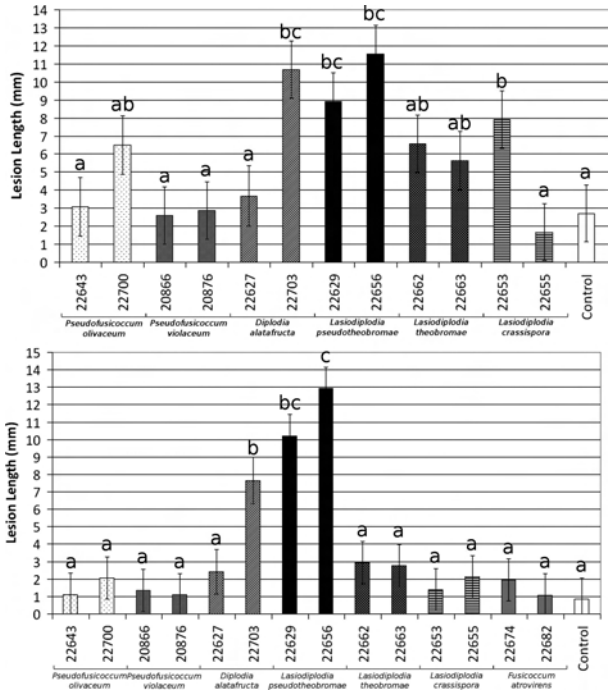

FIG. 9. Mean lesion lengths (mm) resulting from inoculation of *P. angolensis* branches with two isolates of each species of the Botryosphaeriaceae identified in this study and a control after 6 wk. Bars = 95% confidence limits for each isolate.

South Africa) for providing help and advice with the statistical analyses, Prof. Coert Geldenhuys who initially reported the disease situation and provided valuable advice in the course of this study, and Prof. Julia Kerrigan and two anonymous reviewers for their assistance and advice in reviewing this manuscript.

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