Mycobiota associated with anthracnose and dieback symptoms on *Theobroma cacao* L. in Mérida State, Venezuela

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ABSTRACT

Mohali-Castillo, S.R; Woodward, S.; Klopfenstein, N.B.; Kim, Mee-Sook, Stewart, J.E. Mycobiota associated with anthracnose and dieback symptoms on *Theobroma cacao* L. in Mérida State, Venezuela.***Summa Phytopathologica,*** v.49, p.27-42, 2023.

Diverse fungi collected from symptomatic fruit, stem and branch tissues of *Theobroma cacao* in five *T. cacao*-producing localities or municipalities of Mérida State, Venezuela, were identified using both morphological methods and sequencing of multiple loci (ITS, LSU, SSU, TEF1, BTUB, RPB2). *Cophinforma atrovirens*, *Lasiodiplodia brasiliensis* and *Hypoxylon investiens* are reported for the first time on *T. cacao* in Venezuela. Fungi found in association with fruit anthracnose included *Cophinforma atrovirens, Fusarium solani* and *F. oxysporum*, whereas species associated with dieback or sudden death symptoms include *Cophinforma atrovirens, L. theobromae*, *L. brasiliensis* and *H. investiens*. All of the aforementioned fungi are considered putative pathogens of *T. cacao*, which warrant further pathogenicity tests.

Keywords: *Botryosphaeria*, *Cophinforma*, *Fusarium*, *Hypoxylon, Lasiodiplodia*, fungal genera

RESUMEN

Mohali-Castillo, S.R; Woodward, S.; Klopfenstein, N.B.; Kim, Mee-Sook, Stewart, J.E. Micobiota associada a antracnose e sintomas de morte regressiva em *Theobroma cacao* L. no estado de Mérida, Venezuela. ***Summa Phytopathologica,*** v.49, p.27-42, 2023.

Diversos fungos coletados de tecidos sintomáticos de frutos, caules e ramos de *Theobroma cacao* em cinco localidades ou municípios produtores de *T. cacao* no estado de Mérida, Venezuela, foram identificados usando métodos morfológicos e sequenciamento de múltiplos loci (ITS, LSU, SSU, TEF1, BTUB, RPB2). *Cophinforma atrovirens*, *Lasiodiplodia brasiliensis* e *Hypoxylon investiens* foram relatados pela primeira vez em *T. cacao* na Venezuela. Dentre os fungos encontrados em associação com antracnose nos frutos estavam *Cophinforma atrovirens, Fusarium solani* e *F. oxysporum*, enquanto que espécies associadas a sintomas de morte regressiva ou morte súbita incluem *Cophinforma atrovirens, L. theobromae*, *L. brasiliensis* e *H. investiens*. Todos os fungos mencionados acima são considerados patógenos putativos de *T. cacao*, o que justifica testes de patogenicidade adicionais.

Palabras clave: *Botryosphaeria*, *Cophinforma*, *Fusarium*, *Hypoxylon, Lasiodiplodia*, gêneros fúngicos

RESUMO

Mohali-Castillo, S.R; Woodward, S.; Klopfenstein, N.B.; Kim, Mee-Sook, Stewart, J.E. Micobiota asociada con antracnosis y síntomas de muerte regresiva en *Theobroma cacao* L. en el estado Mérida, Venezuela. ***Summa Phytopathologica,*** v.49, p.27-42, 2023.

Se identificaron diversos hongos recolectados desde tejidos sintomáticos de frutos, tallos y ramas de *Theobroma cacao* en cinco localidades o municipios productores de *T.cacao* en el estado Mérida, Venezuela, utilizando tanto métodos morfológicos como secuenciación de múltiples loci (ITS, LSU, SSU, TEF١, BTUB, RPB٢). *Cophinforma atrovirens, Lasiodiplodia brasiliensis* e *Hypoxylon investiens* son reportados por primera vez en *T. cacao* en Venezuela. Los hongos encontrados en asociación con la antracnosis del fruto incluyeron a *Cophinforma atrovirens, Fusarium solani y F. oxysporum*, mientras que las especies asociadas con síntomas de muerte súbita o muerte regresiva incluyeron a *Cophinforma atrovirens, Lasiodiplodia theobromae, L. brasiliensis, Hypoxylon investiens*. Todos los hongos antes mencionados se consideran patógenos putativos de *T. cacao*, que justifican más pruebas de patogenicidad.

Palavras-chave: *Botryosphaeria*, *Cophinforma*, *Fusarium*, *Hypoxylon, Lasiodiplodia*, Géneros de hongos.

The name given by Linnaeus to the genus of *Theobroma cacao* L. [*theos* (God) + *broma* (beverage) = beverage of the Gods] recognized the Mayan belief that the plant had divine origins (5). Cheesman (4) suggested that *T. cacao* has been cultivated in Mexico and Central America for over 2,000 years and that no truly wild populations were present in this region, indicating that *T. cacao* was introduced into

Central America and Mexico. It has been hypothesized that *T. cacao* was naturally present throughout the Amazon Valley and may have subsequently been dispersed along two routes: one leading north and the other one heading west (42). Domestication of *T. cacao* began during this dispersal process in South America, before migrating indigenous human populations spread the seed to Central America and southern Mexico (42). Molecular analyses, combined with the hypotheses of Cheesman (4) and Schultes (42), uphold the theories that *T. cacao* originated in South America and was later introduced to Central America by human activities (26).

Historical records on the origin of *T. cacao* in Venezuela indicate wild *T. cacao* trees were first recorded in Aug 5, 1602, and highlight the discovery of 100,000 *T. cacao* trees close to Maracaibo Lake in western Venezuela (39). Subsequently, propagation of *T. cacao* occurred throughout Venezuela between 1600 and 1700, spreading the cultivar 'sweet cacao' or 'Criollo' (= 'Creole') (39).

The oldest recorded disease affecting *T. cacao* worldwide was documented in Venezuela in the mid-1630s on the "haciendas" upwind of La Guaira, where 'blight' (alhorra) had destroyed over half of all *T. cacao* on the coast within a decade (7, 49). In 1824, the high-yielding *T. cacao* cultivar 'Forastero Trinitario' was introduced to Venezuela. The longer fermentation period required for beans of 'Forastero Trinitario' negatively influenced flavor, resulting in cacao of a lower quality compared to the 'Criollo' cultivar (39, 49). The 'Forastero Trinitario' cultivar was rapidly distributed westward from the Paria peninsula to Barlovento and Tuy, where *T. cacao* 'Criollo' had practically disappeared because of disease pressures. Later, 'Forastero Trinitario' was transported into central Venezuela, but not to the western states of the country (34).

Information on diseases affecting *T. cacao* fruits and trees in Venezuela is typically sparse and dispersed in technical reports or meeting proceedings, and the fungi are mostly described using only morphology. Among the economically most important pathogens on *T. cacao* are *Lasiodiplodia* sp., causing pod rot and dieback in Ghana, Cameroon, Cuba, Ecuador and Venezuela (14, 22, 39, 47), and *Fusarium* sp., as pathogens, endophytes and/or antagonists against other fungal pathogens of plants (12, 22, 39).

Though several fungal diseases have been reported attacking *T. cacao* in Venezuela, little research has been conducted to detect and identify the causal agents since the advent of molecular technologies. For closely related fungi, DNA-based analyses coupled with phylogenetic methods can be used to separate cryptic species. The aim of the present study is to use a combination of morphological and molecular techniques to determine the diversity of fungi associated with anthracnose and dieback of *T. cacao* in five important production localities of Mérida State, Venezuela.

MATERIALS AND METHODS

Isolation of putative fungal pathogens

Stems, branches and fruits with symptoms of diseases, including dieback, sudden death and anthracnose, were collected from *T. cacao* from five municipalities in Mérida State (Table 1, Figure 1). Samples were transported to the laboratory and initially checked for the presence of fungal fruiting bodies; conidia and single spore isolations were performed as previously described (37). For additional fungal isolations, small pieces of stem, branch or fruit tissues (5 mm^2) were excised from the margins of necrotic lesions. Tissue pieces were surface disinfested for 30 s in 70% ethanol and 60 s in 0.5% sodium hypochlorite (NaOCl-bleach); then, they were washed in three changes of sterile water for 60 s each and dried on sterile filter paper. Surface-disinfested samples were plated onto 2% malt extract agar (MEA; Difco Laboratories, Detroit, MI, USA) and incubated at 25 °C for 7 days or until mycelia were observed growing from the plant tissues. Emerging fungal mycelium was sub-cultured to fresh MEA and hyphal tip methods were used to obtain pure cultures.

Mycelial plugs (5-mm) were taken from the periphery of actively growing cultures and transferred to 9-cm Petri dishes containing potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA), MEA or synthetic nutrient-poor agar medium (SNA, 28). Following incubation at room temperature (25 °C) for 7 days, colony characteristics and pigment production were noted; colony diameters were measured after 7- to 10-day growth. Conidia produced on PDA and MEA were mounted in lacto-phenol for measurement of dimensions and morphological analyses.

DNA extraction and sequencing

Total DNA was extracted from 7-day-old cultures, grown on half-strength PDA amended with 100 mg/L streptomycin sulphate (Sigma-Aldrich, USA). Isolates were sub-cultured to sterilized MF-47-mm Millipore membrane filters (Millipore Sigma, Burlington, MA, USA)

Table 1. Municipalities and localities within Mérida State, Venezuela, where fungal samples were collected from *Theobroma cacao* showing different disease symptoms.

Municipality	Capital	Locality	Symptom
Alberto Adriani	El Vigía	Kilometro 15	Dieback or sudden death; canker in the tree
			Anthracnose on fruits
Andrés Bello	La Azulita	La Azulita	Dieback or Sudden death
		La Pueblita	
Antonio Pinto Salinas	Santa Cruz de Mora	San Rafael	Dieback or sudden death
		Los Bocadillos San Miguel	
		Mesa de las Palmas	
		Mesa Bolivar	
Obispo Ramos de Lora	Santa Elena de Arenales	Eloy Paredes-Las Honduras Sector	Dieback or sudden death
Sucre	Lagunillas	Estanques-Santo Domingo Sector	Anthracnose on fruits
		San juan de Lagunillas-Instituto Nacional de	
		Investigaciones Agrícolas (INIA) Sector	Dieback or sudden death

