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Species of *Lasiodiplodia* associated with papaya stem-end rot in Brazil

Mariote S. B. Netto · Iraíldes P. Assunção · Gaus S. A. Lima · Marília W. Marques · Waléria G. Lima · Jean H. A. Monteiro · Valdir de Queiroz Balbino · Sami J. Michereff · Alan J. L. Phillips · Marcos P. S. Câmara

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Abstract This study aims to identify and characterize species of *Lasiodiplodia* associated with stem-end rot of papaya in six different populations in the Northeast of Brazil. Fungal identifications were made using a combination of morphology together with a phylogenetic analysis based on partial translation elongation factor 1-α sequence (EF-1α) and internal transcribed spacers (ITS). Five species of *Lasiodiplodia* were identified: *Lasiodiplodia brasiliense* sp. nov., *L. hormozganensis, L. marypalme* sp. nov., *L. pseudotheobromae* and *L. theobromae.* Only *L. theobromae* had previously been reported in papaya, while all the other species are reported for the first time in association with this host in Brazil and worldwide. *Lasiodiplodia theobromae* was the most prevalent species. All species of *Lasiodiplodia* were pathogenic on papaya fruit, with *L. hormozganensis* being the most virulent.

Keywords Botryosphaeriaceae · *Carica papaya* · Fruit rot · EF1-α · ITS · Phylogeny · Virulence

M. S. B. Netto · M. W. Marques · W. G. Lima · S. J. Michereff · M. P. S. Câmara (✉)
Departamento de Agronomia, Universidade Federal Rural de Pernambuco, 52171-900 Recife, Pernambuco, Brazil
e-mail: mcamara@depa.ufrpe.br

I. P. Assunção · G. S. A. Lima · J. H. A. Monteiro
Centro de Ciências Agrárias, Universidade Federal de Alagoas, 57100-000 Rio Largo, Alagoas, Brazil

V. de Queiroz Balbino
Departamento de Genética, Universidade Federal de Pernambuco, 50732-970 Recife, Brazil

A. J. L. Phillips
Centro de Recursos Microbiológicos, Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal

Introduction

Brazil is the second-largest producer and exporter of papaya (*Carica papaya* L.) worldwide, surpassed only by India. Annual production represents approximately 17 % of total world production, which in 2011 was equivalent to 1.85 million tons (FAO 2013). Most papaya fruits are destined for fresh-market consumption. Any morphological abnormality, especially lesions caused by pathogens, renders the fruit unsuitable for the retail market. Fruit rots caused by fungi are the most common diseases found in the markets (Dantas and Oliveira 2006). Stem-end rot caused by *Lasiodiplodia theobromae* (Pat.) Griff. & Maubl. is an important postharvest disease of this crop in Brazil and worldwide. Disease incidence can reach around 70–80 %, with a resulting reduction in the commercial value of the fruit (Paull et al. 1997; Dantas et al. 2003; Freire et al. 2003; Pereira et al. 2012).

Symptoms of papaya stem-end rot begin at the stem and advance throughout the entire fruit. Stem end-rot arises after harvest on the region of the cut end of the peduncle, affecting the basal part of the fruit, generally at the beginning of maturation. Lesions caused by *L. theobromae* are dark with a wide margin of watery tissue and a surface wrinkled owing to the eruption of the pycnidia. Pockets of growth of mycelium occur in the tissues of the infected parenchyma. In a longitudinal cut of the fruit, the vascular tissue is dark (Ventura et al. 2004).

Lasiodiplodia theobromae is a members of the Botryosphaeriaceae, a genus-rich family in the Dothideomycetes, containing numerous species with a cosmopolitan distribution that occur on a large variety of plant hosts, on which they are found as saprophytes, parasites, and endophytes (von Arx 1987; Slippers and Wingfield 2007; Liu et al. 2012; Wikee et al. 2013). *Lasiodiplodia* species are common, especially in tropical and subtropical regions where they cause a variety of diseases in up to 500 plant hosts

(Punithalingam 1980). The main features that distinguish this genus from other closely related genera are the presence of pycnidial paraphyses and longitudinal striations on mature conidia (Sutton 1980; Phillips et al. 2008).

In Brazil, several other crops of economic importance are affected by *L. theobromae*, especially avocado (*Persea americana* Mill.), banana (*Musa* spp.), barbados cherry (*Malpighia glabra* L.), cashew (*Anacardium occidentale* L.), citrus (*Citrus* spp.), coconut palm (*Cocos nucifera* L.), custard apple (*Annona squamosa* L.), grapevine (*Vitis* sp.), guava (*Psidium guajava* L.), mango (*Mangifera indica* L.), muskmelon (*Cucumis melo* L.), passion fruit (*Passiflora edulis* Sims), soursop (*Annona muricata* L.) and watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) (Tavares 2002; Freire et al. 2003).

The taxonomic history of *L. theobromae* is confused. During the past 150 years this fungus has had many names and has been treated as many different species. This trend ended with the monograph of Punithalingam (1976) which reduced most species to synonymy with *L. theobromae*. In recent years, the use of molecular tools has been offering meaningful advances at the species identification of *Lasiodiplodia* and 16 new species have been reported since 2004 (Pavlic et al. 2004; Burgess et al. 2006; Damm et al. 2007; Alves et al. 2008; Pavlic et al. 2008; Abdollahzadeh et al. 2010; Begoude et al. 2010; Ismail et al. 2012; Úrbez-Torres et al. 2012; Phillips et al. 2013; Slippers et al. 2013).

In Brazil, seven species of *Lasiodiplodia* were reported (Costa et al. 2010; Marques et al. 2013). However, only *L. theobromae* has been reported on papaya (Tavares 2002; Dantas et al. 2003; Freire et al. 2003; Ventura et al. 2004; Dantas and Oliveira 2006; Pereira et al. 2012). However, identifications were based primarily on morphological and cultural data, which is now considered to be unreliable for species discrimination since the morphological characteristics overlap with other species of *Lasiodiplodia* (Costa et al. 2010).

Therefore, considering the recent studies on the taxonomy of the genus *Lasiodiplodia* and the absence of molecular data in the identification of Brazilian isolates, we speculate that other *Lasiodiplodia* species might be associated with stem-end rot of papaya in Brazil. Consequently, the objectives of this study were (1) identify *Lasiodiplodia* isolates using morphological characters and phylogenetic analyses, (2) investigate the prevalence and distribution of the species in the Northeast of Brazil and (3) to evaluate their pathogenicity and virulence in papaya fruit.

Materials and methods

Sampling and fungal isolation

During 2006 and 2007, isolates of *Lasiodiplodia* were obtained from 18 papaya orchards located in Northeastern Brazil. These isolates represented six papaya populations (A to F) according to their geographical origin (Fig. 1). All orchards received at least one spray with methyl benzimidazolecarbamates (MBC), demethylation inhibitors (DMI), quinone outside inhibitor (QoI) or other fungicides. Samples of papaya fruits (20 samples per orchard) showing stem-end rot were recovered from the cultivars Baixinho de Santa Amélia, Calimosa, Formosa, Golden e Hawai. Fruit tissues were surface disinfested in 70 % ethanol for 30 s and 1 % NaOCl for 1 min. Samples were then rinsed in sterile distilled water for 30 s and dried before small pieces (4–5 mm) of tissue were taken from the margin between necrotic and apparently healthy tissue to be plated onto potato dextrose agar (PDA) (Acumedia, Lansing, USA) amended with 0.5 g l^{-1} streptomycin sulfate (PDAS). Plates were incubated at 25 °C in the dark for 3 to 4 days. Fungal colonies emerging from plant tissue pieces that were morphologically similar to species of Botryosphaeriaceae (Sutton 1980; Phillips 2006) were transferred to PDA plates and incubated at 25 °C in the dark, with observation after 3, 7 and 15 days. To obtain single-spore isolates, pycnidia were induced on 2 % water agar (WA) with autoclaved pine needles as a substrate after 3-week incubation at 25 °C under a 12 h daily photoperiod with near-ultraviolet light (Slippers et al. 2004). A single conidium was cut from each isolate under a stereo microscope (Zeiss Stemi DV4; Carl Zeiss, Berlin, Germany) and placed in 250 μl of sterile water to produce a conidial suspension. A 20 μl aliquot was spread on PDAS and incubated at 28 °C in the dark for 24 h. A single-conidia isolate was recovered for an individual sample and transferred to a fresh PDA plate. One-hundred and sixty six isolates were morphologically identified as *Lasiodiplodia* based on morphological characteristics typical of the genus, namely conidiomatal paraphyses, conidia that were initially hyaline and aseptate, but in time developed a single median septum, the wall became dark brown and melanin granules deposited longitudinally on the inner surface of the wall giving the conidia a striate appearance (Sutton 1980; Alves et al. 2008). Stock cultures were stored in PDA slants at 5 °C in the dark.

