Botryosphaeriaceae Species Associated With Cankers and Dieback Symptoms of *Acacia mangium* and *Pinus caribaea* var. *hondurensis* in Venezuela

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Abstract

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Several species in the Botryosphaeriaceae family cause wood stain, cankers, and dieback of trunks and branches in a wide range of forest tree species. The aim of this study was to characterize the botryosphaeriaceous fungi associated with decline symptoms observed in *Acacia mangium* and *Pinus caribaea* var. *hondurensis*, two economically important forest tree species grown in commercial plantations in Venezuela. Fungi isolated from symptomatic samples collected from both hosts in commercial sites were identified based on their morphology and DNA sequences of the internal transcribed spacer region (ITS1-5.8S-ITS2) and part of the β -tubulin and translation elongation factor 1- α genes. *Lasiodiplodia theobromae* and *L. venezuelensis* were routinely isolated from *A. mangium* and *P. caribaea* var. *hondurensis*. Additionally, the novel species *Diplodia guayanensis* was isolated and

characterized from symptomatic and asymptomatic tissues of *A. mangium*. Multigene phylogenetic analyses along with restriction fragment length polymorphism studies further supported the identification of these species. A pathogenicity study was conducted under natural conditions and 12 weeks after inoculation all Botryosphaeriaceae spp. were shown to be highly virulent on *A. mangium*. Contrary, no lesions were observed in the wood of *P. caribaea* var. *hondurensis* when inoculated with *L. theobromae* and *L. venezuelensis*. However, both species were consistently reisolated from the asymptomatic tissue beyond the inoculation point. This study contributes to a better understanding of the role that species in the Botryosphaeriaceae play on disease symptoms and dieback of *A. mangium* and *P. caribaea* var. *hondurensis* from plantations in eastern Venezuela.

Commercial forest plantations in Venezuela are primarily located in the eastern part of the country, where over 615,000 ha have been planted with the species *Pinus caribaea* var. *hondurensis* (Sénécl.) W.H.G. Barret & Golfari since the mid-1970s. *P. caribaea* is of significant importance in Venezuela and plantations with this host provide more than 50% of the country's total round-wood production (ITTO 2005). *Acacia mangium* Willd. plantations were introduced in the early 1990s in Venezuela and occupy an area of approximately 120,000 ha (Briceño 2005). The primary purpose of these plantations is the production of wood and pulp, the latter being mainly used in the fabrication of paper, cardboard, and fiber agglomerates.

Botryosphaeriaceae Theiss. & P. Syd. is a genus-rich family in the Dothideomycetes O. E. Erikss. & Winka that includes endophytic, parasitic, and saprophytic species with a cosmopolitan distribution (Slippers and Wingfield 2007). Species in the Botryosphaeriaceae are plurivorous and several taxa from different genera are well-known pathogens of woody perennial crops and forest tree species causing a broad range of disease symptoms (Slippers et al. 2004; Úrbez-Torres 2011). Among these symptoms, perennial cankers in the vascular system are commonly observed and are probably the most important since they can kill large parts of the plant causing a rapid dieback, which often results in the eventual death of the host. Botryosphaeriaceae spp. are known to occur on a variety of forest tree species

worldwide and are of particular importance on *Acacia* and *Pinus* spp. (Jami et al. 2015; Mohali et al. 2007; Roux and Wingfield 1997). For instance, at least 18 Botryosphaeriaceae spp. have been described from native and nonnative *Acacia* spp. in South Africa with 13 of them occurring just in one host, *A. karroo* (Hayne) Banfi & Galasso, the most commonly occurring native tree in South Africa (Jami et al. 2012, 2015). Similarly, several botryosphaeriaceous taxa are known to cause cankers, tip blight, and shoot dieback in several *Pinus* spp. (Mohali et al. 2007; Swart and Wingfield 1991). Among them, *Diplodia sapinea* (Fr.) Fuckel and *D. scrobiculata* J. de Wet, Slippers & M.J. Wingf. are well-known latent pathogens occurring in native and introduced *Pinus* spp. in the Northern and Southern Hemispheres, respectively (Bihon et al. 2011; Burgess et al. 2004).

Members of the Botryosphaeriaceae, including *Diplodia mutila* (Fr.) Mont., *D. sapinea*, *Botryosphaeria dothidea* (Moug. ex Fr.) Ces. & De Not., and *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. have long been known to occur in Venezuela, where they are commonly isolated from forest tree plantations and agriculture crops showing dieback symptoms (Cedeño et al. 1994; Mohali 1997; Mohali and Encinas 2001; Mohali et al. 2002, 2007, 2009). During the last decade, the introduction of DNA-based molecular identification approaches have significantly contributed to the characterization of other Botryosphaeriaceae spp. associated with cankers and vascular staining of *Eucalyptus*, *Acacia*, and *Pinus* spp. in commercial forest plantations of Venezuela, including *Cophinforma mamane* (D.E. Gardner) A.J.L. Phillips & A. Alves, *Neofusicoccum andinum* (Mohali, Slippers & M.J. Wingf.) Mohali, Slippers & M.J. Wingf., *N. parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips, *N. ribis* (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips, *L. crassispora* T. Burgess & Barber, *L. pseudotheobromae* A.J.L. Phillips, A. Alves & Crous, *L. venezuelensis* T. Burgess, Barber & Mohali, and *Pseudofusicoccum stromaticum* (Mohali, Slippers & M.J. Wingf.) Mohali, Slippers & M.J. Wingf. (Burgess et al. 2006; Castro-Medina et al. 2014; Mohali et al. 2006, 2007). However, these species have been primarily found in forest plantations located in central western states

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and in “Los Andes Cordillera” of Venezuela. Therefore, the aim of the present study is to further investigate the status of Botryosphaeriaceae spp. associated with decline symptoms observed in *A. mangium* and *P. caribaea* var. *hondurensis*, the two most prevalent tree species planted in eastern Venezuela, an area with over 700,000 ha of forest tree plantations of high economic value.