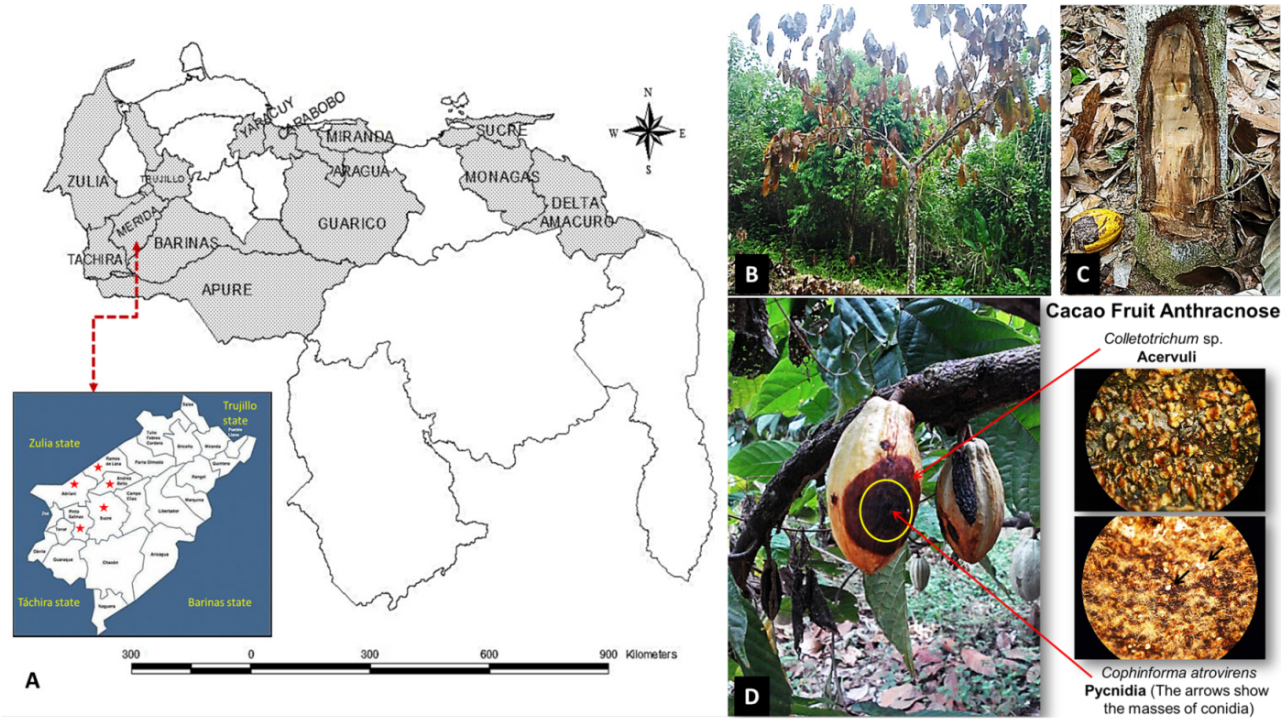

Figure 1. (**A**) Sites of sampling (red stars) for *Theobroma cacao* in the five municipalities in Mérida State, Venezuela; map in grey scale shows additional *T. cacao*-producing states in Venezuela (55); (**B**, **C**) Dieback or sudden death symptoms; (**D**) Anthracnose on *T. cacao* fruits.

overlaid on the surface of the half-strength PDA and incubated at 25 ˚C. DNA was extracted from cultured mycelia using the ZR Fungal/Bacterial DNA MiniPrep (Zymo Research, Irvine, CA, USA), following the manufacturer's protocol. Polymerase chain reactions (PCR) were performed in a reaction mixture comprising 30 ng template genomic DNA, 2.5 µL10 standard *Taq* reaction buffer (New England BioLabs; NEB, Ipswich, MA, USA), 0.5 µL10 mM dNTP (Roche Applied Science, Penzberg, Germany), 1 µL of each 10 µM primer, 0.125 µL (0.6 units) *Taq* DNA polymerase (NEB), and sterile deionized water to a total volume of 25 µL. PCR products were cleaned with ExoSAP-IT® PCR Product Cleanup (Thermo Fisher Scientific, Grand Island, NY, USA) and sequenced at Eurofins Scientific (www.eurofinsus.com).

Six loci were used for DNA-sequence analyses of each isolate: 5.8S rDNA with the two flanking internal transcribed spacers (ITS), a portion of the nuclear ribosomal 18S rRNA gene (small subunit; SSU), a portion of the nuclear rRNA cluster comprising the ITS region plus the D1/D2 variable domains of the ribosomal 28S rRNA gene (large subunit; LSU), and partial sequences of translation elongation factor 1-α (TEF1), β-tubulin (BTUB) and RNA polymerase II second largest subunit (RPB2) genes. Primer sets for amplification included 1) ITS-1F (8) and ITS4 (48) for ITS (*Cophinforma*, *Hypoxylon* and *Lasiodiplodia*); 2) NS1 and NS4 (48) for SSU (*Cophinforma*); 3) ITS1 (48) and NL4 (29) for LSU (*Cophinforma*); 4) EF1-688 and EF1-1252 (1) for TEF1 (*Cophinforma* and *Lasiodiplodia*), EF-1 and EF-2 (31) for TEF1 (*Fusarium*); 5) Bt2a and Bt2b (10) for BTUB (*Cophinforma* and *Lasiodiplodia*); T1 and T22 (30) for BTUB (*Hypoxylon*), and 6) 5F2 and 7eR (21) for RPB2 (*Fusarium*). PCR thermal cycle programs were performed as previously described (8, 21, 29, 30, 31, 48).

Phylogenetic analyses

Sequence data from closely related species for phylogenetic analyses were obtained from GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence alignments, including manual adjustments and concatenation of loci, were conducted using MAFFT (17) within Geneious Pro v. 9.0.5.

Phylogenetic analyses compared DNA sequences of isolates from the current study with sequences from closely related taxa. Phylogenies were estimated for each locus separately and, if necessary, multiple loci were assessed together for each genus. Maximum likelihood (ML) and Bayesian inference (BI) algorithms were used to reconstruct phylogenies for alignments using PhyML (11) and MrBayes v. 3.2.1 (41) in Geneious v6.1.3 (Biomatters Inc.). A best-fit substitution model for each dataset was selected using the Bayesian Information Criterion (BIC) implemented in DT ModSel (24): 1) *Cophinforma* - K80+G for ITS, TIM+G for SSU, K80 for LSU, HKY+I for TEF1, TVMef+I+G for BTUB; 2) *Fusarium* - GTR+I+G for TEF1, TrNef+I+G for RPB2; 3) *Hypoxylon* - TVM+I+G for ITS+BTUB, and 4) *Lasiodiplodia* - TrN+I+G for ITS+BTUB+TEF1.

For ML analyses within PhyML, phylogenies were run with 1,000 bootstrap pseudo-replicates. Bayesian Markov Chain Monte Carlo analyses were carried out using MrBayes (15). Six chains were run for 2,000,000 generations and trees sampled every 100 generations. The first 25,000 trees were discarded as burn-in and the remaining trees used for estimating posterior probabilities (PP) for the majority rule consensus tree.

Phylogenies for multiple loci datasets were estimated using Bayesian analyses implemented in Bayesian Evolutionary Analysis Sampling Trees (BEAST) (6). BEAST does not use concatenation but rather co-estimates the individual gene trees embedded inside the summary species tree. Bayesian Evolutionary Analysis Utility (BEAUti), version 1.7.5, was used to create XML-formatted input files for BEAST v1.7.5. In BEAST, a Markov Chain Monte Carlo algorithm was used to sample the posterior distribution of trees by conducting five independent runs of 100 million generations, each using a constant

size tree prior, strict molecular clock, and uniform priors. Trees were sampled every 1,000 generations and the first 20% discarded as burn-in. Post burn-in trees were combined with the program LogCombiner (BEAST v1.7.5); chains were assumed to converge when the average standard deviation of split frequencies was < 0.01. The maximum clade credibility tree with PP of each node was computed with Tree Annotator (BEAST v 1.7.5). Log files and tree files were visualized in Tracer v1.5 (http://tree.bio.ed.ac.uk/software/tracer/) and FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/), respectively.

RESULTS AND DISCUSSION

Morphological characterization

Morphological characteristics were evaluated to separate and group the fungi at the genus level. Three genera were separated and identified on the basis of morphology: 1) *Cophinforma* sp., conidiophores and paraphyses absent; conidia hyaline, unicellular, rarely becoming septate, mostly fusoid to ellipsoidal, most conidia longer than 30 µm; 2) *Lasiodiplodia* sp., conidia hyaline when young, later becoming 1-septate, dark brown with longitudinal striations, thick-walled, oblong to ellipsoid, straight, broadly rounded at the apex, base truncate; and 3) *Fusarium* sp., chlamydospores abundant, macroconidia septate, apical cell curved and tapering to a point, basal cell foot-shaped. *Hypoxylon* sp. could not be identified by morphology and was identified with DNA sequence-based analyses (Figure 2).

Phylogenetic analyses

ML and BI analyses of all examined loci produced similar results. Representative sequences for fungal isolates from Venezuela are highlighted in bold type in Figures 3-6. GenBank numbers are listed next to each sequence for reference. Posterior probability support for each clade ranged from 1 to 0.80. For internal nodes, however, PP was often lower. Bootstrap (BS) values below 50% are marked with a hyphen (-) on the phylogenetic trees. Fungal genera are presented based on their potential pathogenic importance as species newly reported on *T. cacao*, based on field observations.

Fungal species associated with diseased *T. cacao*

Lasiodiplodia brasiliensis M.S.B. Netto, M.W. Marques & A.J.L.

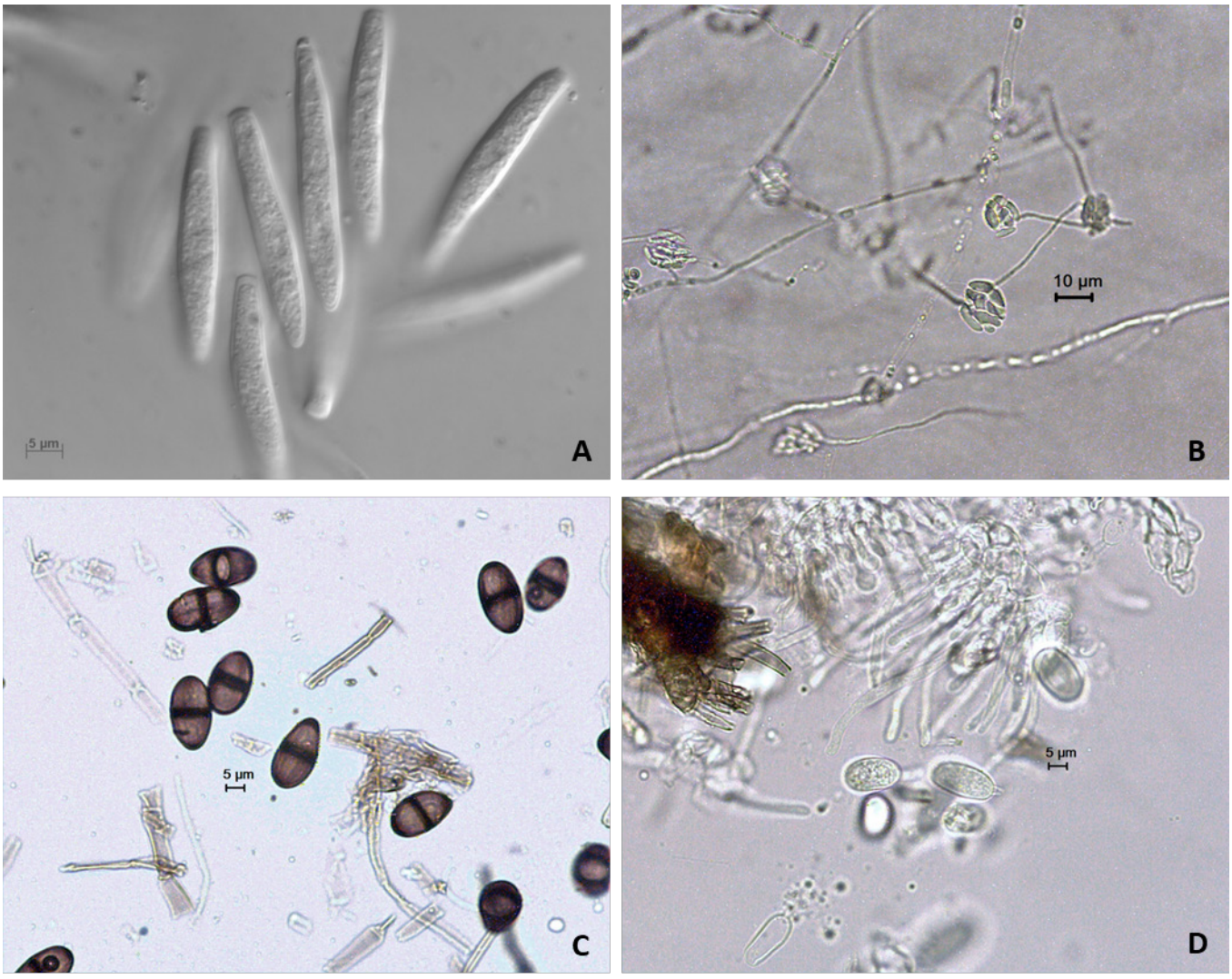

Figure 2. (A) *Cophinforma atrovirens* conidia; (B) *Fusarium oxysporum* microconidia (false heads) on short monophialides; *Lasiodiplodia* sp. mature (C) and inmature (D) conidia. A, C, D, scale bar = 5 µm, and B, scale bar = 10 µm.