DNA isolation, PCR amplification and sequencing

Using a sterile 10 μl pipette tip, a small amount of aerial mycelium was scraped from the surface of a 7 day old culture on PDA at 25 °C and genomic DNA was extracted using the AxyPrep™ Multisource Genomic DNA Miniprep Kit (Axygen Scientific Inc., Union City, USA) following the manufacturer's instructions. A portion of the translation elongation factor 1α (EF1-α) gene was sequenced for all the *Lasiodiplodia* isolates collected from papaya orchards. The internal transcribed spacer (ITS) region of rDNA was sequenced to confirm the identity of representative isolates within each EF1-α identified species. The ITS region was amplified using the primers ITS1 and ITS4 (White et al.

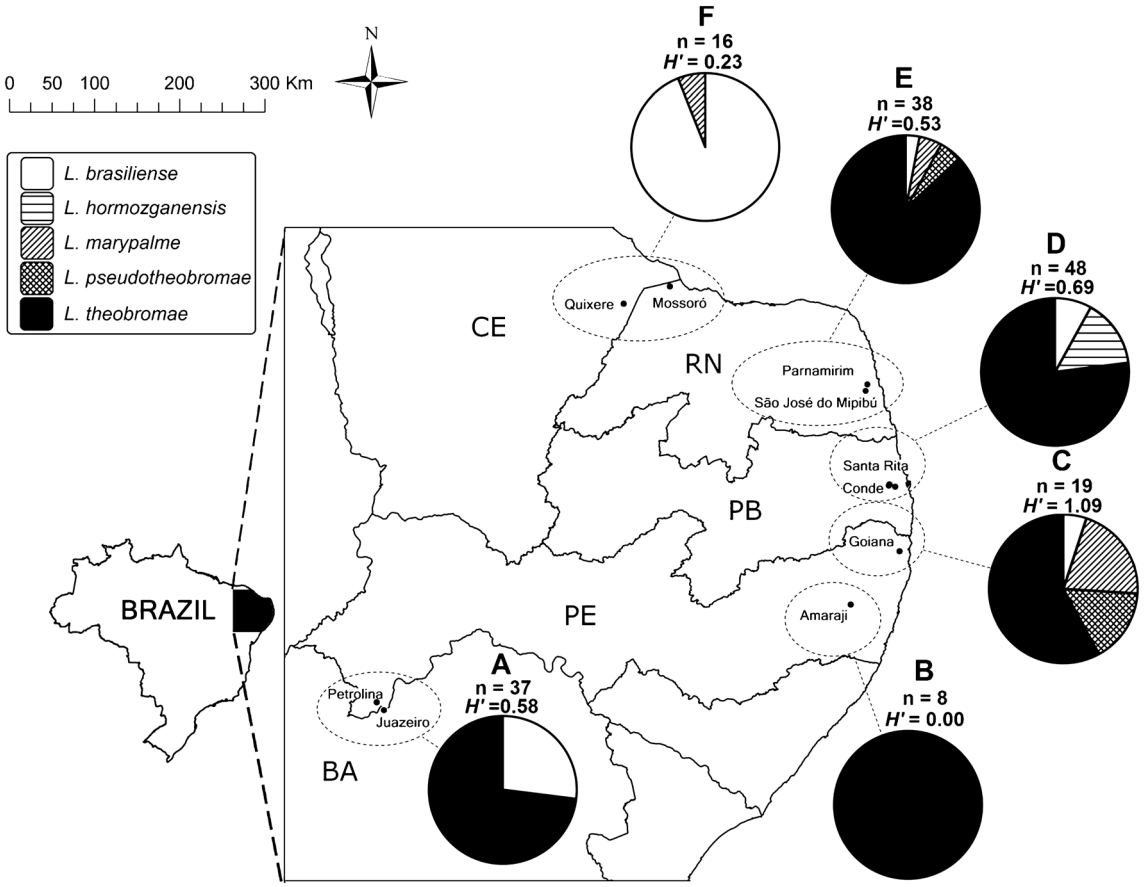

Fig. 1 Collection sites of *Lasiodiplodia* isolates associated with stem-end rot of papaya in six different populations located in the states of Bahia (*BA*), Pernambuco (*PE*), Paraíba (*PB*), Rio Grande do Norte (*RN*) and Ceará (*CE*), Brazil. *Circles* represent association frequency of each species with fruits exhibiting symptoms of stem-end rot in each population sampled, *n* is the number of isolates analyzed in each population, and *H′* is the Shannon-Wiener's diversity index

1990) as described by Slippers et al (2004) and EF1-α gene was amplified using the primers EF1-688F and EF1-1251R (Alves et al. 2008) as described by Phillips et al. (2005). Each 50 μl polymerase chain reaction (PCR) mixture included 21 μl of PCR-grade water, 1 μl of DNA template, 1.5 μM of each primer, and 1 μl of PCR Master Mix (2×) (0.05 u μl^{-1} de *Taq* DNA polimerase, reaction buffer, 4 mM MgCl2, 0.4 mM of each dNTP; Thermo Scientific,Waltham, USA). PCR reactions were carried out in a thermal cycler (Biocycler MJ 96; Applied Biosystems, Foster City, USA). The PCR amplification products were separated by electrophoresis in 1.5 % agarose gels in 1.0× Tris-acetate acid EDTA (TAE) buffer and were photographed under UV light after staining with ethidium bromide (0.5 μg ml^{-1}) for 1 min. The PCR amplification products were separated by electrophoresis in 1.5 % agarose gels in 1.0× Tris-acetate acid EDTA (TAE) buffer and were photographed under UV light after staining with ethidium bromide (0.5 μg ml^{-1}) for 1 min. PCR products were purified using the AxyPrep™ PCR Cleanup Kit (Axygen) following the manufacturer's instructions. ITS and EF1-α regions were sequenced in both directions using a ABI 3730 XL DNA Analyzer (Applied Biosystems) at the Macrogen Inc. (Seoul, Korea).

Phylogenetic analyses

Sequences were aligned with ClustalX v. 1.83 (Thompson et al. 1997), using the following parameters: pairwise alignment parameters (gap opening = 10, gap extension = 0.1) and multiple alignment parameters (gap opening = 10, gap extension = 0.2, transition weight = 0.5, delay divergent sequences = 25 %). Alignments were checked and manual adjustments were made where necessary. Phylogenetic information contained in indels (gaps) was incorporated into the phylogenetic analyses using simple indel coding as implemented by GapCoder (Young and Healy 2003). Sequences of *Lasiodiplodia* type strains obtained from GenBank were included in the analyses (Table 1). *Diplodia seriata* De Not. (CBS 112555) and *D. mutila* Fr. (CBS 112553) were used as outgroup.

Phylogenetic analyses were performed using PAUP v. 4.0b10 (Swofford 2003) for maximum-parsimony and MrBayes v. 3.0b4 (Ronquist and Huelsenbeck 2003) for