Materials and Methods

Sample collection and fungal isolation. Plant material was collected between 2009 and 2010 from forest tree plantations (Maderas del Orinoco C.A.) located in Uverito, Monagas State, Venezuela. Disease symptoms in *A. mangium* were primarily observed in the trunks and were characterized by light-brown cankers with a black exudate occurring in most of the affected trees. The most prevalent disease symptom observed in *P. caribaea* var. *hondurensis* was a blue stain of the wood, which was observed in fallen trees inside the plantations and in trees ready to be processed at the sawmill. In total, 30 *A. mangium* and 20 *P. caribaea* var. *hondurensis* samples from symptomatic trunks and branches were collected. Samples also included asymptomatic wood from both hosts. All samples were taken to the laboratory to perform isolations in order to identify the canker- and blue stain-causing organisms. Samples were first surface sterilized for 1 min in 1.5% sodium hypochlorite and then rinsed twice with sterile distilled water. Small pieces (20 to 25 mm²) of both symptomatic (including the margin between cankered and healthy tissue) and asymptomatic wood tissue were placed on 2% potato dextrose agar (PDA) (Difco, BD Micro Biology Systems, Franklin Lakes, NJ) petri plates supplemented with tetracycline hydrochloride (0.01%) (Sigma-Aldrich, St. Louis, MO) (PDA-tet). Plates were sealed with Parafilm (Pechiney Plastic Packaging, Chicago, IL) and incubated at room temperature (23 ± 2°C) until fungal colonies were observed. Colonies resembling Botryosphaeriaceae spp. (fast growth, white or light green mycelium becoming dark green or olivaceous within few days) were selected and transferred to fresh PDA petri plates. Pure cultures were obtained by hyphal tip excision from the colony margins on PDA. These isolates are maintained in the Department of Plant Pathology fungal collection at the University of California, Davis. Type culture specimens were also deposited in the Centraalbureau voor Schimmelcultures (CBS) fungal collection, Utrecht, The Netherlands.

Morphological characterization. Representative isolates of the different colony types identified as potential Botryosphaeriaceae spp. from pure cultures were transferred to 2% water agar (Difco) petri plates containing double-autoclaved pine needles. Isolates were incubated at 25°C under continuous near-UV light to induce pycnidia formation and sporulation. Conidia morphological characteristics from pycnidia, including cell wall, shape, size, color, and presence of septum were recorded using light microscopy with a Leica DFC Camara Quick Start (Leica Microsystems Imaging Solutions Ltd, Cambridge, U.K.). The length and width of 60 conidia were measured for each taxon and minimum, maximum, mean, standard deviation, and 95% confidence intervals were calculated using JMP Start Statistics software (SAS Institute, Cary, NC).

DNA extraction, PCR amplification, and sequencing. Total genomic DNA from selected Botryosphaeriaceae isolates was extracted from 8- to 10-day-old cultures using the DNeasy Plant Mini Kit (QIAGEN Inc, Valencia, CA). The internal transcribed spacer (ITS) region of the ribosomal RNA operon (ITS1-5.8S-ITS2), and part of the β -tubulin (BT) and translation elongation factor 1- α (TEF) genes were amplified using the ITS4/ITS5, Bt2a/Bt2b, and EF1-688F/EF1-1251R primer sets, respectively (Alves et al. 2008; Glass and Donaldson 1995; White et al. 1990). PCR reactions were carried out in a PTC-100 thermal cycler (MJ Research, Watertown, MA) following the protocol and temperature profiles previously described (Alves et al. 2008; Úrbez-Torres et al. 2008). PCR products were separated by electrophoresis in 1.5% agarose gels stained with ethidium bromide and visualized under UV light. PCR amplicons were purified using the QIAquick PCR purification kit (QIAGEN Inc) and both forward and reverse strands of the ITS, BT, and TEF were sequenced using an ABI Prism 377 DNA Sequencer (Perkin-Elmer, Norwalk, CT) in the sequencing facility at the Division of Biological Sciences, University

of California, Davis. Sequences were manually edited and assembled using Sequencher version 4.1 (Gene Codes, Ann Arbor, MI). Consensus sequences were BLASTed in GenBank to confirm species identity of each isolate and to identify homologous sequences in the database. Representative DNA sequences from each species identified in this study were deposited in GenBank.

Phylogenetic analyses. Fungal sequences were aligned using ClustalW multiple alignment program (Thompson et al. 1994). Manual adjustments of sequence alignment were carried out using BioEdit Sequence Alignment Editor Version 7.0.8 (Tom Hall). GenBank sequences from the different genera including ex-type specimens when possible were selected based on their high similarity with our query sequences using Mega BLAST (Table 1). A multilocus phylogenetic analysis was performed on the concatenated ITS, BT, and TEF datasets using maximum parsimony (MP) with the heuristic search option and the tree-bisection-reconnection (TBR) algorithm in MEGA6 (Tamura et al. 2013). Tree length, consistency index (CI), retention index (RI), and rescaled consistency index (RC) were calculated. The combined ITS, BT, and TEF dataset was further analyzed using the maximum composite likelihood (MCL) method based on the Tamura-Nei model. Tree topology with superior log likelihood value was selected. Bootstrap support was estimated using 1,000 replicates to assess the robustness of each clade in both MP and MLC phylogenetic methods.

PCR-RFLP analysis. A PCR-RFLP fingerprinting technique was applied to identify and group isolates of Botryosphaeriaceae from *A. mangium* and *P. caribaea* var. *hondurensis* that were not sequenced. TEF region sequence data were analyzed using Webcutter 2.0 (<http://rna.lundberg.gu.se/cutter2/>) to identify polymorphisms of restriction enzyme sites among the different Botryosphaeriaceae spp. Part of the TEF locus was amplified from all isolates using primers EF1-688F/EF1-1251R and amplicons were digested with the restriction enzyme (RE) *Cfo*I. The RFLP reaction mixture consisted of 20 μ l RFLP of the PCR product, 0.30 μ l RE, and 2.5 μ l of the matching enzyme buffers. The mixture was incubated at 37°C overnight and the restriction fragments separated on 3% agarose gels (Chen et al. 2011). A standard 100 bp molecular marker was used to estimate the fragment size and gels were visualized as previously described.