Phillips and ***L. theobromae*** (Pat.) Griffon & Maubl.

A total of 28 taxa and 56 isolates were included in the phylogenetic analyses; *Macrophomina phaseolina* was included as the outgroup (Table 2). A concatenation of three gene regions (ITS+TEF1+ BTUB) was used to separate *Lasiodiplodia* isolates from *T. cacao* from other species of *Lasiodiplodia*. Approximately 570 base pairs were sequenced at the ITS, 540 bp at the TEF1, and 430 bp at the BTUB, for a total of 1,540 bp (Figure 3). The concatenated phylogeny highlighted the separation of *L. brasiliensis* and *L. theobromae* (BS/PP = 86%/1.00 and 65%/0.82).

Table 2. GenBank and culture collection accession numbers for *Lasiodiplodia* spp. isolates included in this study. Sequences that represented the isolates from Venezuela in this study are highlighted in bold.

Species	Accession number[a]	Host	Locality	Collector	GenBank accession number[b]		
					ITS	TFE1	BTUB
Lasiodiplodia americana	CERC1960	*Pistachia vera*	USA	T. J. Michailides	KP217058	KP217066	KP217074
L. americana	CERC1961	*Pistachia vera*	USA	T. J. Michailides	KP217059	KP217067	KP217075
L. brasiliensis	CMW 35884	*Adansonia madagascariensis*	Madagascar	M. J. Wingfield	KU887094	KU886972	KU887466
L. brasiliensis	IBL415	*Manilkara zapota*	Brazil	I. B. L. Coutinho	KT151806	KT151800	KT151803
L. brasiliensis	**CSM 11**	***Theobroma cacao***	**Venezuela**	**S. R. Mohali**	**MF436018**	**MF436006**	**MF435998**
L. brasiliensis	**CSM 15**	***Theobroma cacao***	**Venezuela**	**S. R. Mohali**	**MF436019**	**MF436007**	**MF435997**
L. brasiliensis	**CSM 16**	***Theobroma cacao***	**Venezuela**	**S. R. Mohali**	**MF436020**	**MF436008**	**MF435996**
L. brasiliensis	**CSM 232**	***Theobroma cacao***	**Venezuela**	**S. R. Mohali**	**MF436021**	**MF436009**	**MF435995**
L brasiliensis	**CSM 235**	***Theobroma cacao***	**Venezuela**	**S. R. Mohali**	**MF436022**	**MF436010**	**MF435994**
L. caatinguensis	IBL366	*Spondias purpurea*	Brazil	I. B. L. Coutinho/J. S. Lima	KT154760	KT008006	KT154767
L. caatinguensis	IBL381	*Spondias purpurea*	Brazil	I. B. L. Coutinho/J. S. Lima	KT154757	KT154751	KT154764
L. citricola	IRAN1521C	*Citrus* sp.	Iran	A. Shekari	GU945353	GU945339	KU887504
L. citricola	IRAN1522C	*Citrus* sp.	Iran	J. Abdollahzadeh/A. Javadi	GU945354	GU945340	KU887505
L. crassispora	WAC 12533	*Santalum album*	Australia	T.I. Burgess	DQ103550	DQ103557	KU887506
L. crassispora	CMW 13488	*Eucalyptus urophylla*	Venezuela	S.R. Mohali	DQ103552	DQ103559	KU887507
L. egyptiacae	CMM3611	*Jatropha curcas*	Brazil	A. R. Machado	KF234545	KF226691	KF254928
L. egyptiacae	CMM3648	*Jatropha curcas*	Brazil	A. R. Machado	KF234549	KF226705	KF254933
L. euphorbicola	CMM3609	*Jatropha curcas*	Brazil	A.R. Machado/O.L. Pereira	KF234543	KF226689	KF254926
L. euphorbicola	CMM3651	*Jatropha curcas*	Brazil	A.R. Machado/O.L. Pereira	KF234553	KF226711	KF254937
L. exigua	CBS 137785=BL104	*Retama raetam*	Tunisia	B.T. Linaldeddu	KJ638317	KJ638336	KU887509
L. gilanensis	IRAN1501C	Unknown	Iran	J. Abdollahzadeh/A. Javadi	GU945352	GU945341	KU887510
L. gilanensis	IRAN1523C	Unknown	Iran	J. Abdollahzadeh/A. Javadi	GU945351	GU945342	KU887511
L. gonubiensis	CBS 115812	*Syzygium cordatum*	South Africa	D. Pavlic	KF766191	DQ458877	KU887512
L. hormozganensis	IRAN1498C	*Mangifera indica*	Iran	J. Abdollahzadeh/A. Javadi	GU945356	GU945344	KU887514
L. hormozganensis	IRAN1500C	*Olea* sp.	Iran	J. Abdollahzadeh/A. Javadi	GU945355	GU945343	KU887515
Lasiodiplodia iraniensis	IRAN1520C	*Salvadora persica*	Iran	J. Abdollahzadeh/A. Javadi	GU945348	GU945336	KU887516
L. iraniensis	IRAN1502C	*Juglans* sp.	Iran	J. Abdollahzadeh/A. Javadi	GU945347	GU945335	KU887517
L. jatrophicola	CMM3610	*Jatropha curcas*	Brazil	A. R. Machado	KF234544	KF226690	KF254927
L. macrospora	CMM3833	*Jatropha curcas*	Brazil	A.R. Machado/O.L. Pereira	KF234557	KF226718	KF254941
L. mahajangana	CMW27801	*Terminalia catappa*	Madagascar	J. Roux	FJ900595	FJ900641	FJ900630
L. mahajangana	CMW27820	*Terminalia catappa*	Madagascar	J. Roux	FJ900597	FJ900643	FJ900632
L. margaritacea	CBS122519	*Adansonia gibbosa*	Australia	T.I. Burgess	EU144050	EU144065	KU887520
L. mediterranea	CBS 137783	*Quercus ilex*	Italy	B. T. Linaldeddu	KJ638312	KJ638331	KU887521
L. missouriana	UCD2193MO	*Vitis vinifera*	USA	K. Striegler/G. M. Leavitt	HQ288225	HQ288267	HQ288304
Lasiodiplodia missouriana	UCD2199MO	*Vitis vinifera*	USA	K. Striegler/G. M. Leavitt	HQ288226	HQ288268	HQ288305
L. parva	CBS456.78	Cassava-field soil	Colombia	O. Rangel	EF622083	EF622063	KU887523

continue

Table 2. *Continuation*

Species	Accession number[a]	Host	Locality	Collector	GenBank accession number[b]		
					ITS	TFE1	BTUB
L. plurivora	STE-U 5803	*Vitis vinifera*	Africa	F. Halleen	EF445362	EF445395	KU887524
L. pontae	CMM 1277	*Spondias purpurea*	Brazil	J. S. Lima/F. C. O. Freire	KT151794	KT151791	KT151797
L. pseudotheobromae	IBL134	*Annona* x *atemoya*	Brazil	I. B. L. Coutinho/J. S. Lima	KT247481	KT247485	KT247487
L. pseudotheobromae	IBL266	*Anacardium occidentale*	Brazil	I. B. L. Coutinho/J. S. Lima	KT247480	KT247484	KT247486
L. rubropurpurea	WAC12536	*Eucalyptus grandis*	Australia	T.I. Burgess/G. Pegg	DQ103554	DQ103572	KU887530
L. subglobosa	CMM3872	*Jatropha curcas*	Brazil	A.R. Machado/O.L. Pereira	KF234558	KF226721	KF254942
L. subglobosa	CMM4046	*Jatropha curcas*	Brazil	A.R. Machado/O.L. Pereira	KF234560	KF226723	KF254944
L. theobromae	IBL340	*Spondias purpurea*	Brazil	I. B. L. Coutinho/J. S. Lima	KT247466	KT247472	KT247475
L. theobromae	IBL375	*Talisia esculenta*	Brazil	I. B. L. Coutinho/J. S. Lima	KT247467	KT247473	KT247474
L. theobromae	**CSM 33**	***Theobroma cacao***	**Venezuela**	**S. R. Mohali**	**MF436024**	**MF436012**	**MF436004**
L. theobromae	**CSM 34**	***Theobroma cacao***	**Venezuela**	**S. R. Mohali**	**MF436025**	**MF436013**	**MF436003**
L. theobromae	**CSM 36**	***Theobroma cacao***	**Venezuela**	**S. R. Mohali**	**MF436026**	**MF436014**	**MF436002**
L. theobromae	**CSM 53**	***Theobroma cacao***	**Venezuela**	**S. R. Mohali**	**MF436027**	**MF436015**	**MF436001**
L. theobromae	**CSM 54**	***Theobroma cacao***	**Venezuela**	**S. R. Mohali**	**MF436028**	**MF436016**	**MF436000**
Lasiodiplodia theobromae	CSM 57	*Theobroma cacao*	Venezuela	S. R. Mohali	MF436029	MF436017	MF435999
L. venezuelensis	WAC12539	*Acacia mangium*	Venezuela	S.R. Mohali	DQ103547	DQ103568	KU887533
L. viticola	UCD2553AR	*Vitis vinifera*	USA	K. Striegler/G. M. Leavitt	HQ288227	HQ288269	HQ288306
L. viticola	UCD2604MO	*Vitis vinifera*	USA	K. Striegler/G. M. Leavitt	HQ288228	HQ288270	HQ288307
Macrophomina phaseolina	CMM3615	*Jatropha curcas*	Brazil	A. R. Machado	KF234547	KF226693	KF254930
M. phaseolina	PD112	*Prunus dulcis*	USA	TJM, HR, PI	GU251105	GU251237	GU251765

[a] CBS: Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, The Netherlands; IBL: Independent Biological Laboratories Israel. KEFAR MALAL; CMW: Tree Patholgy Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; IRAN: Iranian Fungal Culture Collection, Iranian Research Institute of Plant Protection, Iran; WAC: Department of Agriculture, Western Australia Plant Pathogen Collection, South Perth, Western Australia; STE-U: Culture Collection of the Department of Plant Pathology, University of Stellenbosch, South Africa; CMM: Bradford Art Galleries and Museums U.K. England. BRADFORD; PD: Culture Collection, University of California, Davis, USA; CERC, Culture Collection of China Eucalypt Research Centre, Chinese Academy of Forestry, ZhanJiang, GuangDong, China; CSM: Personal culture collection deposited in the Department of Bioagricultural Sciences & Pest Management, Colorado State University, USA.