Table 1 Isolates of *Lasiodiplodia* species used in this study

Taxon	Culture accession no.[a]	Host	Location	Collector	GenBank accession no.[b]	
					ITS	EF-1α
Diplodia mutila	CBS 112553	*Vitis vinifera*	Portugal	A.J.L. Phillips	AY259093	AY573219
D. seriata	CBS 112555	*V. vinifera*	Portugal	A.J.L. Phillips	AY259093	AY573220
Lasiodiplodia brasiliense	CMM 2184	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484801**	**KC481531**
L. brasiliense	CMM 2185	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484800**	**KC481530**
L. brasiliense	CMM 2186	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484812**	**KC481542**
L. brasiliense	CMM 2188	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484807**	**KC481537**
L. brasiliense	CMM 2212	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484806**	**KC481536**
L. brasiliense	CMM 2248	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484794**	**KC481525**
L. brasiliense	CMM 2249	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484796**	**KC481527**
L. brasiliense	CMM 2251	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484808**	**KC481538**
L. brasiliense	CMM 2253	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484802**	**KC481532**
L. brasiliense	CMM 2255	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484792**	**KC481523**
L. brasiliense	CMM 2256	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484805**	**KC481535**
L. brasiliense	CMM 2257	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484803**	**KC481533**
L. brasiliense	CMM 2258	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484799**	**KC481584**
L. brasiliense	CMM 2259	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484811**	**KC481541**
L. brasiliense	CMM 2260	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484795**	**KC481526**
L. brasiliense	CMM 2266	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484810**	**KC481540**
L. brasiliense	CMM 2292	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484804**	**KC481534**
L. brasiliense	CMM 2312	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484791**	**KC481583**
L. brasiliense	CMM 2313	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484793**	**KC481524**
L. brasiliense	CMM 2314	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484813**	**KC481543**
L. brasiliense	CMM 2315	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484809**	**KC481539**
L. brasiliense	CMM 2318	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484815**	**KC481545**
L. brasiliense	CMM 2319	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484798**	**KC481529**
L. brasiliense	CMM 2320	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484814**	**KC481544**
L. brasiliense	CMM 2321	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484797**	**KC481528**
L. brasiliense	CMM 4015	*Mangifera indica*	Brazil	M.W. Marques	JX464063	JX464049
L. citricola	CBS 124707	*Citrus sp.*	Iran	J. Abdollahzadeh & A. Javadi	GU945354	GU945340
L. citricola	IRAN 1521C	*Citrus sp.*	Iran	A. Shekari	GU945353	GU945339
L. crassispora	CMW 13488	*Eucalyptus urophylla*	Venezuela	S. Mohali	DQ103552	DQ103559
L. crassispora	CMW 14691	*Santalum album*	Australia	T.I. Burgess & G. Pegg	DQ103550	DQ103557
L. egyptiacae	BOT 29	*M. indica*	Egypt	A.M. Ismail	JN814401	JN814428
L. egyptiacae	CBS 130992	*M. indica*	Egypt	A.M. Ismail	JN814397	JN814424
L. gilaniensis	CBS 124704	*Unknown*	Iran	J. Abdollahzadeh & A. Javadi	GU945351	GU945342
L. gilaniensis	IRAN 1501C	*Unknown*	Iran	J. Abdollahzadeh & A. Javadi	GU945352	GU945341
L. gonubiensis	CBS 115812	*Syzigium cordatum*	South Africa	D. Pavlic	DQ458892	DQ458860
L. gonubiensis	CMW 14078	*S. cordatum*	South Africa	D. Pavlic	AY639594	DQ103567
L. hormozganensis	CBS 124709	*Olea sp.*	Iran	J. Abdollahzadeh & A. Javadi	GU945355	GU945340
L. hormozganensis	CMM 2214	*Carica papaya*	Brazil	J.H.A. Monteiro	**KC484837**	**KC481561**
L. hormozganensis	CMM 2215	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484835**	**KC481559**
L. hormozganensis	CMM 2216	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484836**	**KC481560**
L. hormozganensis	CMM 2218	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484833**	**KC481557**
L. hormozganensis	CMM 2219	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484834**	**KC481558**
L. hormozganensis	IRAN 1498C	*M. indica*	Iran	J. Abdollahzadeh & A. Javadi	GU945356	GU945344
L. iraniensis	CBS 124710	*Salvadora persica*	Iran	J. Abdollahzadeh & A. Javadi	GU945348	GU945336
L. iraniensis	IRAN 1502C	*Juglans sp.*	Iran	A. Javadi	GU945347	GU945335

Table 1 (continued)

Taxon	Culture accession no.[a]	Host	Location	Collector	GenBank accession no.[b]	
					ITS	EF-1α
L. mahajangana	CBS 124927	*Terminalia catappa*	Madagascar	J. Roux	FJ900597	FJ900643
L. mahajangana	CMW 27801	*T. catappa*	Madagascar	J. Roux	FJ900595	FJ900641
L. margaritaceae	CBS 122065	*Adansonia gibbosa*	Western Australia	T.I. Burgess	EU144051	EU144066
L. margaritaceae	CBS 122519	*A. gibbosa*	Western Australia	T.I. Burgess	EU144050	EU144065
L. marypalme	CMM 2173	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484839**	**KC481563**
L. marypalme	CMM 2271	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484844**	**KC481568**
L. marypalme	CMM 2272	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484842**	**KC481566**
L. marypalme	CMM 2274	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484841**	**KC481565**
L. marypalme	CMM 2275	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484843**	**KC481567**
L. marypalme	CMM 2289	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484840**	**KC481564**
L. marypalme	CMM 2298	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484838**	**KC481562**
L. missouriana	CBS 128311	*V. vinifera*	Missouri, USA	K. Striegler & G.M. Leavitt	HQ288225	HQ288267
L. missouriana	UCD 2199MO	*V. vinifera*	Missouri, USA	K. Striegler & G.M. Leavitt	HQ288226	HQ288268
L. parva	CBS 456.78	*Cassava-field soil*	Colombia	O. Rangel	EF622083	EF622063
L. parva	CBS 494.78	*Cassava-field soil*	Colombia	O. Rangel	EF622084	EF622064
L. plurivora	CBS 120832	*Prunus salicina*	South Africa	U. Damm	EF445362	EF445395
L. plurivora	STE-U 4583	*V. vinifera*	South Africa	F. Halleen	AY343482	EF445396
L. pseudotheobromae	CBS 116459	*Gmelina arborea*	Costa Rica	J. Carranza-Velásquez	EF622077	EF622057
L. pseudotheobromae	CBS 116459	*G. arborea*	Costa Rica	J. Carranza-Velásquez	EF622077	EF622057
L. pseudotheobromae	CMM 2170	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484830**	**KC481554**
L. pseudotheobromae	CMM 2171	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484832**	**KC481556**
L. pseudotheobromae	CMM 2287	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484831**	**KC481555**
L. pseudotheobromae	CMM 2288	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484829**	**KC481553**
L. pseudotheobromae	IRAN 1518C	*Citrus sp.*	Iran	J. Abdollahzadeh/A. Javadi	GU973874	GU973866
L. rubropurpurea	WAC 12535	*E. grandis*	Queensland	T.I. Burgess & G. Pegg	DQ103553	DQ103571
L. rubropurpurea	WAC 12536	*E. grandis*	Queensland	T.I. Burgess & G. Pegg	DQ103554	DQ103572
L. theobromae	CBS 111530	*Unknown*	Unknown	Unknown	AY622074	AY622054
L. theobromae	CBS 164.96	*Fruit on coral reef coast*	New Guinea	A. Aptroot	AY640255	AY640258
L. theobromae	CMM 2168	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484817**	**KC481572**
L. theobromae	CMM 2179	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484787**	**KC481569**
L. theobromae	CMM 2183	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484824**	**KC481573**
L. theobromae	CMM 2190	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484780**	**KC481518**
L. theobromae	CMM 2193	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484826**	**KC481550**
L. theobromae	CMM 2208	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484776**	**KC481575**
L. theobromae	CMM 2209	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484784**	**KC481578**
L. theobromae	CMM 2210	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484783**	**KC481577**
L. theobromae	CMM 2231	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484775**	**KC481515**
L. theobromae	CMM 2232	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484785**	**KC481521**
L. theobromae	CMM 2235	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484779**	**KC481517**
L. theobromae	CMM 2237	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484819**	**KC481547**
L. theobromae	CMM 2238	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484771**	**KC481512**
L. theobromae	CMM 2239	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484786**	**KC481522**
L. theobromae	CMM 2241	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484790**	**KC481571**
L. theobromae	CMM 2261	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484789**	**KC481579**
L. theobromae	CMM 2262	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484822**	**KC481581**
L. theobromae	CMM 2265	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484772**	**KC481574**
L. theobromae	CMM 2267	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484777**	**KC481576**

Table 1 (continued)

Taxon	Culture accession no.[a]	Host	Location	Collector	GenBank accession no.[b]	
					ITS	EF-1α
L. theobromae	CMM 2268	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484818**	**KC481580**
L. theobromae	CMM 2269	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484821**	**KC481585**
L. theobromae	CMM 2276	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484820**	**KC481548**
L. theobromae	CMM 2278	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484781**	**KC481519**
L. theobromae	CMM 2280	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484773**	**KC481513**
L. theobromae	CMM 2282	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484827**	**KC481551**
L. theobromae	CMM 2294	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484828**	**KC481552**
L. theobromae	CMM 2295	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484774**	**KC481514**
L. theobromae	CMM 2297	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484823**	**KC481582**
L. theobromae	CMM 2303	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484816**	**KC481546**
L. theobromae	CMM 2306	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484788**	**KC481570**
L. theobromae	CMM 2310	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484782**	**KC481520**
L. theobromae	CMM 2327	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484778**	**KC481516**
L. theobromae	CMM 2328	*C. papaya*	Brazil	J.H.A. Monteiro	**KC484825**	**KC481549**
L. venezuelensis	WAC 12539	*Acacia mangium*	Venezuela	S. Mohali	DQ103547	DQ103568
L. venezuelensis	WAC 12540	*A. mangium*	Venezuela	S. Mohali	DQ103548	DQ103569
L. vitícola	CBS 128313	*V. vinifera*	USA	R.D. Cartwright & W.D. Gubler	HQ288227	HQ288269
L. vitícola	UCD 2604MO	*V. vinifera*	USA	K. Striegler & G.M. Leavitt	HQ288228	HQ288270