Pathogenicity studies. A pathogenicity study was conducted under natural conditions using standing trees located in a commercial plantation (Maderas del Orinoco C.A.) in Monagas State, Venezuela. Field inoculations were carried out using two different isolates of each species isolated from symptomatic wood and identified by morphological and molecular studies (Table 1). The pathogenicity study was performed by inoculating the trunks of 2-year-old *A. mangium* and *P. caribaea* var. *hondurensis* trees 150 cm above the soil level. A 5-mm-diameter sterile metal cork borer was used to remove the bark and to expose the cambium layer. Five millimeter diameter mycelial discs from the growing margin of 8-day-old colonies were placed into an exposed wound with the mycelium facing the cambium. Inoculated wounds were sealed with Parafilm to protect them from desiccation and contamination. Ten trees per isolate (60 *A. mangium* and 40 *P. caribaea* var. *hondurensis*) were inoculated and 10 control trees per host were inoculated with noncolonized PDA plugs using the same technique. After 12 weeks, lesion length produced in the cambium from the inoculation point was measured to obtain an indication of the virulence of the isolates. Reisolation was done from the margins of the necrotic lesions as previously described and reisolated fungi were identified by comparing colony and conidial morphological characteristics to the original inoculated isolate. Differences in vascular discoloration length among isolates were determined using one-way analysis of variance (ANOVA) in SAS v. 8.1 (SAS Institute 2005). Treatment means were compared using Tukey's test at the 5% significant level.

Results

Sample collection, fungal isolation, and morphological characterization. Seventy-five and 70 Botryosphaeriaceae-like isolates were obtained from either symptomatic or asymptomatic wood from *A. mangium* and *P. caribaea* var. *hondurensis*, respectively. Botryosphaeriaceae isolates from commercial plantations were first

separated into two groups based on their appearance in culture and conidial morphology. The first group of isolates produced white mycelium, becoming gray to dark gray with age. Conidia from pycnidia developed on pine needles were ovoid, thick-walled, hyaline, and unicellular first, and turning dark brown with one-septum and longitudinal striations as they aged. The second group of isolates was initially characterized by a relatively slow growth white mycelium that became olivaceous in the center. Conidia from pycnidia were ovoid with ends broadly rounded, initially hyaline and unicellular, and becoming dark brown with 1 to 4 septa with age. These morphological characters were compared with those reported in previous studies (Phillips et al. 2013; Urbez-Torres et al. 2008) and isolates from group one and two were tentatively identified to belong to the botryosphaeriaceous genera *Lasiodiplodia* Ellis & Everh. and *Diplodia* Fr., respectively. Measurements taken from different isolates in the *Diplodia* group showed conidia size to be larger than the rest of the *Diplodia* species described in the literature (Table 2). Additionally, these conidia presented up to four septa, which has not been observed in any other *Diplodia* sp. While *Lasiodiplodia* and *Diplodia* isolates were identified from *A. mangium*, only *Lasiodiplodia* isolates were found in *P. caribaea* var. *hondurensis*.

DNA sequencing and phylogenetic analyses. DNA sequences and multilocus phylogenetic analyses allowed the identification of three different species in this study, including *L. theobromae*, *L. venezuelensis*, and a distinct *Diplodia* sp. that did not group with any described

Diplodia sp. from GenBank. The combined ITS, TEF, and BT phylogenetic analysis included 35 taxa with a total of 1,300 positions in the final dataset. MP analysis resulted in seven most parsimonious trees of which one is shown in Figure 1 (TL = 702, CI = 0.676525, RI = 0.819170, RC = 0.667511). *L. theobromae* and *L. venezuelensis* isolates from *A. mangium* and *P. caribaea* var. *hondurensis* from Venezuela grouped in two separate well-supported clades (bootstrap value >98%). *Diplodia* isolates CBS 129749, CBS 129750, and CBS129760 from *A. mangium* from Venezuela fell into a distinct well-supported clade (bootstrap value 97%) and did not group with any known *Diplodia* sp. currently available in GenBank. These isolates were phylogenetically closely related to *D. scrobiculata* (Fig. 1).

PCR-RFLP analysis. A total of 66 Botryosphaeriaceae isolates that were not included in the phylogenetic study were identified by generating restriction maps using the RE *CfoI* (Fig. 2A and B). PCR reactions using primers EF1-688F and EF1-1251R produced single amplicons between 246 and 262 bp. *CfoI*-digested amplicons produced four distinct profiles (Fig. 2A). Twenty-eight isolates represented *L. theobromae* (five from *A. mangium* and 23 from *P. caribaea* var. *hondurensis*), 16 isolates represented *L. venezuelensis* (seven from *A. mangium* and nine from *P. caribaea* var. *hondurensis*), seven isolates represented the previously identified species from *A. mangium*, *L. pseudotheobromae* (Castro-Medina et al. 2014), and 15 isolates represented a distinct *Diplodia* sp. from *A. mangium* (*D. guayanensis*). TEF sequence data from *D. guayanensis* from Venezuela showed

Table 1. Botryosphaeriaceae spp. included in this study for morphological studies, phylogenetic analyses, and pathogenicity tests