[b] See Table 2 for full locus name.

Species of *Lasiodiplodia* are known to cause diseases on *T. cacao* in plantations across diverse global regions. Based on morphological descriptions, *L. theobromae* and *L. citricola* were associated with branch and trunk cankers on *T. cacao* in Nigeria (16), while *L. theobromae* was isolated from rotting *T. cacao* fruits in Ghana and Cuba (22, 46) and from branches and twigs of healthy *T. cacao* in Brazil (12). In addition, microsatellite markers were used to identify *L. theobromae* and *L. pseudotheobromae* associated with dieback symptoms on *T. cacao* in Cameroon (2).

In Venezuela, *L. theobromae* was identified morphologically in association with *T. cacao* showing various symptoms, including fruit rot, dieback and cankers on branches, twigs and roots (35, 39). *Lasiodiplodia theobromae,* identified with ITS sequencing, was also reported as an isolate from *T. cacao* with cushion galls (45); however, ITS sequences appear unreliable as a sole method for identifying currently recognized species of *Lasiodiplodia*. Several well-established cryptic species are recognized in the *Lasiodiplodia* complex, all with nearly identical ITS sequences and/or similar morphology (44). For this reason, multiple loci, including ITS, TEF1, BTUB and RPB2, are necessary for *Lasiodiplodia* species identification, and sequences of these loci are well-represented in databases. Importantly, these loci provide sufficiently stringent phylogenetic signals to distinguish most known cryptic species within *Lasiodiplodia* (43). *Lasiodiplodia brasiliensis,* first described in Brazil as causing stem-end rot of papaya (27), was also reported causing dieback of mango in Peru (40) and postharvest fruit rot of custard apple (*Annona squamosa*) in Brazil (3). Similar methodology was used to isolate *Lasiodiplodia brasiliensis* from branches and twigs of *T. cacao* with dieback or sudden death symptoms in Mérida State. This paper is the first to report *L. brasiliensis* potentially associated with dieback or sudden death on *T. cacao* in Venezuela; however, further studies are needed to confirm pathogenicity of *L. brasiliensis* on *T. cacao*.

Cophinforma atrovirens (Mehl & Slippers) A. Alves & A.J.L. Phillips.

Three or four taxa and 18 isolates (except 20 isolates for ITS sequencing) were included in the phylogenetic analyses using five gene regions, including ITS: 550 bp; TEF1*:* 550 bp; SSU: 1020-1025

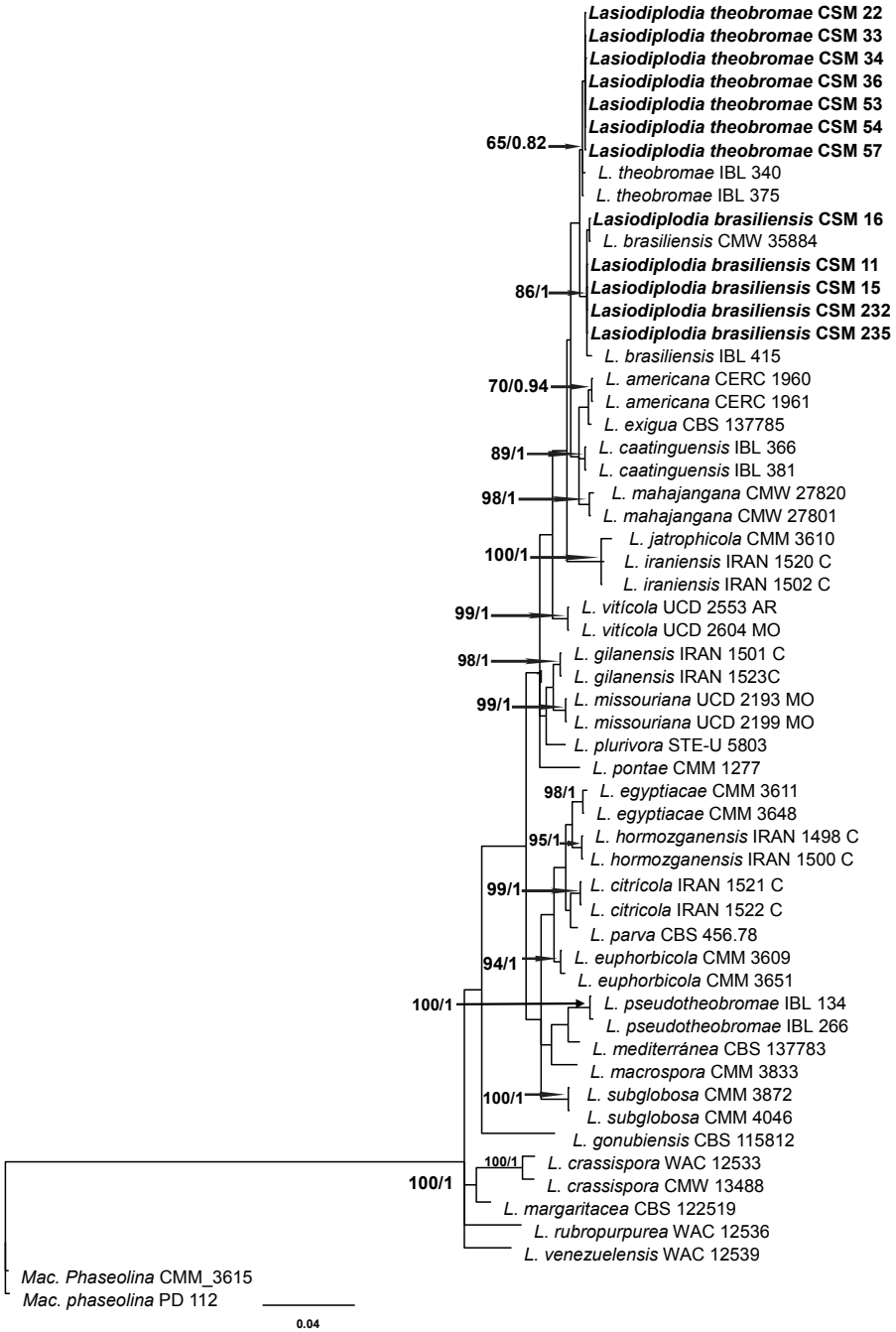

Figure 3. Phylogeny of *Lasiodiplodia* species generated from Bayesian analysis based on the concatenated ITS, BTUB, and TEF1 sequences. The phylogeny was rooted with *Macrophomina phaseolina* (CMM 3615 and PD 112). Node support (bootstrap ≥ 60%:1,000 replicates and Bayesian posterior probabilities ≥ 0.80, BS/PP) are highlighted by arrows. The new isolates from this study are in bold italics.

bp; LSU: 1090 bp, and BTUB: 440 bp. The outgroup taxon for all loci was *Macrophomina phaseolina* (CBS 162.25). *Cophinforma mamane* (= *Botryosphaeria mamane* isolates 97-58 and 97-59) was included in the ITS dataset but not for subsequent phylogenic analyses because sequence data for other loci is lacking for *C. mamane*. The remaining loci (TEF1, SSU, LSU and BTUB) included 18 isolates comprised within three taxa: *C. atrovirens*, *Botryosphaeria dothidea* and *M. phaseolina* (Table 3). Phylogenies of the ITS and TEF1 showed that isolates of *Cophinforma* spp. from *T. cacao* fruits and stem grouped with known isolates of *C. atrovirens* in well-supported clades (BS/PP = -/0.98 and 100%/1.00); phylogenies of the LSU and BTUB loci had moderate levels of support (BS/PP = 82%/0.65 and 80%/0.57); however, no support was identified at the SSU locus (BS/PP = -/-) (Figure 4).

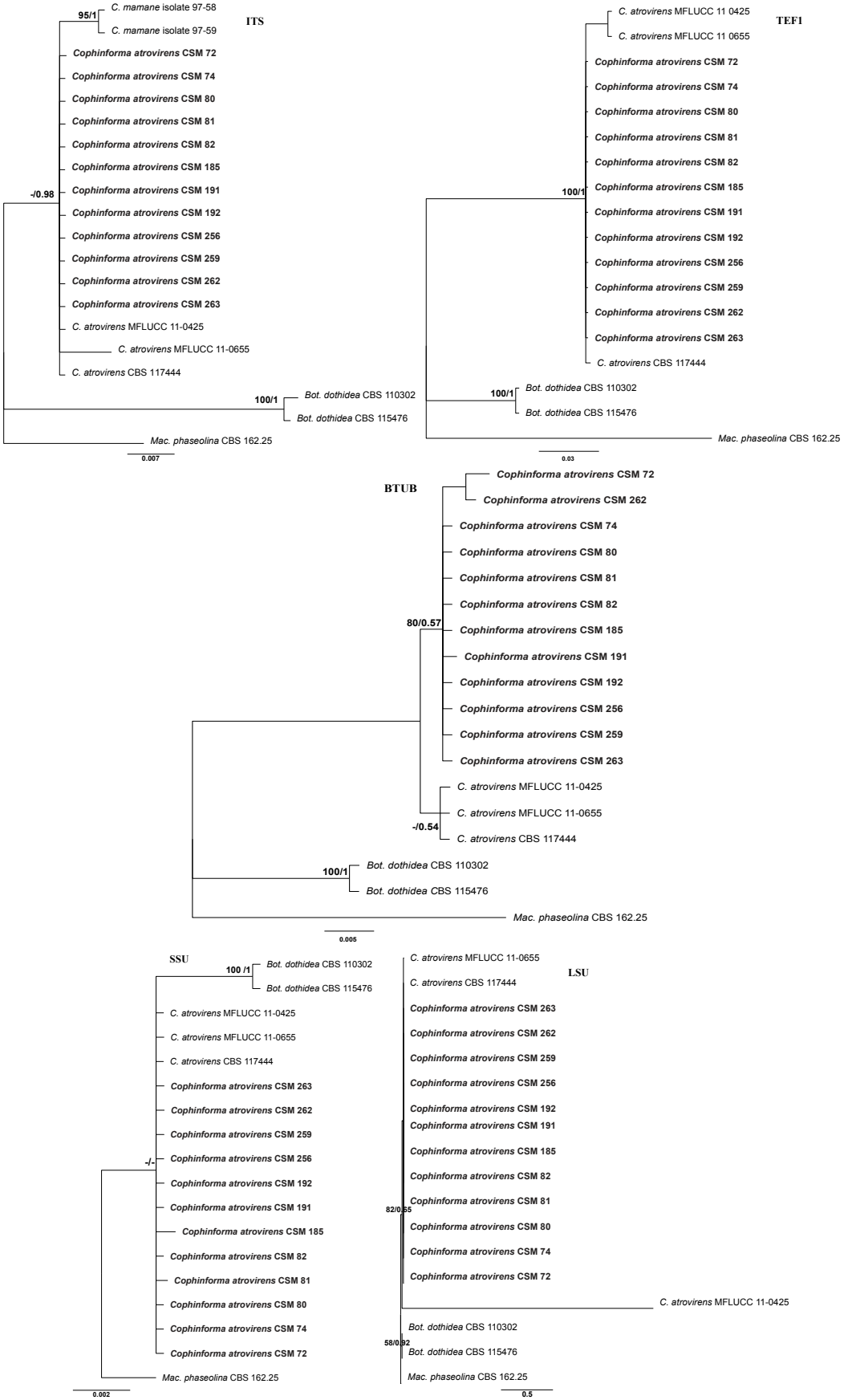

Figure 4. Phylogenies of *Cophinforma* species generated from Bayesian analyses using A: ITS, B: TEF1, C: BTUB, D: LSU, and E: SSU loci. Phylogenies were rooted with *Macrophomina phaseolina* (CBS 162.25). Node support (bootstrap ≥ 50%:1,000 replicates and Bayesian posterior probabilities ≥ 0.50, BS/PP) are highlighted by arrows. The new isolates from this study are in bold italics.