[a] *CBS* Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; *CMW* Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; *WAC* Department of Agriculture Western Australia Plant Pathogen Collection, University of Western Australia, Perth, Australia; *CMM* Culture Collection of Phytopathogenic Fungi "Prof. Maria Menezes", Universidade Federal Rural de Pernambuco, Recife, Brazil; *STE-U* Culture Collection of the Department of Plant Pathology, University of Stellenbosch, Stellenbosch, South Africa; *UCD* Phaff Yeast Culture Collection, Department of Food Science and Technology, University of California, Davis, USA; *BOT* A. M. Ismail, Plant Pathology Research Institute, Giza, Egypt; *IRAN* Culture Collection of the Iranian Research Institute of Plant Protection, Tehran, Iran

[b] Sequence numbers in *bold* were obtained in the present study

Bayesian analyses. Maximum-parsimony analyses were performed using the heuristic search option with 1,000 random taxa addition and tree bisection and reconnection (TBR) as the branch-swapping algorithm. All characters were unordered and of equal weight and gaps were treated as missing data. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the most parsimonious trees was evaluated from 1,000 bootstrap replications (Hillis and Bull 1993). Other measures used were consistency index (CI), retention index (RI) and homoplasy index (HI). In the Bayesian analyses, trees were sampled every 100th generation for a total of 10 000 trees. The first 1 000 trees were discarded as the burn-in phase of each analysis. Posterior probabilities (Rannala and Yang 1996) were determined from a majority-rule consensus tree generated with the remaining 9 000 trees. This analysis was repeated three times starting from different random trees to ensure trees from the same tree space were sampled during each analysis

Phylogenetic trees were viewed with Treeview (Page 1996). Sequences generated in this study were deposited in GenBank (Table 1) and the alignment in TreeBase (S14682). Representative isolates of different *Lasiodiplodia* species obtained in this study were deposited in the Culture Collection of Phytopathogenic Fungi "Prof. Maria Menezes" (CMM) at the Universidade Federal Rural de Pernambuco (Recife, Brazil).

Morphology and cultural characteristics

The 74 *Lasiodiplodia* isolates that were identified in the phylogenetic analysis using the combined data set were used to study colony morphology and conidial characteristics. The color and aerial hyphal growth from isolates were recorded during 15 days of growth on 2 % malt extract agar (MEA) (Acumedia) at 25 °C in the dark. Colony colors were recorded as per Rayner (1970). Characteristics of conidial morphology were observed after placing cultures on 2 % WA containing autoclaved pine needles and incubation under near-ultraviolet light, as previously described. Conidia and other structures were mounted in 100 % lactic acid and digital images recorded with a Leica DFC320 camera on a Leica DMR HC microscope fitted with Nomarski differential interference contrast optics (Leica Microsystems Imaging Solutions Ltd., Cambridge, UK). The length and width of 50 conidia per

isolate were measured with the Leica IM500 measurement module. Mean and standard errors of the conidial measurements, including mean length to width ratio (L/W) of the conidial measurements were calculated. Conidial color, shape, and presence or absence of septa was also recorded.

Isolates were also used to determine the effect of temperature on colony growth of different species. A 3-mm-diameter mycelial plug from the growing margin of a 3-day-old colony was placed in the center of a 90-mm-diameter 2 % MEA plate, and four replicates of each isolate were incubated at temperatures ranging from 10 °C to 35 °C in 5 °C intervals in the dark. After a 2-days incubation period, the colony diameter (mm) was measured in two perpendicular directions. The experiment was done twice. Colony diameters were plotted against temperature and a curve was fitted by a cubic polynomial regression ($y = a + bx + cx^2 + dx^3$). Optimal temperature was estimated from the regression equation and numeric summary with TableCurve™ 2D v. 5.01 (SYSTAT Software Inc., Chicago, USA). Optimum temperature was defined as the temperature that produced the maximum mycelial growth. The colony diameter data at 30 °C were used to calculate the mycelial growth rate (mm/day). One-way analyses of variance (ANOVA) were conducted with data obtained from optimum temperature and mycelial growth rate experiments, and means were compared by Fisher's least significant difference (LSD) test at the 5 % significance level using STATISTIX v. 9.0 (Analytical Software, Tallahassee, USA).

Distribution and diversity of *Lasiodiplodia* species

Based on the number of isolates of each *Lasiodiplodia* species recorded, it was calculated the relative frequency of each species in relation to overall number of isolates and to the total number of isolates within each papaya population (Zak and Willig 2004). The diversity of *Lasiodiplodia* species was estimated in terms of species richness (number of species in the sample) and evenness (dominance of species in the sample) by the Shannon-Wiener's index $H' = \Sigma_j (p_j \ln p_j)$, j = 1…… N_p, where N_p is the number of species identified among these isolates, and p_j is the proportion of individuals in the j^{th} species. The H' values increases with the number of species in a sample or reduces as one or a few species domain in the sample (Shannon and Weaver 1949). To quantify the degree of overlap between the *Lasiodiplodia* species in the papaya populations, a measure of the similarity between pairs of samples was calculated by the Jaccard's index $JI = a/(a + b + c)$, where a represents the number of species occurring in both samples, b represents the number of species restricted to sample 1, and c represents the number of species restricted to sample 2. The JI values ranges from 0 (no species shared) 1 (all species shared) (Kumar and Hyde 2004).

Pathogenicity and virulence in fruits

The isolates used in the morphological characterization were selected for this test. Papaya fruits (cv. Golden) at stage four of maturation (Ministério da Integração Nacional 2000), without visible signs of disease and that had not been treated with fungicides, were washed in running water, surface disinfested in 70 % ethanol for 1 min and 1 % NaOCl for 5 min, then rinsed in sterile distilled water. Since non-wounded treatment caused no lesions of *Lasiodiplodia* (unpublished data), after drying each fruit was wounded at the medium region by pushing the tip of four sterile pins through the surface of the skin to a depth of 3 mm. A mycelial plug (5 mm in diameter) removed from the margin of a 7-day-old PDA culture of each isolate was immediately placed into the wound. For the control, a non-colonized agar plug was used. Inoculated fruits were placed in large plastic containers. Before, the bottom of each container was lined with four paper layers wetted in distilled water to maintain humidity. Each fruit was put on a sterilized Petri dish to avoid direct contact with water. The plastic containers were partially sealed with plastic bags and incubated at 28 °C in the dark. The plastic bags and paper towels were removed after 24 h, and fruits were kept at the same temperature. Isolates were considered pathogenic when the lesioned area advanced beyond the 5-mm diameter initial injury. The virulence of the isolates was evaluated by measurement of the lesion length at 72 h after inoculation in two perpendicular directions on each fruit. The lesion length (l) and lesion width (w) data were combined to determine the lesion area using the Equation $A = \pi lw$, where A is the area of an oval (Sakalidis et al. 2011). The experiment was conducted twice. The experiment was arranged in a completely randomized design with six replicates per treatment (isolate) and one fruit per replicate. The experiment was conducted twice. Differences in virulence caused by *Lasiodiplodia* species were determined by one-way ANOVA and means were compared by LSD test at the 5 % significance level using STATISTIX.

Results

DNA sequencing and phylogenetic analyses

A total of 166 isolates of *Lasiodiplodia* spp. were obtained from papaya fruits. All the isolates were identified based on phylogenetic analysis of the partial translation elongation factor 1α (EF-1α) gene. To confirm the identity of the isolates, the internal transcribed spacer (ITS) sequence was obtained for 74 isolates representing each putative species. The combined ITS and EF-1α data set consists of 110 taxa, including two outgroup. The alignment contained 750 characters, of which 549 were constant while 45 were variable and parsimony uninformative. A heuristic search of the remaining 156

parsimony-informative characters generated 48 equally parsimonious trees with (TL = 357; CI = 0.748; RI = 0.910; HI = 0.252). Maximum-parsimony and Bayesian inference produced nearly identical topologies (Bayesian tree not shown). Sequences of ex-type isolates of *Lasiodiplodia* species from GenBank were included in the analysis together with isolates obtained in this study (Table 1). The combined dataset resulted in 19 well supported clades of which 17 clades corresponded to previously described *Lasiodiplodia* species while two clades did not cluster with any known species, which indicates that these isolates represent a new species. The isolates obtained in this study grouped into five distinct clades. The majority (33 isolates) clustered together in a large clade containing *L. theobromae* (CBS 164.96; CBS111530). The second group with 25 isolates did not cluster with any known species. Five isolates clustered with *L. hormozganensis* (IRAN 1498C; CBS 124709), four with *L. pseudotheobromae* (CBS 116459; IRAN 1518C) and seven isolates formed a clade distinct from any known species (Fig. 2).