Species	Isolate ^a	Host/Origin/Collector	GenBank accession number ^b		
			ITS	TEF	BT
<i>Diplodia africana</i>	CBS120853	<i>Prunus persica</i> /South Africa/U. Damm	EF445343	EF445382	KF766129
<i>D. agrifolia</i>	CBS132777	<i>Quercus agrifolia</i> /U.S.A./S. C. Lynch & A. Eskalen	JN693507	JQ517317	JQ411459
<i>D. alloeocellula</i>	CBS130408	<i>Acacia karroo</i> /South Africa/F. Jami & M. Gyzenhout	JQ239397	JQ239384	JQ239380
<i>D. corticola</i>	CBS112549	<i>Q. suber</i> /Portugal/A. Alves	AY259100	AY573227	DQ458853
<i>D. cupressi</i>	CBS168.87	<i>Cupressus sempervirens</i> /Israel/Z. Solel	DQ458893	DQ458878	DQ458861
<i>D. guayanensis</i>	CBS129749 ^c	<i>A. mangium</i> /Venezuela/F. Castro-Medina & S. R. Mohali	<i>JX545106</i>	<i>JX545126</i>	<i>JX545146</i>
<i>D. guayanensis</i>	CBS129750^c	<i>A. mangium</i> /Venezuela/F. Castro-Medina & S. R. Mohali	<i>JX545108</i>	<i>JX545128</i>	<i>JX545148</i>
<i>D. guayanensis</i>	CBS129760	<i>A. mangium</i> /Venezuela/F. Castro-Medina & S. R. Mohali	<i>JX545109</i>	<i>JX545129</i>	<i>JX545149</i>
<i>D. mutila</i>	CBS112553	<i>Vitis vinifera</i> /Portugal/A. J. L. Phillips	AY259093	AY573219	JQ659295
<i>D. olivarum</i>	CBS121887	<i>Olea europaea</i> /Italy/S. Frisullo	EU392302	EU392279	HQ660079
<i>D. sapinea</i> (A)	CBS 393.84	<i>P. nigra</i> /Netherlands/H. A. van der Aa	DQ458895	DQ458880	DQ458863
<i>D. sapinea</i> (C)	CBS 109725	<i>P. patula</i> /South Africa/M. J. Winfield	DQ458896	DQ458881	DQ458864
<i>D. rosulata</i>	CBS116470	<i>P. africana</i> /Ethiopia/A. Gure	EU430265	EU430267	EU673132
<i>D. scrobiculata</i>	CMW 189	<i>P. banksiana</i> /U.S.A./M. A. Palmer	AY253292	AY624253	AY624258
<i>D. scrobiculata</i>	CBS 109944	<i>P. greggii</i> /Mexico/M. J. Wingfield	DQ458899	DQ458884	DQ458867
<i>D. scrobiculata</i>	CBS 113423	<i>P. greggii</i> /Mexico/M. J. Wingfield	DQ458900	DQ458885	DQ458868
<i>D. seriata</i>	CBS112555	<i>V. vinifera</i> /Portugal/A. J. L. Phillips	AY259093	AY573219	DQ458856
<i>D. tsugae</i>	CBS418.64	<i>Tsuga heterophylla</i> /Canada/A. Funk	DQ458888	DQ458873	DQ458855
<i>Lasiodiplodia crassisporea</i>	CBS110492	Unknown/Unknown/Unknown	EF622086	EF622066	EU673134
<i>L. missouriana</i>	CBS128311	<i>V. vinifera</i> /U.S.A./J. R. Urbez-Torres	HQ288225	HQ288267	HQ288304
<i>L. parva</i>	CBS494.78	<i>Cassava-field soil</i> /Colombia/O. Rangel	EF622084	EF622064	EU673114
<i>L. pseudotheobromae</i>	CBS447.62	<i>Citrus aurantium</i> /Suriname/C. Smulders	EF622081	EF622060	EU673112
<i>L. pseudotheobromae</i>	CBS 116459	<i>Gmelina arborea</i> /Costa Rica/J. Carranza-Velásquez	EF622077	EF622057	EU673111
<i>L. pseudotheobromae</i>	CBS129752	<i>A. mangium</i> /Venezuela/F. Castro-Medina & S. R. Mohali	<i>JX545091</i>	<i>JX545111</i>	<i>JX545131</i>
<i>L. pseudotheobromae</i>	UCD-A1	<i>A. mangium</i> /Venezuela/F. Castro-Medina & S. R. Mohali	<i>JX545092</i>	<i>JX545112</i>	<i>JX545132</i>
<i>L. theobromae</i>	CAA006	<i>V. vinifera</i> /U.S.A./T. J. Michailides	DQ458891	DQ458876	DQ458859
<i>L. theobromae</i>	UCD-A28	<i>A. mangium</i> /Venezuela/F. Castro-Medina & S. R. Mohali	<i>JX545095</i>	<i>JX545115</i>	<i>JX545135</i>
<i>L. theobromae</i>	CBS129751 ^c	<i>A. mangium</i> /Venezuela/F. Castro-Medina & S. R. Mohali	<i>JX545096</i>	<i>JX545116</i>	<i>JX545136</i>
<i>L. theobromae</i>	CBS129754 ^c	<i>P. caribaea</i> /Venezuela/F. Castro-Medina & S. R. Mohali	<i>JX545099</i>	<i>JX545119</i>	<i>JX545139</i>
<i>L. venezuelensis</i>	CBS118739	<i>A. mangium</i> /Venezuela/S. R. Mohali	DQ377904	EU673305	EU673129
<i>L. venezuelensis</i>	CBS129753 ^c	<i>A. mangium</i> /Venezuela/F. Castro-Medina & S. R. Mohali	<i>JX545100</i>	<i>JX545120</i>	<i>JX545140</i>
<i>L. venezuelensis</i>	UCD-P1	<i>P. caribaea</i> /Venezuela/F. Castro-Medina & S. R. Mohali	<i>JX545103</i>	<i>JX545123</i>	<i>JX545143</i>
<i>L. venezuelensis</i>	CBS129755 ^c	<i>P. caribaea</i> /Venezuela/F. Castro-Medina & S. R. Mohali	<i>JX545104</i>	<i>JX545124</i>	<i>JX545144</i>
<i>L. venezuelensis</i>	UCD-P13	<i>P. caribaea</i> /Venezuela/F. Castro-Medina & S. R. Mohali	<i>JX545105</i>	<i>JX545125</i>	<i>JX545145</i>
<i>L. viticola</i>	CBS128313	<i>V. vinifera</i> /U.S.A./J. R. Urbez-Torres	HQ288227	HQ288269	HQ288306
<i>Botryosphaeria dothidea</i>	CMW 8000	<i>Prunus</i> sp./Switzerland/B. Slippers	AY23949	AY236898	AY236927
<i>Neofusicoccum parvum</i>	CMW 9081	<i>Populus nigra</i> /New Zealand/G. J. Samuels	AY236943	AY236888	AY236917

^a Isolate numbers in bold represent ex-type specimens.

^b Sequences in italics were generated in this study. ITS: Internal transcribed spacer (ITS1-5.8S-ITS2), TEF: translation elongation factor 1-alpha, BT: β -tubulin.