Table 3. GenBank and culture collection accession numbers of *Cophinforma* spp. isolates included in this study. Sequences that represented the isolates from Venezuela in this study are highlighted in bold.

Species	Accession number[a]	Host	Locality	Collector	GenBank accession number[b]				
					ITS	TEF1	BTUB	SSU	LSU
Cophinforma atrovirens	MFLUCC 11-0425	*.Eucalyptus* sp	Thailand	M. Doilom	JX646800	JX646865	JX646848	JX646833	JX646817
C. atrovirens	MFLUCC 11-0655	*.Eucalyptus* sp	Thailand	M. Doilom	JX646801	JX646866	JX646849	JX646834	JX646818
C. atrovirens	CBS 117444	*Eucalyptus urophylla*	Venezuela	S. R. Mohali	KF531822	KF531801	KF531802	KF531821	DQ377855
C. atrovirens	**CSM 72**	***Theobroma cacao***	**Venezuela**	**S.R Mohali**	**MF436087**	**MF436099**	**MF436111**	**MF436134**	**MF436146**
C. atrovirens	**CSM 74**	***Theobroma cacao***	**Venezuela**	**S.R Mohali**	**MF436088**	**MF436100**	**MF436112**	**MF436133**	**MF436145**
C. atrovirens	**CSM 80**	***Theobroma cacao***	**Venezuela**	**S.R Mohali**	**MF436089**	**MF436101**	**MF436113**	**MF436132**	**MF436144**
C. atrovirens	**CSM 81**	***Theobroma cacao***	**Venezuela**	**S.R Mohali**	**MF436090**	**MF436102**	**MF436114**	**MF436131**	**MF436143**
C. atrovirens	**CSM 82**	***Theobroma cacao***	**Venezuela**	**S.R Mohali**	**MF436091**	**MF436103**	**MF436115**	**MF436130**	**MF436142**
C. atrovirens	**CSM 185**	***Theobroma cacao***	**Venezuela**	**S.R Mohali**	**MF436092**	**MF436104**	**MF436116**	**MF436129**	**MF436141**
C. atrovirens	**CSM 191**	***Theobroma cacao***	**Venezuela**	**S.R Mohali**	**MF436093**	**MF436105**	**MF436117**	**MF436128**	**MF436140**
C. atrovirens	**CSM 192**	***Theobroma cacao***	**Venezuela**	**S.R Mohali**	**MF436094**	**MF436106**	**MF436118**	**MF436127**	**MF436139**
C. atrovirens	**CSM 256**	***Theobroma cacao***	**Venezuela**	**S.R Mohali**	**MF436095**	**MF436107**	**MF436119**	**MF436126**	**MF436138**
C. atrovirens	**CSM 259**	***Theobroma cacao***	**Venezuela**	**S.R Mohali**	**MF436096**	**MF436108**	**MF436120**	**MF436125**	**MF436137**
C. atrovirens	**CSM 262**	***Theobroma cacao***	**Venezuela**	**S.R Mohali**	**MF436097**	**MF436109**	**MF436121**	**MF436124**	**MF436136**
C. atrovirens	**CSM 263**	***Theobroma cacao***	**Venezuela**	**S.R Mohali**	**MF436098**	**MF436110**	**MF436122**	**MF436123**	**MF436135**
C. mamane	97-58	*Sophora chrysophylla*	Hawaii	D.E. Gardner	AF246929	-	-	-	-
C. mamane	97-59	*S. chrysophylla*	Hawaii	D.E. Gardner	AF246930	-	-	-	-
Botryosphaeria dothidea	CBS 110302	*Vitis vinifera*	Portugal	A.J.L. Phillips	AY259092	AY573218	EU673106	EU673174	EU673243
B. dothidea	CBS 115476	*.Prunus* sp	Switzerland	B. Slippers	AY236949	AY236898	AY236927	EU673173	AY928047
Macrophom. phaseolina	CBS 162.25	*.Eucalyptus* sp	Uganda	Goig T	KF531826	KF531803	KF531805	KF531824	DQ377905

[a] CBS: Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, The Netherlands; MFLUCC: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; CSM: Personal culture collection deposited in the Department of Bioagricultural Sciences & Pest Management, Colorado State University, USA.
[b] See Table 2 for full locus name.
- : Data not found.

The genus *Cophinforma* was introduced within *Botryosphaeriaceae* by Liu et al. (20) as a monotypic genus that comprised *C. eucalypti*. Phillips et al. (37), however, showed that two species previously included in *Botryosphaeria* were better accommodated in *Cophinforma*: *Botryosphaeria mamane* as *Cophinforma mamane*, and *Cophinforma eucalypti* renamed as *Cophinforma atrovirens*. The conidia produced by *Cophinforma* are longer than those of any known species of *Botryosphaeria*. Although *Botryosphaeria* and *Cophinforma* share other morphological similarities, these genera are phylogenetically distinct. Based on phylogenetic relationships, *C. mamane* and *C. atrovirens* are currently recognized within *Cophinforma* (37).

Little published information is available for *Cophinforma* spp. *Cophinforma atrovirens* (= *Cophinforma eucalypti* = *Fusicoccum atrovirens*) was reported as a saprophyte on a dead branch of *Eucalyptus* sp. in Thailand (20), whereas *C. atrovirens* (as *F. atrovirens*) was isolated from asymptomatic branches and twigs of *Pterocarpus angolensis* in South Africa (23). In Venezuela, *C. mamane* (as *B. mamane*) was isolated from stems and branches of *Acacia mangium* and *Eucalyptus* hybrids (25), and it was also isolated from *Sophora chrysophylla* in Hawaii (9). In the present study, *C. atrovirens* was isolated from fruit anthracnose- and stem/branch dieback-associated tissues of *T. cacao*, and it was accompanied by *L. theobromae* and *L. brasiliensis* in Mérida State. Despite these findings, the capacity of *C. atrovirens* to cause disease is not verified. This report is the first showing that *C. atrovirens* is associated with fruit anthracnose, dieback or sudden death on *T. cacao* in Venezuela; however, inoculation work is required to conclusively determine if *C. atrovirens* is a pathogen of *T. cacao*.

Fusarium oxysporum Schlechtendahl emend. Snyder & Hansen. and ***Fusarium solani*** (Martius) Appel & Wollenweber emend. Snyder & Hansen/sexual morph ***Neocosmospora solani*** (Mart.) L. Lombard & Crous.

A total of 41 isolates comprised within 26 taxa of *Fusarium* spp. were used in the phylogenetic analyses with *F. lyarnte* RBG 5331 as the outgroup (Table 4). A total of 1,680 bp were sequenced at the TEF1 (710 bp) and RPB2 (970 bp) loci to determine species identity of 13 isolates of *Fusarium* from *T. cacao*. Phylogenetic analyses of the TEF1 and RPB2 showed that 11 isolates belonged to *Neocosmospora solani*, while two isolates belonged to *Fusarium oxysporum*; both species clades possessed well-supported nodes (BS/PP = 100%/1.00; Figure 5).

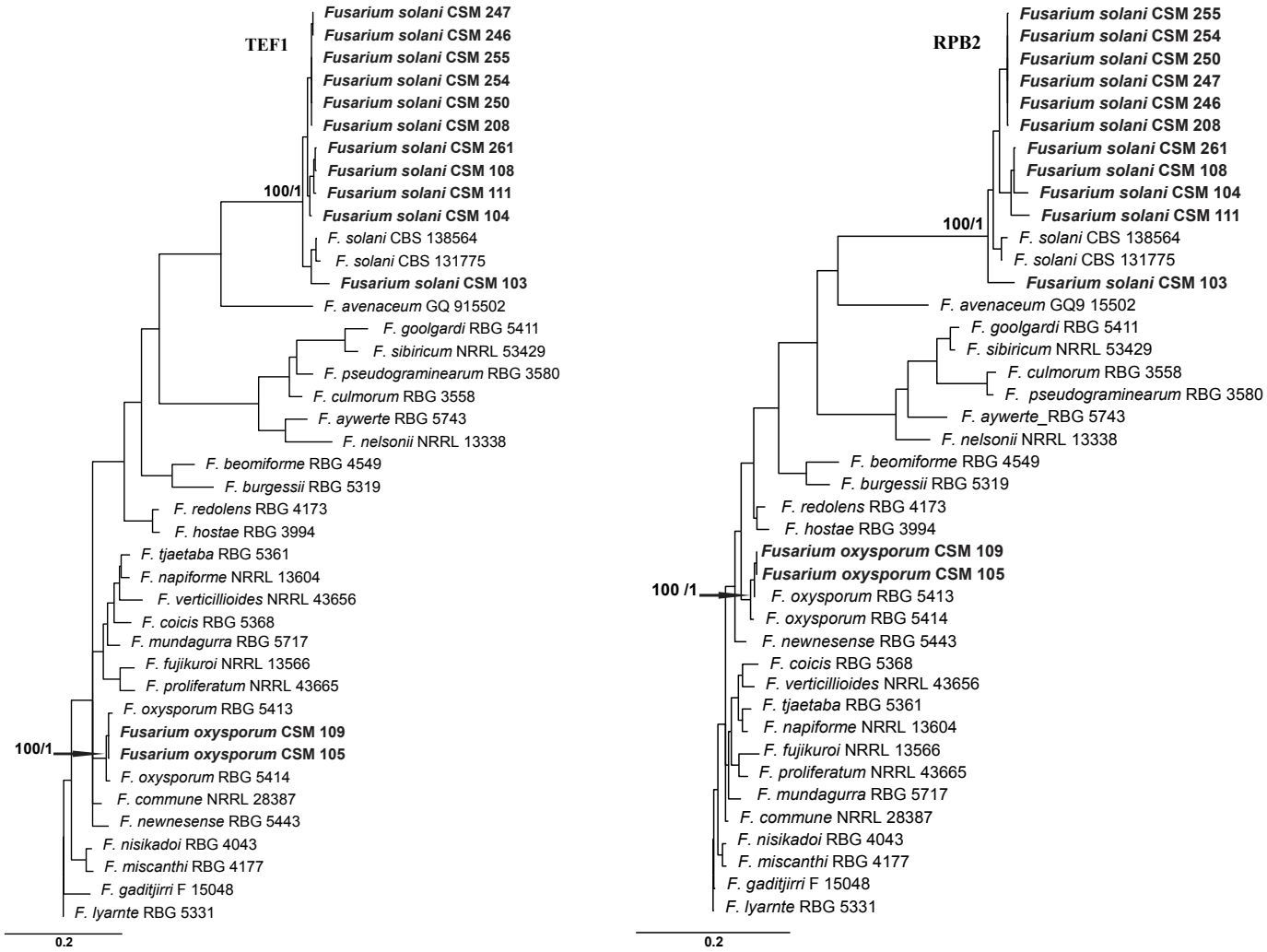

Figure 5. Phylogenies of *Fusarium* species generated from Bayesian analysis based on A: TEF1 and B: RPB2 sequences. Phylogenies were rooted with *Fusarium lyarnte* (RBG 5331). Node support (bootstrap ≥ 90%:1,000 replicates and Bayesian posterior probabilities ≥ 0.90, BS/PP) are highlighted by arrows. The new isolates from this study are in bold italics.