Fig. 2 One of 48 most parsimonious trees (TL=357; CI=0.748; RI=0.910; HI=0.252) obtained from combined ITS and EF1-α sequence data. Maximum parsimony bootstrap support values from 1,000 replications and Bayesian posterior probability scores are shown at the nodes. Ex-type isolates are in *bold*. The Bar represents 10 changes

Morphology and cultural characteristics

The 74 *Lasiodiplodia* isolates [*L. hormozganensis* (25), *L. pseudotheobromae* (4), *L. theobromae* (33), *Lasiodiplodia* sp. 1 (25) and *Lasiodiplodia* sp. 2 (7)] that were identified based in the phylogenetic analysis using the combined data were further characterized by colony morphology and conidial characteristics. All isolates produced anamorph structures on the pine needles on WA within 2–4 weeks. No teleomorph structures were observed during this study. All species showed morphological features typical of the genus, namely slowly maturing conidia with thick walls and longitudinal striations (Punithalingam 1976, 1980). All isolates grew rapidly on PDA, covering the entire surface of the Petri dishes within 4 days. The aerial mycelium was initially white, turning dark greenish-grey or greyish after 4–5 days at 25 °C in the dark. The species of *Lasiodiplodia* found in this study show differences in conidial size. The conidial dimensions found in *L. pseudotheobromae* and *L. theobromae* are outside of the range previously described for these species in the literature (Table 2).

Taxonomy

Lasiodiplodia brasiliense M.S.B. Netto, M.W. Marques & A.J.L. Phillips **sp. nov**. MycoBank MB807525; Fig. 3a–e

Etymology: The name refers to Brazil, the country where this fungus was first found.

Ascomata not seen. *Conidiomata*: stromatic, pycnidial, produced on pine needles on WA within 2–4 weeks, superficial, dark brown to black, covered with dense mycelium, mostly uniloculate, solitary, globose, thick-walled, non-papillate with a central ostiole. *Paraphyses*: hyaline, cylindrical, aseptate, rounded at apex. *Conidiophores*: absent. *Conidiogenous cells*: holoblastic, discrete, hyaline, smooth, thin-walled, cylindrical. *Conidia:* initially hyaline, aseptate, ellipsoid to ovoid, with granular content, rounded at apex, base mostly truncate, wall <2 μm, becoming pigmented, verruculose, ellipsoid to ovoid, 1-septate with longitudinal striations, 22.7–29.2 × 11.7–17.0 μm ($\overline{x} = 26.01 \pm 1.36 \times 14.64 \pm 1.16$, $n = 50$).

Culture characteristics: aerial mycelia becoming smoke-grey (21’’’’f) to olivaceous-grey (21’’’’i) at the surface and Mouse grey (17’’’’i) to olivaceous grey (19’’’’i). Colonies reaching 80 mm on MEA after 2 days in the dark at 25 °C. Optimum temperature for mycelial growth: 31.9 ± 2.04 °C, covering the surface of 90 mm diam. Petri dishes within 3 days on MEA in the dark

Substrate: *Carica papaya, Mangifera indica*

Known Distribution: Brazil (Pernambuco, Paraíba, Rio Grande do Norte, Ceará).

Holotype: Brazil, Pernambuco, Farm Dan (40° 41′ 49″, 9° 22′ 35″), on *Mangifera indica* stems, 2010, coll. M.W. Marques, holotype URM (85580) a dry culture on pine needles, ex-holotype living culture CMM 4015 = URM 7118.

Specimen examined: Brazil, Rio Grande do Norte, Ipanguaçu, Farm Sede (36°51′ 05″, 5°28′53″), on *Mangifera indica* fruit, 2010, coll. M.W. Marques (CMM 4011). Brazil, Pernambuco, Petrolina, Farm AGS Frutas (40°28′ 09.8″, 9°18′ 25.3″), on *Carica papaya* fruit, 2007, coll. J.H.A. Monteiro (CMM 2320). Brazil, Ceará, Quixere, Farm Frutacor (37° 51′ 24.1″, 05° 05′ 09.4″), on *Carica papaya* fruit, 2007, coll. J.H.A. Monteiro (CMM 2257). Brazil, Ceará, Quixere, Farm Frutacor (37° 22′ 06.4″, 04° 54′ 13.4″), on *Carica papaya* fruit, 2007, coll. J.H.A. Monteiro (CMM 2249). Brazil, Rio Grande do Norte, Ipanguaçu, Farm São João (36°53′03,4″, 5°31′28,8″), on *Mangifera indica* stems, Jan 2010, coll. M.W. Marques (CMM 4010). Brazil, Ceará, Quixere, Farm Frutacor (37° 22′ 06.4″, 04° 54′ 13.4″), on *Carica papaya* fruit, 2007, coll. J.H.A. Monteiro (CMM 2249). Brazil, Pernambuco, Petrolina, Farm AGS Frutas (40°28′ 09.8″, 9°18′25.3″), on *Carica papaya* fruit, 2007, coll. J.H.A. Monteiro (CMM 2314).

Notes — Phylogenetically *Lasiodiplodia brasiliense* is closely related to *L. viticola*, but conidia of *L. brasiliense*, 22.7–29.2 × 11.7–17.0 μm, are longer and wider than those of *L. viticola* 18.2–20.5 × 8.8–10.1 μm. *Lasiodiplodia brasiliense* differs from its closest phylogenetic neighbor, *L. viticola*, by unique fixed alleles in one loci based on alignments of the separate loci deposited in TreeBase as study

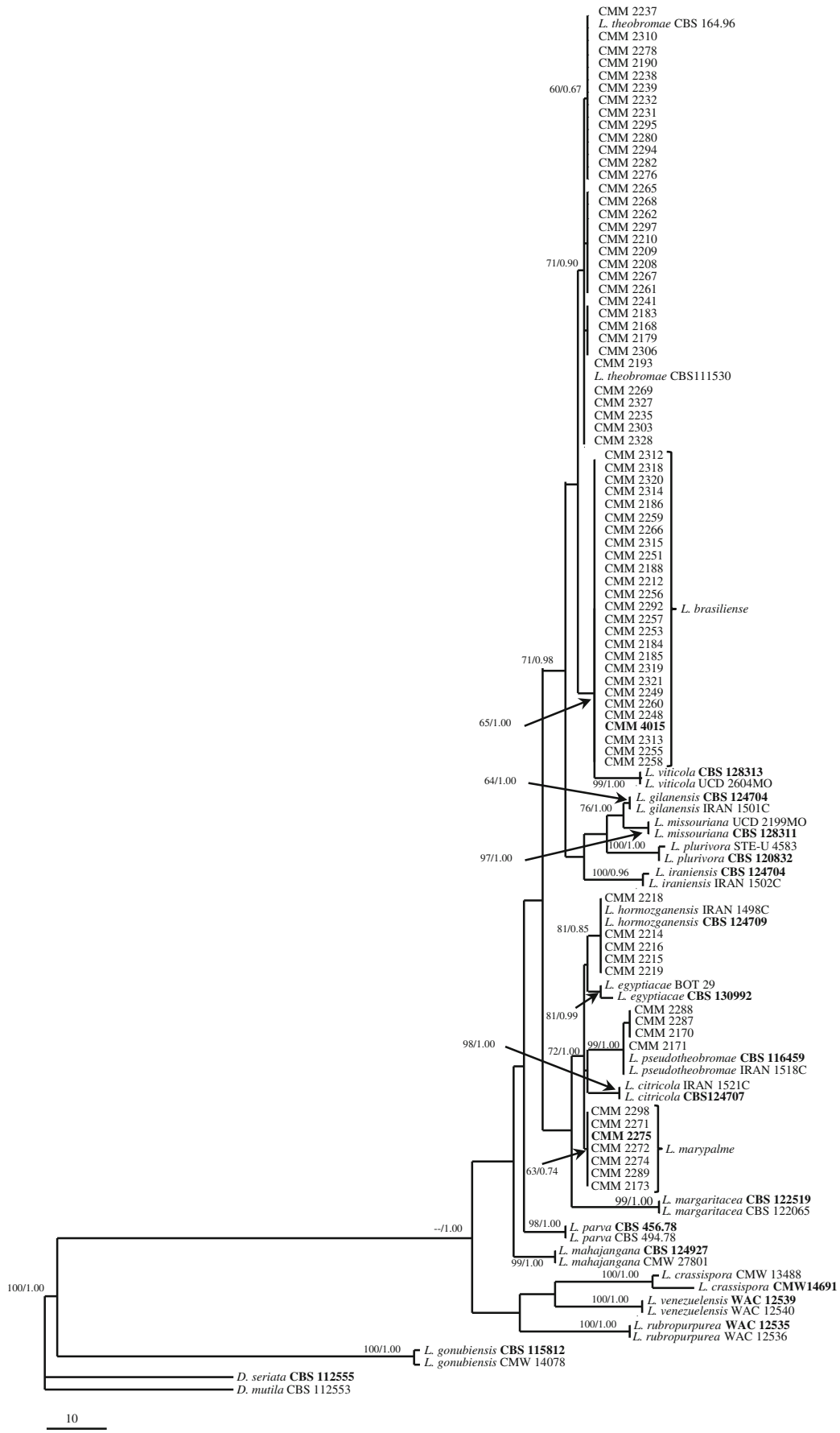
CMM 2237
L. theobromae CBS 164.96
CMM 2310
CMM 2278
CMM 2190
CMM 2238
CMM 2239
CMM 2232
CMM 2231
CMM 2295
CMM 2280
CMM 2294
CMM 2282
CMM 2276
CMM 2265
CMM 2268
CMM 2262
CMM 2297
CMM 2210
CMM 2209
CMM 2208
CMM 2267
CMM 2261
CMM 2241
CMM 2183
CMM 2168
CMM 2179
CMM 2306
CMM 2193
L. theobromae CBS111530
CMM 2269
CMM 2327
CMM 2235
CMM 2303
CMM 2328
CMM 2312
CMM 2318
CMM 2320
CMM 2314
CMM 2186
CMM 2259
CMM 2266
CMM 2315
CMM 2251
CMM 2188
CMM 2212
CMM 2256
CMM 2292
CMM 2257
CMM 2253
CMM 2184
CMM 2185
CMM 2319
CMM 2321
CMM 2249
CMM 2260
CMM 2248
CMM 4015
CMM 2313
CMM 2255
CMM 2258
L. brasiliense
L. viticola CBS 128313
L. viticola UCD 2604MO
L. gilanensis CBS 124704
L. gilanensis IRAN 1501C
L. missouriana UCD 2199MO
L. missouriana CBS 128311
L. plurivora STE-U 4583
L. plurivora CBS 120832
L. iraniensis CBS 124704
L. iraniensis IRAN 1502C
CMM 2218
L. hormozganensis IRAN 1498C
L. hormozganensis CBS 124709
CMM 2214
CMM 2216
CMM 2215
CMM 2219
L. egyptiacae BOT 29
L. egyptiacae CBS 130992
CMM 2288
CMM 2287
CMM 2170
CMM 2171
L. pseudotheobromae CBS 116459
L. pseudotheobromae IRAN 1518C
L. citricola IRAN 1521C
L. citricola CBS124707
CMM 2298
CMM 2271
CMM 2275
CMM 2272
CMM 2274
CMM 2289
CMM 2173
L. marypalme
L. margaritacea CBS 122519
L. margaritacea CBS 122065
L. parva CBS 456.78
L. parva CBS 494.78
L. mahajangana CBS 124927
L. mahajangana CMW 27801
L. crassispora CMW 13488
L. crassispora CMW14691
L. venezuelensis WAC 12539
L. venezuelensis WAC 12540
L. rubropurpurea WAC 12535
L. rubropurpurea WAC 12536
L. gonubiensis CBS 115812
L. gonubiensis CMW 14078
D. seriata CBS 112555
D. mutila CBS 112553
60/0.67
71/0.90
71/0.98
65/1.00
64/1.00
99/1.00
76/1.00
100/1.00
97/1.00
100/0.96
81/0.85
81/0.99
98/1.00
99/1.00
72/1.00
63/0.74
99/1.00
98/1.00
--/1.00
99/1.00
100/1.00
100/1.00
100/1.00
100/1.00
100/1.00
100/1.00
10

