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Analysis of phylogeny, distribution, and pathogenicity of *Botryosphaeriaceae* species associated with gummosis of *Anacardium* in Brazil, with a new species of *Lasiodiplodia*

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ABSTRACT

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Introduction

Cashew (*Anacardium occidentale*) is a tropical evergreen crop cultivated worldwide with a centre of origin in the Amazonian forest of Brazil. In contrast to the other seven species within the genus *Anacardium*, only cashew (*A. occidentale*) is an economically important nut crop, with both an edible hypo carp (apple) and nutritious kernel arising from a drupe (Aliyu 2012). It is important as an export commodity, with considerable consumption in Europe and the USA. Brazilian production in 2013 reached 259 900 t, from a production area of 708 430 ha. In 2016, 12 165 t of cashew nuts were exported generating about US$ 79 M. The north-eastern region of Brazil is responsible for 99 % of the country's production (Agrianual 2015), with the cashew industry in rural areas recognized to be of considerable socio-economic importance (Moreira *et al.* 2013).

Of the numerous diseases that compromise cashew production, cashew gummosis, which is caused by *Lasiodiplodia theobromae*, is considered one of the most important diseases for the cashew industry (Cysne *et al.* 2010). This fungal species was first reported on cashew in 1990 (Freire 1991), and was soon recognized as one of the most important diseases of the crop in north-eastern Brazil (Freire *et al.* 2002; Moreira *et al.* 2013). The main symptoms of this disease comprise the appearance of cankers along the trunk or branches, which develop over time and release a characteristic resin-like gum. Gummosis subsequently results in reduced water and nutrient transport, branch dieback, inflorescence blight, reduction in photosynthesis, and eventual plant death (Freire *et al.* 2002; Moreira *et al.* 2013).

To date only *L. theobromae* has been found associated with cashew gummosis (Freire *et al.* 2002; Cardoso *et al.* 2004; Muniz *et al.* 2012; Moreira *et al.* 2013). However, identification of causal agents was based on analysis of fungal morphology and cultural characteristics, which are today considered insufficient for species identification in the genus *Lasiodiplodia* (Phillips *et al.* 2013).

Lasiodiplodia is a member genus of the Botryosphaeriaceae, a family in the Dothideomycetes. This family contains numerous fungal species which occur as saprophytes, parasites or endophytes on a diverse range of plant hosts (Slippers & Wingfield 2007; Phillips *et al.* 2013). In addition to cashew in Brazil, genera of Botryosphaeriaceae such as *Botryosphaeria*, *Fusicoccum*, *Macrophomina*, *Neofusicoccum*, *Neoscytalidium*, and *Pseudofusicoccum* (Marques *et al.* 2013b; Machado *et al.* 2014) have been reported to cause disease in several other economically important crops including avocado (*Persea americana*), banana (*Musa* spp.), barbados cherry (*Malpighia glabra*), cacao (*Theobromae cacao*), castor bean (*Ricinus communis*), citrus (*Citrus* spp.), coconut palm (*Cocos nucifera*), custard apple (*Annona squamosa*), grapevine and table grape (*Vitis* spp.), guaraná (*Paullinia cupana*), guava (*Psidium guajava*), mango (*Mangifera indica*), muskmelon (*Cucumis melo*), papaya (*Carica papaya*), passion fruit (*Passiflora edulis*), physic nut (*Jatropha curcas*), sour sop (*Annona muricata*), and watermelon (*Citrullus lanatus*) (Costa *et al.* 2010; Marques *et al.* 2013a; Machado *et al.* 2014; Netto *et al.* 2014; Correia *et al.* 2016).

Although the taxonomy of the Botryosphaeriaceae has until recently been based upon morphology of asexual morphs, more recent phylogenetic inference based upon analysis of sequence data for target DNA loci has had considerable impact on the systematics of the Botryosphaeriaceae, with increased resolution enabling discrimination of species with overlapping morphological characteristics (de Wet *et al.* 2008; Phillips *et al.* 2013).