Table 4. GenBank and culture collection accession numbers of *Fusarium* spp. included in this study. Sequences that represented the isolates from Venezuela in this study are highlighted in bold.

Species	Accession number[a]	Host	Locality	Collector	GenBank accession number[b]	
					TEF1	RPB2
Fusarium avenaceum	FRC R-09495	-	-	-	GQ915502	GQ915486
F. aywerte	RBG5743	-	Australia	RBG	KP083250	KP083278
F. beomiforme	RBG4549	-	Australia	RBG	HQ667157	HQ646396
F. burgessii	RBG5319	Soil	Australia	B.Wang/A. Fraser	HQ667149	HQ646392
F. coicis	RBG5368	*Coix gasteenii*	Australia	RBG	KP083251	KP083274
F. commune	NRRL28387	*Dianthus caryophyllus*	The Netherlands	NRRL	AF246832	JX171638
F. culmorum	RBG3558	-	Australia	RBG	HQ667167	HQ646401
F. fujikuroi	NRRL13566	*Oryza sativa*	Taiwan	NRRL	AF160279	EF470116
F. gaditjirri	F15048	*Heteropogon triticeus*	Australia	L.W. Burgess	AY639634	HQ662690
F. goolgardi	RBG5411	*Xanthorrhoea glauca*	Australia	D.M Robinson/M.H. Laurence	KP101123	KP083280
F. hostae	RBG3994	-	Australia	RBG	HQ667160	HQ646390

continue

Table 4. *Continuation*

Species	Accession number[a]	Host	Locality	Collector	GenBank accession number[b]	
					TEF1	RPB2
F. lyarnte	RBG5331	-	Australia	RBG	EF107118	HQ662691
F. miscanthi	RBG4177	-	Australia	RBG	HQ667166	HQ662693
F. moniliforme	NRRL43656	Contact lens	USA	NRRL	EF452987	EF470026
F. mundagurra	RBG5717	From soil Carnarvon Gorge National Park	Australia	S.M. Dunstan/M.H. Laurence	KP083256	KP083276
F. napiforme	NRRL13604	*Pennisetum typhoides*	Namibia	A.Lübben	AF160266	EF470117
F. nelsonii	NRRL13338	Soil	Australia	NRRL	GQ505402	GQ505466
F. newnesense	RBG5443	-	Australia	RBG	KJ397074	KP083277
F. nisikadoi	RBG4043	-	Australia	RBG	HQ667165	HQ662692
F. oxysporum	RBG5413	-	Australia	RBG	HQ667161	HQ646385
F. oxysporum	RBG5414	-	Australia	RBG	HQ667163	HQ646388
F. oxysporum	**CSM 105**	***Theobroma cacao***	**Venezuela**	**S. R. Mohali**	**MF436042**	**MF447156**
F. oxysporum	**CSM 109**	***Theobroma cacao***	**Venezuela**	**S. R. Mohali**	**MF436041**	**MF447155**
F. proliferatum	NRRL43665	Contact lens	USA	NRRL	EF452996	EF470035
F. pseudograminearum	RBG3580	-	Australia	RBG	HQ667168	HQ646400
Fusarium redolens	RBG4173	-	Australia	RBG	HQ667159	HQ646391
F. sibiricum	NRRL53429	*Avena sativa*	Russia	L. S. Malinovskaya/E.A.Pirjazeva	HM744683	HQ154471
F. solani	CBS 138564	Homo sapiens	Turkey	-	KT272100	KT272102
F. solani	CBS 131775	wheat	Iran	M. Davari	JX118990	JX237778
F. solani	**CSM 103**	***Theobroma cacao***	**Venezuela**	**S. R. Mohali**	**MF436040**	**MF436050**
F. solani	**CSM 104**	***Theobroma cacao***	**Venezuela**	**S. R. Mohali**	**MF436039**	**MF436052**
F. solani	**CSM 108**	***Theobroma cacao***	**Venezuela**	**S. R. Mohali**	**MF436038**	**MF436051**
F. solani	**CSM 111**	***Theobroma cacao***	**Venezuela**	**S. R. Mohali**	**MF436037**	**MF436053**
F. solani	**CSM 208**	***Theobroma cacao***	**Venezuela**	**S. R. Mohali**	**MF436036**	**MF436049**
F. solani	**CSM 246**	***Theobroma cacao***	**Venezuela**	**S. R. Mohali**	**MF436035**	**MF436048**
F. solani	**CSM 247**	***Theobroma cacao***	**Venezuela**	**S. R. Mohali**	**MF436034**	**MF436047**
F. solani	**CSM 250**	***Theobroma cacao***	**Venezuela**	**S. R. Mohali**	**MF436033**	**MF436046**
F. solani	**CSM 254**	***Theobroma cacao***	**Venezuela**	**S. R. Mohali**	**MF436032**	**MF436045**
F. solani	**CSM 255**	***Theobroma cacao***	**Venezuela**	**S. R. Mohali**	**MF436031**	**MF436044**
F. solani	**CSM 261**	***Theobroma cacao***	**Venezuela**	**S. R. Mohali**	**MF436030**	**MF436043**
F. tjaetaba	RBG5361	*Sorghum interjectum*	Australia	J.L. Walsh	KP083263	KP083275

[a] RBG: Royal Botanic Gardens Trust, Sydney, New South Wales, Australia; NRRL: Agricultural Research Service Culture Collection, Peoria, Illinois, USA; F: University of Sydney, Sydney, New South Wales, Australia; CBS: Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, The Netherlands; CSM: Personal culture collection deposited in the Department of Bioagricultural Sciences & Pest Management, Colorado State University, USA.
[b] See Table 2 for full locus name.
- : Data not found.

Taxa within the genus *Fusarium* are known as pathogens, endophytes and/or antagonists against other fungal pathogens of plants (12). *Fusarium decemcellulare*, the cause of cushion disease, is a weak pathogen that requires injuries to infect *T. cacao* (14). Cushion disease occurs in Sri Lanka, West Africa and the Western Hemisphere, and it causes economically important damage in Colombia, Costa Rica and Nicaragua (13). *Fusarium decemcellulare* has also been reported in Cuba and Venezuela, where it occurs with other diseases and pathogens of *T. cacao*, including fruit rots and stem/branch/root cankers associated with *Phytophthora* spp., *Lasiodiplodia* spp. and *M. perniciosa* (22, 36, 38, 39).

In the present study, isolates of *F. oxysporum* and *F. solani* were obtained from *T. cacao* fruits with anthracnose symptoms. In Mérida State, these same *Fusarium* species were also isolated from *T. cacao* tissues with dieback or sudden death symptoms in association with *L. brasiliensis* and *L. theobromae*.

Fusarium oxysporum, *F. solani* and other *Fusarium* species, such as *F. incarnatum*, *F. equiseti*, *F. camptoceras*, *F. crokwellense*, *F. moniliforme*, *F. proliferatum* and *F. decemcellulare*, were previously identified on *T. cacao* in Venezuela, based on morphological and ITS

sequence data (39, 45, 47). The use of the ITS region alone, however, does not provide sufficient resolution to distinguish some species of *Fusarium* that diverged relatively recently from common ancestors (32), and ITS is less informative than TEF1, RPB2 and BTUB (33) for phylogenetic analyses. The current paper confirmed the presence of *F. oxysporum* and *F. solani* on *T. cacao* in Venezuela based on multilocus sequencing.

Hypoxylon investiens (Schwein.) M. A. Curtis.

Two gene regions, ITS and BTUB, were used to identify the two isolates of *Hypoxylon* collected from *T. cacao*. A total of 44 isolates representing 41 *Hypoxylon* species were used in phylogenetic analyses, and *Nemania serpens* voucher 235 was used as the outgroup (Table 5). A total of 2,108 bp were sequenced: 1,448 bp for BTUB and 660 pb for ITS. Using a concatenated dataset of the BTUB and the ITS regions, a well-supported node grouped the two isolates collected from *T. cacao* into the *Hypoxylon investiens* clade (BS/PP = 100%/1), strongly supporting this identity for these isolates (Figure 6).

Table 5. GenBank and culture collection accession numbers of *Hypoxylon* spp. included in this study. Sequences that represented the isolates from Venezuela in this study are highlighted in bold.

Species	Accession number[a]	Host	Locality	Collector	GenBank accession number[b]	
					ITS	BTUB
Hypoxylon addis	MUCL 52797	-	Ethiopia	Holotype	KC968931	KC977287
H. brevisporum	(voucher 36 (JDR	on fallen tree	Puerto Rico	D. J. Lodge	JN660821	AY951705
H. carneum	voucher YMJ 30	*.Malus* sp	France	J. Fournier	JN660822	AY951706
H. cercidicola	CBS 119009	*Fraxinus excelsior*	France	J. Fournier	KC968908	KC977263
H. cinnabarinum	voucher YMJ 8	Branch	Mexico	F. San Martin	JN979409	AY951709
H. crocopeplum	voucher YMJ 11	Decorticated rotten wood	USA	J. D. Rogers	JN979410	AY951710
H. dieckmannii	voucher YMJ 45	Wood	Mexico	F. San Martin	JN979412	AY951712
H. erythrostroma	MUCL 53759	-	Martinique	-	KC968910	KC977296
H. fendleri	YMJ 92092016	Wood	Taiwan	Y. M. Ju	JN979418	AY951718
H. fraxinophilum	MUCL 54176	*Fraxinus excelsior*	France	J. Fournier	KC968938	KC977301
H. fuscopurpureum	voucher YMJ 67	*.Quercus* sp	USA	J. D. Rogers	JN979421	AY951721
H. fuscum	voucher YMJ 91010205	*Alnus formosana*	Taiwan	Y. M. Ju	JN979423	AY951723
H. griseobrunneum	MUCL 53754	On wood and bark	Martinique	J. Fournier	KC968909	KC977289
H. haematostroma	MUCL 53301	Dicot bark	Martinique	J. Fournier	KC968911	KC977291
H. hypomiltum	CBS 129036	corticated branch	Guadeloupe	J. Fournier	KC968914	KC977298
H. investiens	CBS 118183	-	Malaysia	T. Læssoe	KC968925	KC977270
H. investiens	**CSM 173**	***Theobroma cacao***	**Venezuela**	**S. R. Mohali**	**MF435993**	**MF435991**
H. investiens	**CSM 174**	***Theobroma cacao***	**Venezuela**	**S. R. Mohali**	**MF435992**	**MF435990**
H. isabellinum	CBS 129035	*Swietenia macrophylla*	Martinique	J. Fournier	KC968935	KC977295
H. jecorinum	voucher YMJ 39	*Diospyros texana* Scheele	Mexico	F. San Martin	JN979429	AY951731
H. laminosum	CBS 129032	Bamboo	Martinique	J. Fournier	KC968934	KC977292
H. lividicolor	voucher YMJ 70	On decorticated wood	Taiwan	Holotype	JN979432	AY951734
H. macrosporum	voucher YMJ 47	*.Populus* sp	Canada	B. E. Callan	JN979434	AY951736
H. monticulosum	MUCL54626	-	Martinique	MUCL	KC968939	KC977302
H. nicaraguense	CBS 117739	-	Burkina Faso	J. H. Petersen	AM749922	KC977272
H. ochraceum	MUCL 54625	(bark (coastal xerophilic forest	Martinique	MUCL	KC968937	KC977300
H. papillatum	ATCC 58729	-	USA	Holotype	KC968919	KC977258
Hypoxylon perforatum	MUCL 54174	-	Japan	MUCL	KC968936	KC977299
H. polyporus	MUCL 49209	-	Ivory Coast	MUCL	AM749941	KC977283