Table 2 Comparison of conidial dimensions of *Lasiodiplodia* species examined in this study and previous studies

Species	Conidial size (μm)	L/W ratio	References
Lasiodiplodia brasiliense	22.7–29.2×11.7–17.0	1.8	Present study
L. hormozganensis	19–22.8×10.7–11.7	1.9	Present study
	19.6–23.4×11.7–13.3	1.7	Abdollahzadeh et al. 2010
L. marypalme	18.0–24.4×9.8–15.3	1.9	Present study
L. pseudotheobromae	21.2–25.8×12.5–13.9	1.8	Present study
	23.5–32.0×14.0–18.0	1.7	Alves et al. 2008
L. theobromae	20.7–22.7×11.7–14.1	1.8	Present study
	23.6–28.8×13–15.4	1.9	Alves et al. 2008

S14682: ITS positions 8 (T), 12 (A), 16 (C), 22 (GAP), 28 (GAP), 41 (GAP) 89 (T) and 336 (A);

Lasiodiplodia marypalme M.S.B. Netto, M.W. Marques, A.J.L. Phillips & M.P.S. Câmara **sp. nov**. MycoBank MB807526; Fig. 4a–d

Etymology: named in honor of Mary E. Palm, United States Department of Agriculture - APHIS, for her contribution to mycology.

Ascomata not seen. *Conidiomata*: stromatic, pycnidial, produced on pine needles on WA within 2–4 weeks, superficial, dark brown to black, covered with dense mycelium, mostly uniloculate, solitary, globose, thick-walled, non-papillate with a central ostiole. *Paraphyses*: hyaline, cylindrical, aseptate, rounded at apex. *Conidiophores:* absent. *Conidiogenous cells*: holoblastic, discrete, hyaline, smooth, thin-walled, cylindrical. *Conidia*: initially hyaline, aseptate, ellipsoid to ovoid, with granular content, rounded at apex, base mostly truncate, wall <2 μm, becoming pigmented, verruculose, ellipsoid to ovoid, 1-septate with longitudinal striations, 19.1–28.5 × 10–15.3 μm, ($\overline{x} = 21.2 \pm 3.2 \times 11.4 \pm 1.6$, $n = 50$)

Culture characteristics: *Colonies* with abundant aerial mycelia reaching to the lid of Petri plate, aerial mycelia becoming mouse grey (13””i) to pale mouse grey (17””i), at the surface and grey olivaceous (21””b) to olivaceous black (27””m) at the reverse. Colonies reaching 39 mm on MEA after 2 days in the dark at 25 °C. Optimum temperature for mycelial growth: 32.8 ± 2.12 °C, covering the surface of 90 mm diam. Petri dishes within 3 days on MEA in the dark

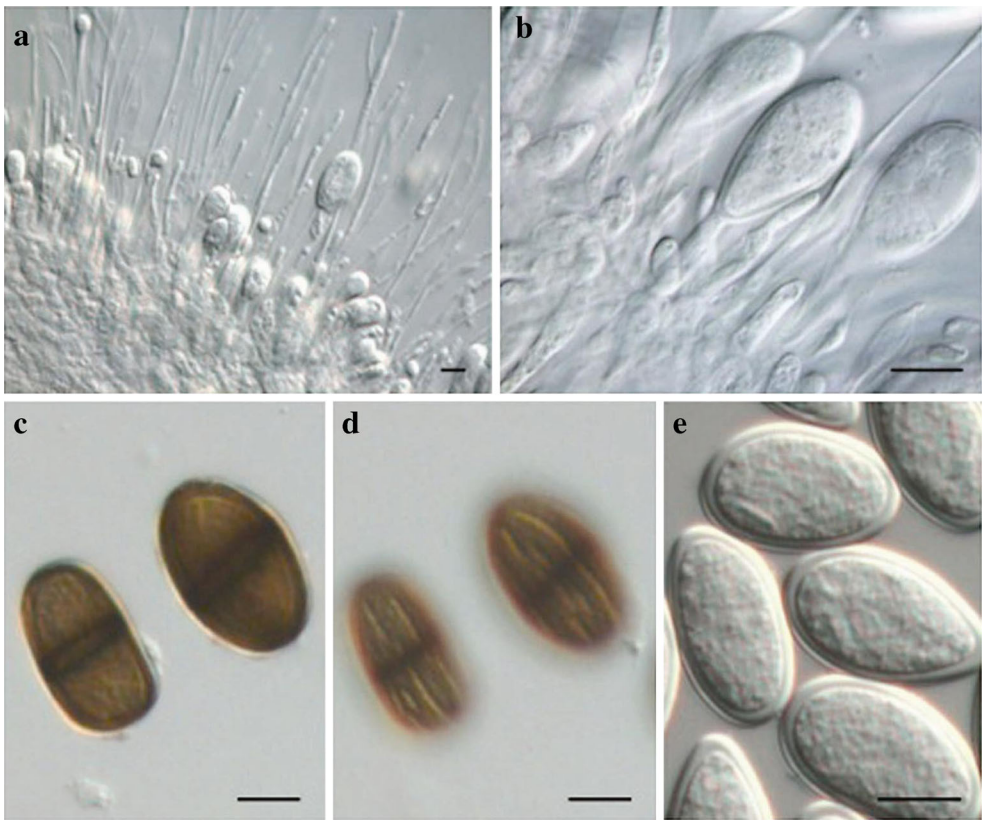

Fig. 3 *Lasiodiplodia brasiliense* holotype. **a**–**b** Conidia developing on conidiogenous cells between paraphyses. **c**–**d** Mature conidia in two different focal planes to show the longitudinal striations. **e** Hyaline, immature conidia. Scale bars: **a**–**e** = 10 μm