^c Isolates used in the pathogenicity study.

different restriction patterns with *CfoI* enzyme using the Webcutter 2.0 program when compared with other phylogenetically closely related *Diplodia* spp. such as *D. sapinea* (B), *D. scrobiculata*, and *D. intermedia*, all of which showed the same restriction patterns (Fig. 2B). PCR-RFLP results along with the multilocus phylogenetic analysis and the observations recorded in the morphological study supported *Diplodia* isolates from *A. mangium* from Venezuela to be different from known species, and thus they are newly described in this study.

Taxonomy. *Diplodia guayanensis*. F. Castro-Medina, J. R. Úrbez-Torres, S. R. Mohali & W. D. Gubler sp. nov. MycoBank: 812480 (Fig. 3).

Etymology. The name refers to the Guayana Region in Venezuela where this fungus was first collected.

Conidiomata. Pycnidia dark, submerged in agar or on the surface of pine needles, dark brown to black, covered with mycelium, single, (100-) 150 (-250) μm diameter, ostiole central, circular. Conidiogenous cells hyaline, discrete, spherical to cylindrical, holoblastic with limited proliferation precurrent, or proliferating at same level giving rise to periclinal thickenings. Conidia initially hyaline, becoming pigmented light brown to dark brown with age, unicellular to 1 to 4 septa, clavate to truncate, thick-walled, granular, (33.5-) 40.6 to 42.4 (-56) $\times$ (12-) 15.8 to 16.7 (-18.5), $n = 60$ (av. $\pm$ SD = $41 \pm 1.8 \times 16 \pm 1 \mu\text{m}$, L/W ratio = 2.5).

Culture characteristics. Colonies on PDA with moderately dense mycelium, initially white turning light olive in the center within 8 days, and becoming dark olive with age, colonies with sinuate edges. Optimum temperature for colony growth at 25°C.

Substrate. *Acacia mangium*.

Known distribution. Eastern Venezuela.

Holotype. Venezuela, Monagas, Uverito, isolated from trunk of *A. mangium* showing vascular discoloration of the wood in January 2010, F. Castro-Medina & S. R. Mohali, ex-type culture CBS 129750.

Additional specimens examined. Venezuela, Monagas, Uverito, isolated from *A. mangium* showing vascular discoloration in the trunk in June 2010, F. Castro-Medina & S. R. Mohali, CBS 129749, CBS 129760.

Notes. DNA sequence analyses revealed *D. guayanensis* to be closely related to *D. scrobiculata*. *D. guayanensis* can be distinguished from *D. scrobiculata* for having larger conidial size (42 $\times$ 16.5 μm versus 39 $\times$ 14 μm) (Table 2) and the presence of up to 4 septa (*D. scrobiculata*

conidia mostly aseptate but up to 3 septa when present). *D. guayanensis* differs from *D. sapinea* (Fr.) Fuckel by the presence of septa.

Pathogenicity test. *A. mangium* trees inoculated with Botryosphaeriaceae isolates obtained from this host showed bark swelling around the inoculation points and necrosis of the vascular system below the bark 12 weeks after inoculation (Fig. 4A to C). Additionally, black exudation was observed when the outer bark was removed from inoculation points. *D. guayanensis*, *L. theobromae*, and *L. venezuelensis* isolates on *A. mangium* caused substantial vascular discoloration that ranged between 278.45 (CBS 129753) and 185.55 mm (CBS 129750) (Fig. 5). All three species were successfully reisolated from the lesions observed. On *P. caribaea* var. *hondurensis*, *L. theobromae* and *L. venezuelensis* caused lesions no different than the noncolonized PDA controls 12 weeks after inoculation (Figs. 4E and 5). However, both species were consistently reisolated from asymptomatic tissue approximately 2 cm beyond the necrotic wood observed around the inoculation point. *A. mangium* and *P. caribaea* var. *hondurensis* trees inoculated with noncolonized PDA showed no symptoms and the small necrotic lesions observed around the inoculation point represented wood reactions to the wound as none of the Botryosphaeriaceae spp. were reisolated from them (Fig. 4E).

Discussion

Morphological studies, DNA sequencing and multilocus phylogenetic analyses, RFLP fingerprinting, and pathogenicity studies allowed the identification and characterization of three Botryosphaeriaceae spp. from commercial forest plantations in eastern Venezuela. These species included *L. theobromae* and *L. venezuelensis* from both *A. mangium* and *P. caribaea* var. *hondurensis* and the novel species *D. guayanensis* from *A. mangium*.

Species within the *Lasiodiplodia* genus were the most common botryosphaeriaceous taxa isolated from commercial tree forest plantations in eastern Venezuela and among them, *L. theobromae* was the most prevalent fungus isolated from *A. mangium* trees showing cankers and dieback symptoms and from blue stain of the wood observed in *P. caribaea* var. *hondurensis* fallen trees and in log yards at sawmills. *L. theobromae* is a plurivorous ascomycete primarily found in temperate, tropical, and subtropical regions, where it has been reported to cause cankers, root and fruit rots, and leaf spots in a wide range of annual and perennial hosts, including both native and introduced *Acacia* and *Pinus* spp. (Farr and Rossman 2016; Punithalingam 1980).

Table 2. Comparison of conidial dimensions of *Diplodia guayanensis* against previously described *Diplodia* spp.