Despite the pathogenic importance attributed to Botryosphaeriaceae on diverse host plants, there have been no phylogenetic analyses of this family on cashew. Given the increasing economic importance of cashew gummosis and the recent reports of new species of Botryosphaeriaceae occurring on tropical plants, it is possible that a number of species of this family may be associated with cashew gummosis in Brazil. For effective disease management, a clear understanding of disease aetiology is essential for determination of the distribution of individual species and their disease epidemiology. In this context, the objectives of this study were (i) to identify species of Botryosphaeriaceae associated with cashew gummosis in Brazil, (ii) to determine the prevalence and distribution of each species, and (iii) to characterize isolates in terms pathogenicity and virulence using excised cashew green shoots.

Materials and methods

Isolation of fungal material

During 2013 and 2014, samples were obtained from 30 *Anacardium* orchards, located in six states of Brazil (Alagoas, Ceará, Minas Gerais, Pernambuco, Piauí, and Rio Grande do Norte). In each orchard, a total of 15 *Anacardium* trees exhibiting gummosis symptoms were selected for isolation of fungal material. Symptomatic shoot material at the interface between necrotic and apparently uninfected tissue was surface sterilized using 70 % ethanol for 30 s followed by 1 % NaOCl for 1 min. Following rinsing once with sterile distilled water for 30 s, material was then dried and 4–5 mm fragments plated onto potato dextrose agar (PDA) (Acumedia, Lansing, USA) containing 0.5 g l^{-1} streptomycin sulphate (PDAS). Following incubation at 25 °C in the dark for a period of 3–4 d, all colonies showing morphological characteristic typical of the Botryosphaeriaceae (Sutton 1980; Phillips 2006) were plated onto PDA and incubated at 25 °C in the dark and observed over a period 15 d. Pycnidial production was induced following growth on 2 % water agar (WA) and autoclaved pine needles (PNA) as carbon source. After a 3-week incubation period at 25 °C under a 12 h daily photoperiod with near-ultraviolet light (Slippers *et al.* 2004a), individual pycnidia were from each isolate were examined under a stereo-microscope (Zeiss Stemi DV4; Carl Zeiss, Berlin, Germany), and transferred in 250 µl of sterile distilled water. A 20 µl aliquot of the resultant conidial suspension was spread onto PDAS and incubated at 28 °C in the dark for 24 h. Single spore isolates were prepared for each sample through transfer onto fresh PDA plates. Based upon morphological characteristics typical of the genus, a total of 138 isolates were identified as members of the Botryosphaeriaceae. All isolates were preserved on PDA slants at 5 °C in the dark.

Molecular-based amplification

For identification of the Botryosphaeriaceae, a region of the translation elongation factor 1α (EF1-α) gene was amplified and sequenced for all isolates collected from the cashew orchards. The rDNA internal transcribed spacer (ITS) regions was employed to support species identity based on EF1-α gene sequence data, with a portion of the β-tubulin gene for the fusicoccum-like representative isolates. Total DNA was extracted from aerial mycelium from 7-day-old cultures grown on PDA at 25 °C using an AxyPrep™ Multisource Genomic DNA Miniprep Kit (Axygen Scientific Inc., Union City, USA) according to the manufacturer's instructions.

The target region of the *EF1-α* gene was amplified using primer pairs EF-688F and EF-1251R (Alves *et al.* 2008), as described by Phillips *et al.* (2005). The rDNA ITS region was amplified using universal primers ITS1 and ITS4 (White *et al.* 1990) according to Slippers *et al.* (2004b). A portion of the β-tubulin (*TUB*) gene was amplified using the primers BT2a and BT2b (Glass & Donaldson 1995). Each PCR reaction contained 1 μl of total DNA, 1.5 μM of each primer, 25 μl of 2× PCR Master Mix (Thermo Scientific, Waltham, USA), containing 0.05 U of *Taq* DNA polymerase, 2× reaction buffer, 4 mM $MgCl_2$, and 0.4 mM dNTPs. Reaction volumes were completed to 50 μl volumes using PCR-grade water. Temperature cycling was conducted with a thermo cycler (Biocycler MJ 96; Applied Biosystems, Foster City, USA). PCR products were photodocumented under UV light after staining 1.5 % agarose gels with ethidium bromide (0.5 μg ml^{-1}) for 1 min. Purification of PCR products was performed with the AxyPrep™ PCR Cleanup Kit (Axygen), according to the manufacturer's instructions. The rDNA ITS, EF1-α, and β-tubulin regions were forward and reverse sequenced with an ABI 3730 XL DNA Analyzer (Applied Biosystems).