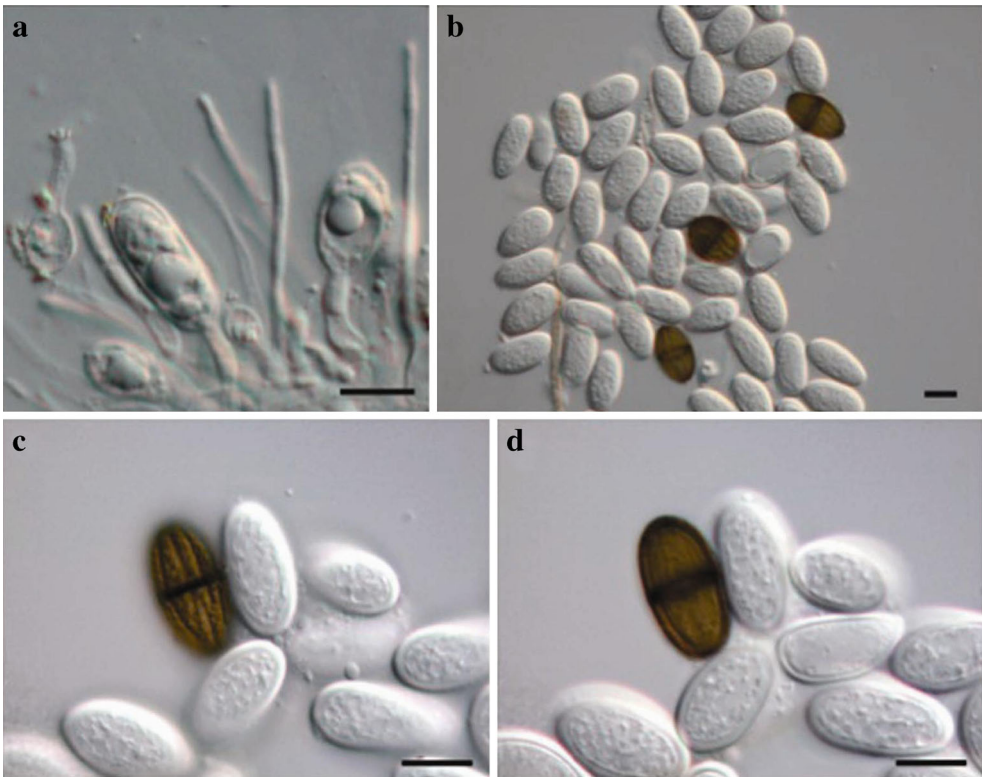

Fig. 4 *Lasiodiplodia marypalme* holotype. **a** Conidia developing on conidiogenous cells between paraphyses. **b** Hyaline immature conidia and mature conidia. **c**–**d** Mature conidia in two different focal planes to show the longitudinal striations. Scale bars: **a**–**d**=10 μm

Substrate: *Carica papaya*

Known Distribution: Brazil (Pernambuco, Rio Grande do Norte).

Holotype: Brazil, Pernambuco, Goiana, Farm Ubu 92 (34° 56′ 16.5″, 7° 42′ 32.6″), on *Carica papaya* fruit, 2007, coll. J.H.A. Monteiro, holotype URM (85579) a dry culture on pine needles, ex-holotype living culture CMM 2275 = URM 7117.

Specimen examined: Brazil, Pernambuco, Goiana, Farm Ubu 92 (34° 56′ 16.5″, 07° 42′ 32.6″), on *Carica papaya* fruit, 2007, coll. J.H.A. Monteiro (CMM 2272). Brazil, Pernambuco, Goiana, Farm Ubu 92 (34° 56′ 16.5″, 07° 42′ 32.6″), on *Carica papaya* fruit, 2007, coll. J.H.A. Monteiro (CMM 2271). Brazil, Pernambuco, Goiana, Farm Ubu 92 (34° 56′ 16.5″, 07° 42′ 32.6″), on *Carica papaya* fruit, 2007, coll. J.H.A. Monteiro (CMM 2274). Brazil, Rio Grande do Norte, Parnamirim, Farm Arco Verde (35° 16′ 36.4″, 05° 56′ 30.2″), on *Carica papaya* fruit, 2006, coll. J.H.A. Monteiro (CMM 2173). Brazil, Rio Grande do Norte, São José do Mipibú, Farm Vale do Lírio (35° 17′ 48.8″, 06° 00′ 50.2″), *Carica papaya* fruit, 2007, coll. J.H.A. Monteiro (CMM 2298). Brazil, Rio Grande do Norte, São José do Mipibú, Farm Vale do Lírio (37° 22′ 06.4″, 04° 54′ 13.4″), on *Carica papaya* fruit, 2007, coll. J.H.A. Monteiro (CMM 2289).

Notes — Phylogenetically *Lasiodiplodia marypalme* is closely related to *L. pseudotheobromae* and *L. citricola*, but conidia of *L. citricola*, 22.5–26.6 × 13.6–17.2 μm and *L. pseudotheobromae L. pseudotheobromae* 23.5–32 × 14–18, are longer and wider than those of *L. marypalme*, 18.0–24.4 × 9.8–15.3 μm. *Lasiodiplodia marypalme* differs from its closest phylogenetic neighbor, *L. pseudotheobromae* and *L. citricola*, by unique fixed alleles in two loci based on alignments of the separate loci deposited in TreeBase as study S14682: ITS positions 12 (A), 68 (C), 86 (T) and 409 (C); EF-1α positions 19(C) and 43 (T), 51 (T), 128 (GAP), 166 (G), 185 (A), 220 (G), 221 (C) and 229 (T).

Distribution and diversity of *Lasiodiplodia* species

Lasiodiplodia theobromae was the predominant species isolated from papaya fruits (69.9 %) followed by *L. brasiliense* (18.7 %), *L. hormozganensis* (4.2 %), *L. marypalme* (4.2 %) and *L. pseudotheobromae* (3.0 %). The distribution of *Lasiodiplodia* species differed between the six papaya populations of Northeastern Brazil. *Lasiodiplodia theobromae* was the predominant species in five populations (A, B, C, D and E), but was not found in population F. *Lasiodiplodia brasiliense* was found in all populations, except in the population B, and was the predominant species in population F. *Lasiodiplodia marypalme* were found in populations C, E and F, while *L. hormozganensis* was found only in population D. *Lasiodiplodia pseudotheobromae* was found in populations C and E (Fig. 1).

A comparison of the Shannon-Wiener's diversity index (H') showed the highest diversity in the population E (H' = 1.09), followed by the populations C (H' = 0.69), A (H' = 0.0.58) and E (H' = 0.53). The population B had the lowest

diversity (H' = 0.00), being dominated by only one species (*L. theobromae*) (Fig. 1).

The comparison between the *Lasiodiplodia* species recovered from different papaya populations was computed using a Jaccard's index for possible pairs of populations. The highest overlap (JI = 1.00) was observed for the *Lasiodiplodia* species from populations C and E, followed by populations A and D (JI = 0.67). The lowest value of similarity (JI = 0.00) was observed between populations B and F, but two nearby populations (B–C) also showed low species overlap (JI = 0.25).

Mycelial growth

All species of *Lasiodiplodia* used in this study grew at 10 °C. There were no significant differences ($P > 0.05$) in the optimum temperature for mycelial growth and mycelial growth rate among the *Lasiodiplodia* species. The optimum temperature varied from 31.4 °C (*L. hormozganensis*) to 32.8 °C (*L. marypalme*), while the mycelial growth rate ranged from 35.7 mm/day (*L. hormozganensis*) to 40.6 mm/day (*L. pseudotheobromae*). The mycelial growth rates for new species *L. brasiliense* and *L. marypalme* were 36.6 mm/day and 39.7 mm/day, respectively.

Pathogenicity and virulence in fruits

All isolates of *Lasiodiplodia* were pathogenic to papaya fruits, resulting in visible lesions 72 h after inoculation. Observed symptoms on the fruit surface were dark brown necrotic lesions with roughly circular shape around the inoculation sites. There were significant ($P \leq 0.05$) differences in virulence among the species, wherein *L. hormozganensis* was most virulent, causing the largest lesion (29.0 mm). The other species (*L. brasiliense*, *L. marypalme, L. pseudotheobromae* and *L. theobromae*) were less virulent, with lesions varying from 14.7 mm to 16.4 mm and did not differ significantly from each other (Fig. 5).