Species	Conidial dimensions (μm)	Reference
<i>D. africana</i>	(17-) 29.3 (-34) $\times$ (10-) 13 (-15) ^a [avg. 29.2 $\times$ 13]	Damm et al. 2007
<i>D. agrifolia</i>	(21.5-) 27 (-36.5) $\times$ (12-) 14 (5-18) ^a [avg. 27.7 $\times$ 14.7]	Lynch et al. 2013
<i>D. alatafructa</i>	(22.4-) 24.6 to 29.2 (-32.9) $\times$ (9.3-) 11.0 to 13.8 (-15.8) ^b [avg. 26.9 $\times$ 12.4]	Mehl et al. 2011
<i>D. allocellula</i>	(20-) 23.3 (-30) $\times$ (9-) 11.3 (-14.5) ^a	Jami et al. 2012
<i>D. bulgarica</i>	(22.5-) 25.5 (-28) $\times$ (14.5-) 16.8 (-18.5) ^a [avg. 25.4 $\times$ 16.8]	Phillips et al. 2013
<i>D. corticola</i>	(23.5-) 26 to 34.5 (-46) $\times$ (9-) 12 to 16 (-18.5) ^b [avg. 29.9 $\times$ 13.6]	Alves et al. 2004
<i>D. cupressi</i>	(21.5-) 23.5 to 28.5 (-30.5) $\times$ (12.0-) 13.5 to 15.0 (-16.0) ^b [avg. 24.9 $\times$ 14.2]	Alves et al. 2006
<i>D. fraxini</i>	(26.5-) 29 to 31.5 (-33) $\times$ (11-) 12.5 to 14 (-17.5) ^b [avg. 29.8 $\times$ 13.5]	Alves et al. 2014
<i>D. guayanensis</i>	(33.5-) 40.6 to 42.4 (56) $\times$ (12-) 15.8 to 16.7 (-18.5)^b [avg. 41.5 $\times$ 16]	This study
<i>D. intermedia</i>	(24.6-) 29 to 33.5 (-36.9) $\times$ (10-) 11 to 16 (-17.5) ^b [avg. 30.6 $\times$ 13.3]	Phillips et al. 2013
<i>D. malorum</i>	(24-) 29 (-36) $\times$ (12-) 15.5 (-18.5) ^a [avg. 28.1 $\times$ 14.4]	Phillips et al. 2013
<i>D. mutila</i>	(23.5-) 24.5 to 27 (-27.5) $\times$ (12.5-) 13 to 14 (-14.5) [avg. 25.4 $\times$ 13.4]	Phillips et al. 2013
<i>D. neojuniperi</i>	(17-) 18 to 21 (-22) $\times$ (9-) 10 (-11) ^a	Trakunyingcharoen et al. 2015
<i>D. olivarium</i>	(21.5-) 24.8 (-28.5) $\times$ (10-) 12.3 (-14.5) ^a [avg. 24.4 $\times$ 12.4]	Phillips et al. 2013
<i>D. sapinea</i>	(25.5-) 30.5 to 52.5 (-54) $\times$ (10-) 12.5 to 20 (-21) ^b [avg. 40.8 $\times$ 15.5]	Palmer et al. 1987
<i>D. pseudoseriata</i>	(23-) 25.5 to 26.5 (-30.5) $\times$ (10-) 11.5 to 12 (-14) ^b	Pérez et al. 2010
<i>D. quercivora</i>	(23-) 28 (-30.5) $\times$ (11.5-) 14 (-14.5) ^a [avg. 28.1 $\times$ 13.8]	Linaldeddu et al. 2013
<i>D. rosulata</i>	(21-) 28.5 (-36) $\times$ (10-) 14.3 (-19.5) ^a [avg. 28 $\times$ 14.5]	Gure et al. 2005
<i>D. scrobiculata</i>	(37.5-) 39.5 (-41.5) $\times$ (13-) 14 (-15.5) ^a	de Wet et al. 2003
<i>D. seriata</i>	(21.5-) 24.5 (-28) $\times$ (11-) 13 (-15.5) [avg. 24.9 $\times$ 12.9]	Phillips et al. 2013
<i>D. tsugae</i>	36 to 41 $\times$ 18 to 22 ^c	Phillips et al. 2013

^a Dimensions represent the mode with minimum and maximum in parenthesis.

^b Dimensions represent the lower and upper 95% confidence limits, with minimum and maximum in parenthesis.

^c Dimensions represent the minimum and maximum.

For instance, *L. theobromae* has been associated with symptomatic and/or asymptomatic wood tissues of *A. cincinnata*, *A. crassicarpa*, *A. holosericea*, and *A. mangium* in Brazil (Mendes et al. 1998); *A. mearnsii* and *A. karroo* in South Africa (Jami et al. 2015; Roux and Wingfield 1997); and *A. mangium* in Indonesia (Tarigan 2008; Tarigan et al. 2011). Furthermore, *L. theobromae* has been reported from *P. caribaea* in Brazil (Mendes et al. 1998), Honduras (Richardson

1990), and Nicaragua (Rees 1988); and from *P. elliotii* in Brazil (Maciel et al. 2015) and South Africa (Cilliers et al. 1995; Mohali et al. 2005). *L. theobromae* is widespread in Venezuela and has been associated with disease symptoms in over 100 plant hosts, including economic important horticultural crops such as citrus (*Citrus aurantifolia* and *Citrus × sinensis*) (Cedeño and Palacios-Pru 1992), coffee (*Coffea arabica*), mango (*Mangifera indica*), and papaya

Fig. 1. One of seven equally parsimonious trees resulting from a total of total of 1,300 positions in the final ITS, BT, and TEF combined dataset. Tree length (TL) = 702, consistency index (CI) = 0.676525, retention index (RI) = 0.819170, retention composite index (RC) = 0.667511. Isolates in bold represent Botryosphaeriaceae spp. isolated in this study. Isolates in italics and underline represent ex-type specimens. Bootstrap values with 1,000 replications are shown on branches for maximum parsimony (MP).

(*Carica papaya*) (Farr and Rossman 2016). In Venezuela, *L. theobromae* has long been known to cause shoot blight and dieback of *Pinus oocarpa* Schiede ex Schltdl., blue stain of the wood of Caribbean pine (*P. caribaea*), and it has been isolated from both symptomatic and

asymptomatic tissues of *Eucalyptus urophylla* S.T. Bake $\times$ *E. grandis* Hill ex Maiden hybrids (Cedeño et al. 1996; Mohali et al. 2002, 2005, 2007). Furthermore, and similar to the results obtain in the current study, previous surveys conducted throughout commercial forest

Fig. 2. Restriction fragment length polymorphism analyses. **A**, Agarose gel showing *CfoI* restriction fragments of translation elongation factor 1- α (TEF) PCR products of *Diplodia guayanensis*, *Lasiodiplodia pseudotheobromae*, *L. theobromae*, and *L. venezuelensis* from forest plantations in eastern Venezuela. Asterisks indicate positive controls of each species group. Molecular marker = 1,500 bp. **B**, Restriction maps for the restriction enzyme *CfoI* to the TEF locus of Botryosphaeriaceae spp. from Venezuela and closely related species. Fragments sizes (numbers indicate sizes in bp) were inferred from sequence data.