Species	Accession number[a]	Host	Locality	Collector	GenBank accession number[b]	
					ITS	BTUB
H. porphyreum	CBS 119022	*Quercus petraea*	France	J. Fournier	KC968921	KC977264
H. pulicicidum	MUCL53764	on rotten wood	Martinique	MUCL	JX183077	JX183073
H. pulicicidum	CBS 122622	on rotten wood (coastal meso-(philic forest	Martinique	J. Fournier	JX183075	JX183072
H. rickii	(YMJ 25 (JDR	Wood	Mexico	F. San Martin	JQ009313	AY951750
H. samuelsii	MUCL 51843	on dicot. wood and bark	Guadeloupe	C. Lechat	KC968916	KC977286
Hypoxylon shearii var. minor	voucher YMJ 29	on branch of Quercus	Mexico	Isotype	EF026142	AY951753
H. subgilvum	voucher YMJ 88113007	-	Taiwan	Y. M. Ju	JQ009315	AY951755
H. sublenormandii	CBS 138917	-	Sri Lanka	-	KM610291	KM610303
H. submonticulosum	CBS 115280	-	France	-	KC968923	KC977267
H. cf. subticinense	MUCL 53752	-	French Guiana	MUCL	KC968913	KC977297
H. tortisporum	STMA06079	-	Thailand	not yet deposited	KF234420	KF300546
H. trugodes	(voucher YMJ 57 (JDR	Wood	Taiwan	Y. M. Ju	JQ009319	AY951759
H. ulmophilum	(voucher YMJ 350 (JDR	*Ulmus japonica*	Russia	L. Vasilyeva	JQ009320	AY951760
Nemania serpens	(voucher 235 (HAST	Soil	Canada	G. Barron	GU292820	GQ470223

[a] CBS: Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, The Netherlands; ATCC: American Type Culture Collection, Virginia, USA; MUCL: Université Catholique de Louvain Belgium. LOUVAIN-LA-NEUVE; JF: Jonkershoek Forestry Research Centre South Africa. Western Cape Province. STELLENBOSCH; YMJ and J.R: Ju and Rogers cultures accumulated from previous studies; CSM: Personal culture collection deposited in the Department of Bioagricultural Sciences & Pest Management, Colorado State University, USA.
[b] See Table 2 for full locus name.
- : Data not found

Hypoxylon is one of the largest genera in *Xylariaceae*, including 159 currently accepted taxa with a global distribution, the highest diversity of which occurs in the tropics (19). The sexual morphs are usually associated with dead hardwood species, often occurring along with their respective asexual morphs (19). *Hypoxylon* is most diverse in warmer climates, and *Hypoxylon* spp. can live as saprophytes or as facultative parasites on stressed or diseased hosts (18). In this study, *H. investiens* was isolated from stems and branches of *T. cacao* in Mérida State, where it appeared to co-exist with other fungi as a potential endophyte; however, more studies are needed to confirm the ecological role of *H. investiens* in association with *T. cacao*. *Hypoxylon investiens* is reported for the first time on *T. cacao* in Venezuela, and identification of this fungus increases our knowledge of the mycoflora on *T. cacao*.

Historically, fungi reported from *T. cacao* plantations in Venezuela were identified using morphological characteristics or, more recently, through ITS sequence data. This paper presents the first detailed study examining putative disease-causing fungi on *T. cacao* from Venezuela based on morphology and DNA sequence-based analyses of multiple loci. DNA sequencing enabled the identification of several fungi that are reported for the first time in Venezuela.

The current study demonstrates a large diversity of fungi, especially ascomycetes, associated with diverse disease symptoms on *T. cacao* in plantations of Mérida State, Venezuela. Since samples were collected only in western Venezuela, it is likely that collections from other *T. cacao*-producing areas in eastern Venezuela could further increase the known diversity of fungi associated with *T. cacao*.

Declaration of competing interest

The authors declare no potential conflict of interest for this study, and this study has not been published previously.

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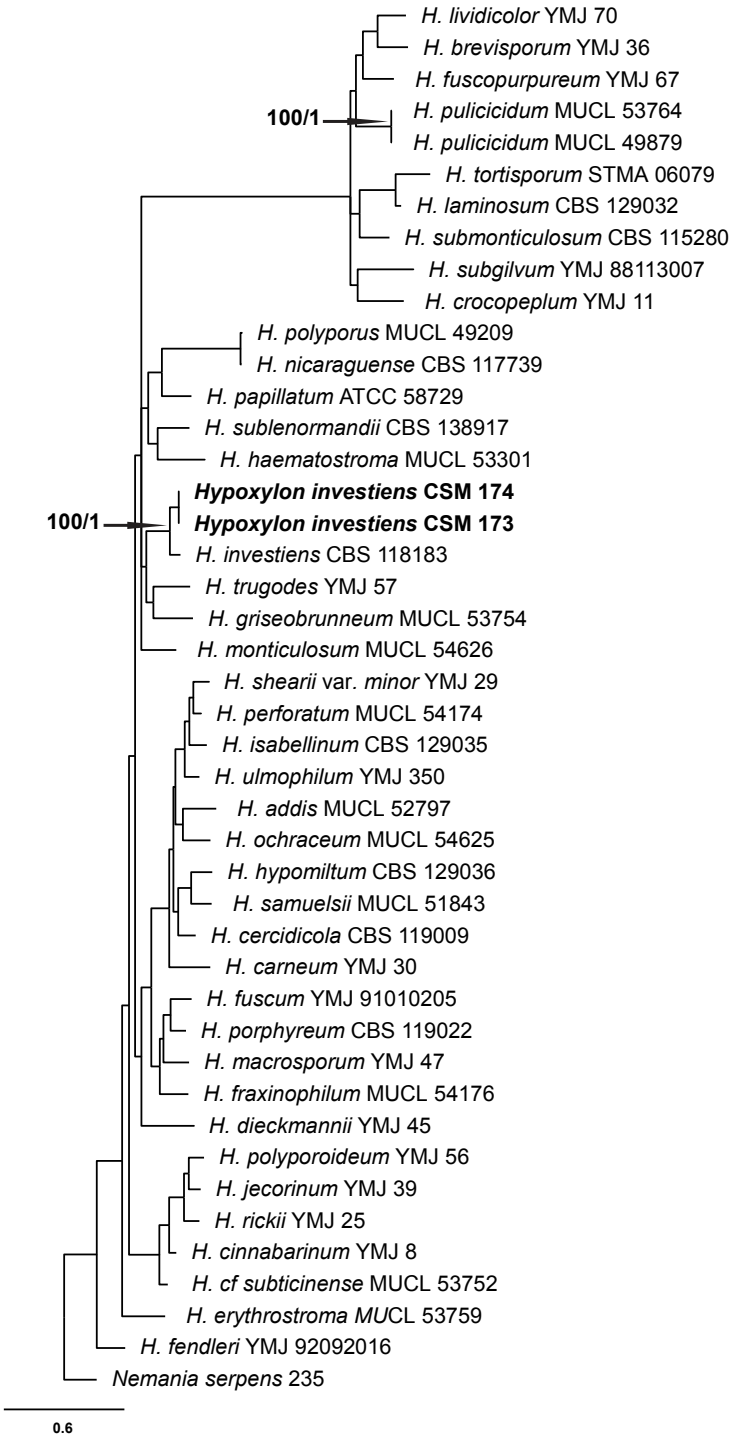

Figure 6. Phylogeny of *Hypoxylon* species generated from Bayesian analysis based on combined ITS and BTUB sequences. The phylogeny was rooted with *Nemania serpens* (235). Node support (bootstrap ≥ 90%:1,000 replicates and Bayesian posterior probabilities ≥ 0.90, BS/PP) are highlighted by arrows. The new isolates from this study are in bold italics.

REFERENCES

1. Alves, A.; Crous, P.W.; Correia, A.; Phillips, A.J.L. Morphological and molecular data reveal cryptic speciation in *Lasiodiplodia theobromae*. **Fungal Diversity**, Kunming, v.28, p.1-13, 2008.
2. Begoude, B.A.D.; Slippers, B.; Perez, G.; Wingfield, M.J.; Roux, J. High gene flow and outcrossing within populations of two cryptic fungal pathogens on a native and non-native host in Cameroon. **Fungal Biology,** Netherlands, v.116, n.3, p.343-353, 2012.
3. Cardoso, J.E.; Lima, J.S.; Viana, F.M.P.; Ootani, M.A.; Araújo, F.S.A.; Fonseca, W.L.; Lima, C.S.; Martins, M.V.V. First report of *Lasiodiplodia brasiliense* causing postharvest fruit rot of custard apple (*Annona squamosa*) in Brazil. **Plant Disease Notes**, St. Paul, v.101, n.8, p.1542, 2017.
4. Cheesman, E.E. Notes on the nomenclature, classification and possible relationships of cacao populations. **Tropical Agriculture**, Trinidad and