Discussion

This study represents the first survey of species of *Lasiodiplodia* associated with stem-end rot of papaya in Brazil in an extensive collection of isolates, and integrating morphology, pathology and molecular data. Five species of *Lasiodiplodi*a were identified as causing stem-end rot in papaya: *L. brasiliense*, *L. hormozganensis, L. marypalme, L. pseudotheobromae* and *L. theobromae*. Except for *L. theobromae*, all the other species are reported for the first time on papaya worldwide.

In this work, *L. theobromae* was the most frequently isolated species associated with stem-end rot of papaya (69.9 %), and was the most widespread *Lasiodiplodia* species in Northeastern Brazil. Similar results were obtained when the frequency of *Lasiodiplodia* species associated with dieback and stem-end rot of mango was investigated in the semi-arid region of Northeastern Brazil (Marques et al. 2013). This species is considered a pantropical pathogen occurring in a wide range of hosts (Punithalingam 1980; Burgess et al. 2006). In Brazil, only *L. theobromae* had been reported in papaya, perhaps due to the fact that in the earlier studies molecular methods were not used to confirm the etiology of stem-end rot. In this work, four more species were found in this host. Worldwide, several species have been described in the *L. theobromae* complex, mostly because of the increase in the application of DNA sequence data, but also because of the increased sampling of relatively unexplored areas, including Venezuela (Burgess et al. 2006), Australia (Pavlic et al. 2008), Iran (Abdollahzadeh et al. 2010), Egypt (Ismail et al. 2012), Brazil (Marques et al. 2013), Oman and United Arab Emirates (Al-Sadi et al. 2013).

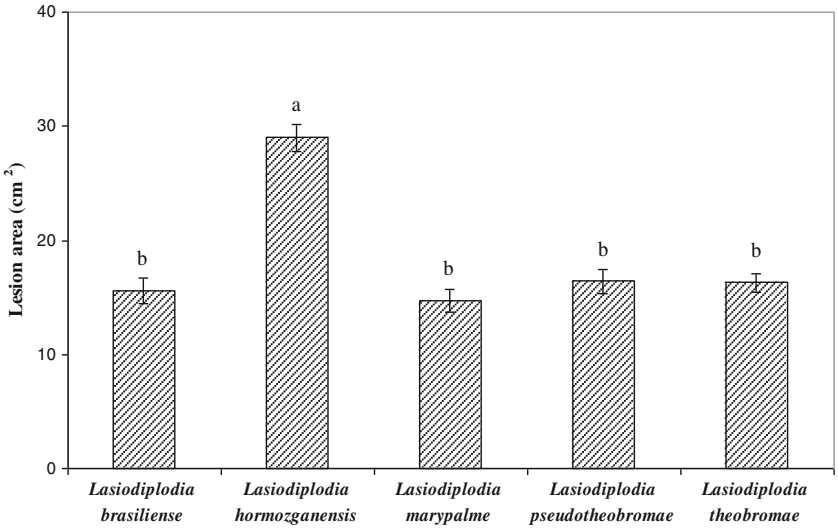

Fig. 5 Mean lesion areas (cm^2) caused by five *Lasiodiplodia* species associated with papaya stem-end rot in Northeastern Brazil, 72 h after inoculation with mycelium colonized agar plugs onto wounded fruits of Golden cultivar. Bars above columns are the standard error of the mean. Columns with same letter do not differ significantly according to Fisher's LSD test ($P \leq 0.05$)

Lasiodiplodia brasiliense is recognized as a new species in the genus *Lasiodiplodia*, closely related to *L. viticola*. However, six nucleotide in the ITS region distinguish *L. brasiliense* from *L. viticola*. Considering the phylogenetic data, the papaya isolates of *L. brasiliense* formed a clade strongly supported in the Bayesian analysis (1.00) but with only moderate support in MP (65 %). Although no difference was found in the nucleotide sequence of EF1-α gene, *L. brasiliense* can also be distinguished from *L. viticola* based on conidial size, which are longer and larger than those described for this species (Urbéz-Torres et al. 2012). In this work, *L. brasiliense* was the second most frequent species in papaya orchards in Northeastern Brazil (18.7 %), being pathogenic to this host and not differing in virulence from *L. marypalme, L. pseudotheobromae* and *L. theobromae*.

The second previously undescribed species, *L. marypalme*, is phylogenetically most closely related to *L. citricola* and *L. pseudotheobromae*. However, 7 and 9 nucleotide of ITS and EF1-α regions distinguish *L. marypalme* from *L. citricola* and *L. pseudotheobromae*, respectively. Considering the phylogenetic data, the papaya isolates of *L. marypalme* formed a clade moderated supported both in the Bayesian analysis (0.74) and MP (63 %). Conidia of *L. marypalme* are smaller than those described for *L. citricola* (Abdollahzadeh et al. 2010) and *L. pseudotheobromae* (Alves et al. 2008).

Lasiodiplodia hormozganensis was recently described in Iran associated with mango and *Olea* sp. (Abdollahzadeh et al. 2010), in Australia associated with *Adansonia digitata* L. (Sakalidis et al. 2011), in Brazil associated with mango (Marques et al. 2013) and in Oman associated with *Citrus*, date palm (*Phoenix dactylifera* L.) and mango (Al-Sadi et al. 2013). This work represents the first report of this species causing stem-end rot in *C. papaya* worldwide. In this study, *L. hormozganensis* was the most virulent species in papaya fruit, proving to be a potential threat to this crop. Similar results were found by Sakalidis et al. (2011) and Marques et al. (2013) where *L. hormozganensis* isolates produced the largest lesions in mango branches and fruits, respectively.

Another species associated with stem-end rot of papaya in Brazil was *L. pseudotheobromae*. This species was described from *Acacia*, *Citrus*, *Coffea*, *Gmelina* and *Rosa* species, and differs from *L. theobromae* in its bigger conidia that are more ellipsoid and do not taper as strongly towards the base (Alves et al. 2008). Worldwide, *L. pseudotheobromae* has been reported on numerous hosts (Alves et al. 2008; Phillips et al. 2008; Begoude et al. 2010, Pérez et al. 2010, Wright and Harmon 2010; Zhao et al. 2010; Abdollahzadeh et al. 2010; Sakalidis et al. 2011; Ismail et al. 2012), but in Brazil it has been reported only on *Vitis* spp. (Correia et al. 2013) and mango (Marques et al. 2013). This work represents the first report of this species on papaya worldwide. This shows an increase in the spread of the fungus, suggesting that *L. pseudotheobromae*, like *L. theobromae,* has a worldwide distribution and a wide host range. Regarding the pathogenicity, *L. pseudotheobromae* was the most virulent species in mango fruits in Australia (Sakalidis et al. 2011), in mango seedlings in Egypt (Ismail et al. 2012) and in young trees of *Terminalia catappa* L. in Cameroon (Begoude et al. 2011). However, in the present work, compared with other species, *L. pseudotheobromae* had relatively low level of virulence in papaya fruit, similar to that observed when this species was inoculated in mango fruits in Brazil (Marques et al. 2013). The divergent results indicate that there is a great variability in virulence within this species and that the Brazilian isolates represent a population with low virulence.

Regarding cultural characteristics, the optimum temperature for mycelial growth for *Lasiodiplodia* species from papaya varied between 31.4 °C and 32.8 °C. In addition, all the species in this study grew at 10 °C. This growth at low temperature corroborates the work of Abdollahzadeh et al. (2010) and Marques et al. (2013), and is in contrast to other studies that show only *L. pseudotheobromae* as capable of growing at this temperature (Alves et al. 2008; Ismail et al. 2012). As can be observed, cultural characteristics may vary widely among isolates of the same species and therefore are of limited value in the determination of species.

Regarding the distribution of species in the sampled populations, a greater diversity of *Lasiodiplodia* species was observed in populations C and E, while population B presented lower species diversity. *Lasiodiplodia theobrome* was present in five of the six populations and it was the species that had the largest number of isolates (116). Based on what was observed in the Jaccard's similarity index, populations with close proximity presented low similarity values, indicating that there is no relationship between the geographic proximity of papaya populations and the overlapping of *Lasiodiplodia* species.

This paper reports five species of the genus *Lasiodiplodia* associated with stem-end rot of papaya in Northeastern Brazil. Although *L. theobromae* is the most frequent species it is not the only etiological agent as was previously believed. All the species found in Northeastern Brazil have potential to cause disease to papaya, but *L. hormozganensis* was the most virulent species. Information about this species is scarce due to its recent descriptions (Abdollahzadeh et al. 2010). Studies are needed on the epidemiology and impact on papaya production together with information referring to ecology, distribution, host range and fungicide sensitivity of all species of *Lasiodiplodia* found in this study.

The results of this study will certainly be crucial to a better formulation of control strategies and genetic improvement programs for the papaya crop.

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