Fig. 3. *Diplodia guayanensis* isolate CBS 129750 macroscopic and microscopic phenotypic characteristics. **A**, Colony after 21 days on potato dextrose agar in 9-cm petri dish incubated at 20°C. **B**, Pycnidia sporulating on autoclaved pine needles. **C**, Hyaline conidia developing on conideogenous cells. **D**, Unicellular, hyaline, and light-brown conidia. **E**, Mature dark-brown conidia with two and three septa. **F**, Mature conidia with four septa. Pictures of conidia were taken at 40 $\times$. Scale bars: B = 150 μ m; C-E = 15 μ m; F = 5 μ m.

plantations in the western states of Falcon, Portuguesa, and Cojedes showed *L. theobromae* to be the most prevalent fungus isolated from both *A. mangium* and *P. caribaea* var. *hondurensis* (Mohali et al. 2007). Though *L. theobromae* was already known to occur on these hosts in Venezuela, results from the current study are highly important as they show this species to also occur in eastern Venezuela. *L. theobromae* is known to significantly reduce the strength of low density tropical hardwoods and consequently has been shown to reduce wood value by up to 50% (Mohali et al. 2007). Therefore, *L. theobromae* could represent an important threat to wood production in eastern Venezuela, an economically important region with some of the largest commercial forest plantations in the country.

L. venezuelensis was the second most commonly isolated fungus in this study and was found in a similar number of samples collected from either *A. mangium* or *P. caribaea* var. *hondurensis*. *L. venezuelensis* was first described from *A. mangium* living wood tissue in the north-western state of Portuguesa, Venezuela (Burgess et al. 2006). Compared with other *Lasiidiplodia* spp. and primarily to *L. theobromae*, the type species of the genus, *L. venezuelensis* has a restricted distribution as it has not been reported from elsewhere in the world to date. Results from this study show the distribution of *L. venezuelensis* to expand not only to forest plantations of *A. mangium* in eastern Venezuela but also to occur in a new host, *P. caribaea* var. *hondurensis*. *L. pseudotheobromae* was the least abundant Botryosphaeriaceae spp. found in this study and it was confirmed to only occur in *A. mangium* samples by RFLP patterns. *L. pseudotheobromae* was first described by Alves et al. (2008) and discovered as a cryptic species within *L. theobromae* using morphological and molecular data. Since then, this species is shown to have a cosmopolitan distribution and has been reported to cause cankers and dieback in over 70 different hosts (Farr and Rossman 2016), including horticultural crops such as citrus (Abdollahzadeh et al. 2010), coffee (Trakunyingcharoen et al. 2015), grapevines (*Vitis vinifera* L.) (Correia et al. 2016), mango (Marques et al. 2013), and papaya (Netto et al. 2014). Moreover, *L. pseudotheobromae*

has also been associated with native and/or introduced tree species such as *Eucalyptus grandis* in Uruguay (Pérez et al. 2010), English walnut (*Juglans regia*) in China (Li et al. 2016), and *Pterocarpus angolensis* and *Terminalia catappa* in South Africa (Begoude et al. 2010; Mehl et al. 2011). With regard to *Acacia* spp., *L. pseudotheobromae* was first reported from trunk cankers of *A. mangium* in Venezuela and shown to be highly virulent in this host (Castro-Medina et al. 2014). Later, it was found to also occur in healthy wood tissues of *A. karroo* in South Africa, where pathogenicity studies indicated to be weakly virulent in this host (Jami et al. 2015). Results from the

Fig. 5. Mean of vascular discoloration length 12 weeks after inoculation with *Diplodia guayanensis*, *Lasiidiplodia theobromae*, and *L. venezuelensis* in young *Acacia mangium* and *Pinus caribaea* var. *hondurensis* in a commercial plantation under natural conditions using fresh mycelium grown on agar medium. Control treatment was inoculated with noncolonized agar plugs. Mean lesion lengths followed by the same letter are not significantly different according to Tukey's test ($P = 0.05$). Bars represent standard error of the mean.

Fig. 4. Symptoms observed in *Acacia mangium* and *Pinus caribaea* var. *hondurensis* 12 weeks after inoculation. **A**, Bark swelling in *A. mangium* after inoculation with *Diplodia guayanensis* isolate CBS 129750. **B** and **C**, Vascular discoloration and necrotic wood tissue observed upward and downward the point of inoculation caused by *D. guayanensis* on *A. mangium*. **D**, No lesions were observed in *P. caribaea* var. *hondurensis* after inoculation with *L. theobromae* and *L. venezuelensis*. **E**, Control inoculations on *A. mangium* with malt extract agar plugs produced no lesions.

current study suggest *L. pseudotheobromae* to have a broader distribution in forest plantations of *A. mangium* throughout Venezuela and hence, this species may play a more important role on *A. mangium* dieback than originally thought.