Tobago, v.21, n.8, p.144-159, 1944.
5. Dillinger, T.L.; Barriga, P.; Escárcega, S.; Jimenez, M.; Lowe, D. S.; Grivetti, L. E. Food of the Gods: Cure for humanity? A cultural history of the medicinal and ritual use of chocolate. **The Journal of Nutrition**, Rockville, MD, v.130, n.8, p.2057S-2072S, 2000.
6. Drummond, A.J.; Suchard, M.A.; Xie, D.; Rambaut, A. Bayesian phylogenetics with BEAUti and the BEAST 1.7. **Molecular Biology and Evolution**, Oxford, v.29, n.8, p.1969-1973, 2012.
7. Ferry, R.J. The colonial elite of Caracas: Formation and crisis 1567-1767. **Journal of Social History**, Oxford, v.27, n.1, p.158-159, 1989.
8. Gardes, M.; Bruns, T.D. ITS primers with enhanced specificity for basidiomycetes application to the identification of mycorrhizae and rusts. **Molecular Ecology**, Hoboken, New Jersey, v.2, p.113-118, 1993.
9. Gardner, D.E. *Botryosphaeria mamane* sp. nov. associated with witches'-brooms on the endemic forest tree *Sophora chrysophylla* in Hawaii. **Mycologia**, London, v.89, n.2, p.298-303, 1997.
10. Glass, N.L.; Donaldson, G. Development of primer sets designed for use with PCR to amplify conserved genes from filamentous ascomycetes. **Applied and Environmental Microbiology**, USA, v.61, n.4, p.1323-1330, 1995.
11. Guindon, S.; Gascuel, O. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. **Systematic Biology**, Oxford, v.52, n.5, p.696-704, 2003.
12. Hanada, R.E.; Pomella, A.W.; Costa, H.S.; Bezerra, J.L.; Loguercio, L.L.; Pereira, J.O. Endophytic fungal diversity in *Theobroma cacao* (cacao) and *T. grandiflorum* (cupuacu) trees and their potential for growth promotion and biocontrol of black-pod disease. **Fungal Biology,** Netherlands, v.114, n.11-12, p.901-910, 2010.
13. Hansen, A.J. Fusaria as agents of cacao green point cushion gall in the Caribbean and in Latin America. **The Plant Disease Reporter**, Maryland, v.50, n.4, p.229-233, 1966.
14. Holliday, P. **Fungus Diseases of Tropical Crops**. Cambridge: Cambridge University Press, 1980. 622p.
15. Huelsenbeck, J.P.; Ronquist, F. MRBAYES: Bayesian inference of phylogeny. **Bioinformatics**, Oxford, v.17, n.8, p.754-755, 2001.
16. Jaiyeola, I.; Akinrinlola, R.; Ige, G.; Omoleye, O.; Oyedele, A.; Odunayo, B.; Emehin, O.; Bello, M.; Adesemoye, T. Bot canker pathogens could complicate the management of *Phytophthora* black pod of cocoa. **African Journal of Microbiology Research,** Nigeria, v.8, p.3094-3100, 2014.
17. Kearse, M.; Moir, R.; Wilson, A.; Stones-Havas, S.; Cheung, M.; Sturrock, S.; Buxton, S.; Cooper, A.; Markowitz, S.; Duran, C.; Thierer, T.; Ashton, B.; Mentjies, P.; Drummond, A. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. **Bioinformatics**, Oxford, v.28, n.12, p.1647-1649, 2012.
18. Kuhnert, E.; Fournier, J.; Peršoh, D.; Jennifer, J.; Luangsa-Ard, D.; Stadler, M. New *Hypoxylon* species from Martinique and new evidence on the molecular phylogeny of *Hypoxylon* based on ITS rDNA and β-tubulin data. **Fungal Diversity**, Kunming, v.64, p.181–203, 2014.
19. Li, G.J.; Hyde, K.D.; Zhao, R.L.; Hongsanan, S. Fungal diversity notes 253-366: taxonomic and phylogenetic contributions to fungal taxa. **Fungal Diversity**, Kunming, v.78, p.1-237, 2016.
20. Liu, J.-K.; Phookamsak, R.; Doilom, M.; Wikee, S.; Li, Y.-M.; Ariyawansha, H.; Boonmee, S.; Chomnunti, P.; Dai, D.-Q.; Bhat, J. D.; Romero, A. I.; Zhuang, W.-Y.; Monkai, J.; Gareth Jones, E. B.; Chukeatirote, E.; Ko, T.W.K.; Zhao, Y.-C.; Wang, Y.; Hyde, K.D. Towards a natural classification of *Botryosphaeriales*. **Fungal Diversity**, Kunming, v.57, p.149-210, 2012.
21. Liu, Y.J.; Whelen, S.; Hall, B.D. Phylogenetic relationships among ascomycetes: evidence from an RNA polymerse II subunit. **Molecular Biology and Evolution**, Oxford, v.16, n.12, p.1799-1808, 1999.
22. Martínez de la Parte, E.; Pérez Vicente, L. Incidencia de enfermedades fúngicas en plantaciones de cacao de las provincias orientales de Cuba. **Revista de Protección Vegetal**, Mayabeque, Cuba, v.30, n.2, p.87-96, 2015.
23. Mehl, J. W. M.; Slippers, B.; Roux, J.; Wingfield, M. J. *Botryosphaeriaceae* associated with *Pterocarpus angolensis* (kiaat) in South Africa. **Mycologia**, London, v.103, n.3, p.534-553, 2011.
24. Minin, V.; Abdo, Z.; Joyce, P.; Sullivan, J. Performance-based selection of likelihood models for phylogeny estimation. **Systematic Biology**, Oxford, v.52, n.5, p.674-683, 2003.
25. Mohali, S.R.; Slippers, B.; Wingfield, M.J. Identification of *Botryosphaeriaceae* from *Eucalyptus*, *Acacia* and *Pinus* in Venezuela. **Fungal Diversity**, Kunming, v.25, p.103-125, 2007.
26. Motamayor, J.C.; Risterucci, A.M.; Lopez, P.A.; Ortiz, C.F.; Moreno, A.; Lanaud, C. Cacao domestication I: the origin of the cacao cultivated by the Mayas. **Heredity**, Glasgow, Scotland, v.89, n.5, p.380-386, 2002.
27. Netto, M.S.; Assuncao, I.P.; Lima, G.S.; Marques, M.W.; Lima, W.G.; Monteiro, J.H.A.; Balbino, V.Q.; Michereff, S.J.; Phillips, A.J.L.; Camara, M.P.S. Species of *Lasiodiplodia* associated with papaya stem-end rot in Brazil. **Fungal Diversity**, Kunming, v.67, p.127-141, 2014.
28. Nirenberg, H. Studies on the morphological and biological differentiation in the *Fusarium* section Liseola. Mitteilungen, Berlin-Dahlem, n.169, p.1-117, 1976.
29. O'Donnell, K. *Fusarium* and its near relatives. In: Reynolds, D.R.; Taylor, J.W. (ed.). **The fungal holomorph**: Mitotic, meiotic and pleomorphic speciation in fungal systematics. Wallingford: CABI Agriculture and Bioscience, 1993. p.225-233.
30. O'Donnell, K.; Cigelnik, E. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. **Molecular Phylogenetics and Evolution**, Oxford, v.7, n.1, p.103-116, 1997.
31. O'Donnell, K.; Kistler, H. C.; Cigelnik, E.; Ploetz, R.C. Multiple evolutionary origins of the fungus causing Panama disease of banana: concordant evidence from nuclear and mitochondrial gene genealogies. **Proceedings of the National Academy of Sciences**, Washington, DC, v.95, n.5, p.2044-2049, 1998.
32. O'Donnell, K.; Rooney, A.P.; Proctor, R.H.; Brown, D.W.; McCormick, S.P.; Ward, T.J.; Frandsen, R.J.N.; Lysøe, E.; Rehner, S.A.; Aoki, T.; Robert, V.A.R.G.; Crous, P.W.; Groenewald, J.Z.; Kang, S.; Geiser, D.M. Phylogenetic analyses of RPB1 and RPB2 support a middle Cretaceous origin for a clade comprising all agriculturally and medically important fusaria. **Fungal Genetics and Biology**, USA, v.52, p.20-31, 2013.
33. O'Donnell, K.; Sutton, D.A.; Rinaldi, M.G.; Gueidan, C.; Crous, P.W.; Geiser, D.M. Novel multilocus sequence typing scheme reveals high genetic diversity of human pathogenic members of the *Fusarium incarnatum-F. equiseti* and *F. chlamydosporum* species complexes within the United States. **Journal of Clinical Microbiology**, Washington, DC, v.47, n.12, p.3851-3861, 2009.
34. Palma, M. El cultivo del cacaotero en la Región Central. El Agricultor Venezolano (Caracas, Ven). **Ministerio de Agricultura y Cría**, Caracas, v.18 n.196, p.28-33, 1953.
35. Parra, D.; Pérez, S.; Sosa, D.; Rumbos, R.; Gutiérrez, B.; Moya, A. Avances en las investigaciones venezolanas sobre enfermedades del cacao. **Revista de Estudios Transdisciplinarios**, Caracas, v.1, n.2, p.56-75, 2009.
36. Pérez, L.; Martínez de la Parte, E.; Cantillo, T. First Report in Cuba of green point gall of cocoa cushion caused by *Albonectria rigidiuscula* (*Fusarium decemcellulare*). **Fitosanidad**, La Habana, v.16, n.2, p.19-25, 2002.
37. Phillips, A.J.L.; Alves, A.; Abdollahzadeh, J.; Slippers, B.; Wingfield, M.J.; Groenewald, J.Z.; Crous, P.W. The *Botryosphaeriaceae*: genera and species known from culture**. Studies in Mycology**, Utrecht, v.76, p.51-167, 2013.
38. Ploetz, R.C. *Fusarium*-Induced Diseases of Tropical, Perennial Crops. **Phytopathology**, St. Paul, v.96, n.6, p.648-652, 2006.
39. Reyes, H.; Capriles de Reyes, L. **El Cacao en Venezuela**. Caracas: Chocolates El Rey C.A, 2000. 270p.
40. Rodriguez-Galvez, E.; Guerrero, P.; Barradas, C.; Crous, P.W.; Alves, A. Phylogeny and pathogenicity of *Lasiodiplodia* species associated with dieback of mango in Peru. **Fungal Biology**, Netherlands, v.121, n.4, p.452-465, 2017.
41. Ronquist, F.; Teslenko, M.; van der Mark, P.; Ayres, D.; Darling, A.; Höhna, S.; Larget, B.; Liu, L.; Suchard, M.; Huelsenbeck, J. MrBayes v.3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. **Systematic Biology**, Oxford, v.61, n.3, p.539-542, 2012.
42. Schultes, R.E. Amazonian cultigens and their northward and westward migrations in pre-Columbian times. In: Stone, D. (ed.). **Pre-Columbian plant migration**. Cambridge: Harvard University Press, 1984. v.76, p.69-83.
43. Slippers, B.; Crous, P.W.; Jami, F.; Groenewald, J.Z.; Wingfield, M.J. Diversity in the *Botryosphaeriales*: Looking back, looking forward. **Fungal Biology**, Netherlands, v.121, n.4, p.307-321, 2017.
44. Slippers, B.; Roux, J.; Wingfield, M.J.; Van Der Walt, F.J.J.; Jami, F.; Mehl, J.W.M.; Marais, G.J. Confronting the constraints of morphological taxonomy in the fungi: a *Botryosphaeriaceae* case study. **Persoonia**, Netherlands, v.33, p.155-168, 2014.

45. Sosa del Castillo, D.; Parra, D.; Noceda, C.; Perez-Martinez, S. Co-occurrence of pathogenic and non-pathogenic *Fusarium decemcellulare* and *Lasiodiplodia theobromae* isolates in cushion galls disease of cacao (*Theobroma cacao* L.). **Journal of Plant Protection Research**, Poznań, v.56, n.2, p.129-138, 2016.
46. Twumasi, P.; Ohene-Mensah, G.; Moses, E. The rot fungus *Botryodiplodia theobromae* strains cross infect cocoa, mango, banana and yam with significant tissue damage and economic losses. **African Journal of Agricultural Research**, Nigeria, v.9, n.6, p.613-619, 2014.
47. Urdaneta, L.M.; Delgado, A.E. Identificación de la micobiota del filoplano del cacaotero (*Theobroma cacao* L.), en el municipio Carraciolo Parra Olmedo, estado Mérida, Venezuela. **Revista de la Facultad de Agronomía**, Caracas, v.24, n.1, p.47-68, 2007.
48. White, T.J.; Bruns, T.; Lee, S.; Taylor, J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenies. In: Innis, M.A.; Gelfand, D.H.; Sninsky, J.J.; White, T.J. (ed.). **PCR protocols**: A guide to methods and applications. San Diego: Academic Press, 1990. p.315-322.
49. Zhang, D.; Motilal, L. Origin, Dispersal, and Current Global Distribution of Cacao Genetic Diversity. In: Bailey, B.; Meinhardt, L. (ed.). **Cacao Diseases**, **A History of Old Enemies and New Encounters**. Switzerland: Springer International Publishing, 2016. p.3-31.

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