Morphological and molecular data obtained in this study supported the description of the novel species *D. guayanensis* from symptomatic wood tissues of *A. mangium* in Venezuela, which increases to 34 the total number of *Diplodia* spp. currently described and with DNA sequence data available in GenBank. *D. guayanensis* was shown to be morphologically and phylogenetically closely related to *D. scrobiculata* and to a lesser extent to *D. sapinea*. Morphologically, *D. guayanensis* presented the largest conidia of all known *Diplodia* spp. Additionally, most conidia from different isolates developed up to four septa, a morphological character not observed yet from any other described *Diplodia* sp. *D. sapinea* and *D. scrobiculata* conidia are mostly aseptate but when septa are present, they rarely formed one or three septa, respectively (Palmer et al. 1987). Though these characters appeared to be robust to discriminate among these species, morphological aspects among species in the Botryosphaeriaceae (primarily conidial size and septation) are known to be quite plastic and overlap depending on host and culture medium and thus, they need to be used with caution (Crous et al. 2006; Phillips et al. 2013). However, conidial size and septation have been widely used along with molecular data to discriminate among botryosphaeriaceous taxa, particularly among *Diplodia* spp. For instance, Lynch et al. (2013) showed that *D. agri-folia* S.C. Lynch & A. Eskalen could be separated from its phylogenetically closely related species *D. mutila* (Fr.) Mont. by its larger and wider conidia. Similarly, *D. allocellula* Jami, Gryzenh, Slippers & M.J. Wingf., morphologically very similar to *D. seriata* De Not., *D. alatrafructa* Mehl & Slippers, and *D. pseudoseriata* C.A. Pérez, Blanchette, Slippers & M.J. Wingf. can be distinguished from this species by having generally smaller conidia (Phillips et al. 2013). Furthermore, the smaller conidia and apparent preference for Rosaceae hosts can be used to discriminate between *D. intermedia* A.J.L. Phillips, J. Lopes & A. Alves and its very closely related species *D. sapinea* (Phillips et al. 2013). Several *Diplodia* spp. and in particular *D. sapinea* and *D. scrobiculata* are well-known to occur as latent pathogens in *Pinus* spp. in different parts of the world (Burgess et al. 2004; de Wet et al. 2003; Punithalingam and Waterson 1970; Swart et al. 1985). Interestingly, *D. guayanensis* was only isolated from *A. mangium* and not from *P. caribaea* var. *hondurensis* in this study. Host specificity could be included as a distinguish feature for *D. guayanensis*; however, further investigations are still needed to support this trait. Despite phenotypical differences, species delimitation within most botryosphaeriaceous genera based solely on cultural and morphological characteristics is challenging. Thus, molecular analyses are nowadays being routinely used for species identification. In the current study, species discrimination was supported not only by morphological characters but also by phylogenetic studies and RFLP analyses. Moreover, RFLP analyses allowed us to determine the dominant Botryosphaeriaceae sp. (*L. theobromae*) occurring in forest plantations in eastern Venezuela. In the case of the novel species *D. guayanensis*, it formed a well-supported distinct clade in the phylogenetic tree and presented distinctive RFLP patterns when compared against its closely related species *D. sapinea* and *D. scrobiculata*. RFLP analyses have long been shown to successfully differentiate botryosphaeriaceous taxa within several genera (Mohali et al. 2007; Slippers et al. 2004). Additionally, RFLP is a rapid, robust, inexpensive, and user-friendly tool that can be used not only for species profiling but also in epidemiological studies to better understand species dominance and distribution.

Pathogenicity studies showed *D. guayanensis*, *L. theobromae*, and *L. venezuelensis* to be highly virulent on *A. mangium*. The current study also showed the presence of *L. pseudotheobromae* in symptomatic *A. mangium* trees in eastern Venezuela but this species was not included in the pathogenicity test as a similar study showed it recently to be very virulent on this host in Venezuela (Castro-Medina et al. 2014). Though *L. venezuelensis* was known to occur in *A. mangium* and *P. caribaea* var. *hondurensis* since mid-2000 in western Venezuela, its pathogenicity was not determined

(Burgess et al. 2006; Mohali et al. 2007). Accordingly, pathogenicity proof is shown for the first time in *A. mangium*, which resulted in *L. venezuelensis* to be the most virulent species among those tested in this study. *L. theobromae* has been known to occur in *A. mangium* in Brazil since the late 1990s (Mendes et al. 1998) and was first reported from this host in Venezuela in 2007 (Mohali et al. 2007). Recently, *L. theobromae* has been reported, along with *Ceratocystis acaciivora* Tarigan & M. van Wyk, as the main cause of death of young *A. mangium* trees in central Sumatra, Indonesia (Tarigan 2008; Tarigan et al. 2011). These latter results are in agreement with the pathogenicity scores obtained in the current study, which showed *L. theobromae* to be also very virulent in *A. mangium* in young trees in Venezuela. Similar to our results, a recent study also showed *L. theobromae* to be the most virulent botryosphaeriaceous species in *A. karroo*, a different species within the same family, in South Africa (Jami et al. 2015). The results obtained in the current study are of significant importance and confirm *L. theobromae* and *L. venezuelensis*, two widely spread species in Venezuela, as a major threat to *A. mangium* plantations in the country. Additionally, the novel species *D. guayanensis* is shown to also play an important role in the health status of this host in Venezuela. Interestingly, both *L. venezuelensis* and *L. theobromae* did not cause lesions when inoculated in *P. caribaea* var. *hondurensis*, but they were routinely reisolated from asymptomatic wood, which may indicate the latent pathogen status of these species in this host. It is well known that several species in the Botryosphaeriaceae family are latent pathogens residing asymptotically in tree hosts for long periods of time until stress conditions turn them in aggressive pathogens (Jami et al. 2013, Slippers and Wingfield 2007). This has long been demonstrated with species such as *B. dothidea*, *D. sapinea*, and *D. scrobiculata* in *Pinus* spp. (Burgess et al. 2004; Flowers et al. 2003; Smith et al. 1996; Stanosz et al. 1995). Recently, Jami et al. (2015) identified 13 Botryosphaeriaceae spp. from healthy tissues of *A. karroo* in South Africa, including *L. theobromae*, which clearly showed this species to occur as a latent pathogen in this host. *L. theobromae* has been reported to occur asymptotically in *Pinus* spp. seedlings in nurseries in Brazil (Maciel et al. 2015) and South Africa (Cilliers et al. 1995), which has been highlighted as a potential source for introduction of infected trees into forest plantations. However, and contrary to what we know in other forest tree hosts, there is still a lack of information about the status of *Lasiodiplodia* spp. as latent pathogens in *Pinus* spp. Forestry is still one of the most important economic engines in Venezuela. However, no studies have been conducted to determine the potential threat that Botryosphaeriaceae spp. may have as latent pathogens within plantations of exotic or introduced forest tree species in Venezuela.

The results of this study show the relevant role that Botryosphaeriaceae spp., including the newly described *D. guayanensis*, play on the health status of the two most important forest tree species grown in commercial plantations in Venezuela and adds newly information on the occurrence of *L. venezuelensis* as latent pathogens in *P. caribaea* var. *hondurensis*. Accordingly, this study provides the foundation to further investigate the impact, biology, and epidemiology of these fungi in forest plantations in Venezuela with the aim to develop and implement effective management strategies.

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