Phylogenetic analyses

Alignment of sequence data was conducted using ClustalX v. 1.83 (Thompson *et al.* 1997), with the following parameters: pair wise alignment (gap opening = 10, gap extension = 0.1); multiple alignment (gap opening = 10, gap extension = 0.2, transition weight = 0.5, delay divergent sequences = 25 %). Sequences of two isolates of each species of Botryosphaeriaceae including the type strains available in GenBank were also included in the analyses and outgroup (Table 1). Only the type species of *Lasiodiplodia pontae* was included in the analysis because the two other strain available [isolate IBL 14 (GenBank accession number: ITS-KT151795; EF-1α-KT151792) and isolate IBL 18 (GenBank accession number: ITS-151796; EF-1α-KT151793)] did not cluster in the type strain clade (Coutinho *et al.* 2016). Simple indel coding, as implemented by GapCoder (Young & Healy 2003), was employed for incorporation of phylogenetic information present in indels (gaps) into the phylogenetic analyses. Tree robustness was evaluated following 1000 bootstrap replications (Hillis & Bull 1993). Sequence alignments were deposited in TreeBASE (http://www.treebase.org/) under accession number S19242 for *Lasiodiplodia*, S19243 for *Neofusicoccum*, and S19241 for *Pseudofusicoccum*. Phylogenetic analyses were conducted using the programme GENEIOUS v. 7.1.8 (Kearse *et al.* 2012). Maximum likelihood estimation was conducted using a plugin for PhyML (Guindon *et al.* 2010) and Bayesian analyses using MrBayes v. 3.0b4 (Ronquist & Huelsenbeck 2003). Bayesian analysis was performed by four independent runs with the Markov Chain Monte Carlo (MCMC) algorithms (Larget & Simon 1999). Data were partitioned according to locus, with nucleotide substitution model parameters for each partition set as described below. Four parallel MCMC chains were run, with a heating scheme set at 0.3, under a general time-reversible (GTR) substitution model with rate variation of gamma-distribution (G), and proportion of invariable site (I) (Rodríguez *et al.* 1990). Trees were sampled every 1000th generation from a total of 10 000 trees, with the first 2500 trees discarded as representing the burn-in phase of each analysis. The remaining 7500 trees (stationary distribution) were employed for determination of posterior probabilities (Rannala & Yang 1996) and mapping onto the majority-rule consensus tree. FigTree v.1.4.2 (Rambaut 2009) was employed for tree visualization. Representative cultures of the species identified in this study were deposited in the Culture Collection of Phytopathogenic Fungi 'Prof. Maria Menezes' (CMM) at the Universidade Federal Rural de Pernambuco, Brazil.

Morphology and cultural characteristics

Colony morphology and conidial characteristics were examined for a total of 33 representative isolates among the Botryosphaeriaceae species identified following phylogenetic analysis. Colony colour and aerial hyphae were recorded after 15 d of growth on 2 % malt extract agar (MEA) (Acumedia) at 25 °C in the dark. Colony colours were examined according to Rayner (1970). Conidial morphology characteristics were examined after growth under near-ultraviolet light on PNA, as previously described. Digital images for conidia and other structures mounted in 100 % lactic acid were recorded using a Leica DFC320 camera fitted to a Leica DMR HC microscope with Nomarski differential interference contrast optics (Leica Microsystems Imaging Solutions Ltd., Cambridge, UK). The Leica IM500 measurement module was employed to determine the length and width of 50 conidia per isolate, with mean values and standard errors calculated for all measurements. Conidial shape, colour, and septation were also recorded.

The effect of temperature on colony growth was examined across the different species identified. Four replicates were included per isolate, with experiments performed in duplicate. Mycelial plugs isolated from the growing margin of 3-day-old colonized plates were transferred onto 2 % MEA plates and incubation the dark at temperatures ranging from 5 °C to 35 °C, with 5 °C intervals. After a 2-day period, colony diameters (mm) were measured in two perpendicular directions. Mycelial growth rate (mm d^{-1}) was estimated based on colony diameters following growth at 30 °C. In order to estimate the optimal growth temperature, recorded colony diameters were plotted against temperature and a curve fitted through cubic polynomial regression ($y = a + bx + cx\ 2 + dx\ 3$) using the programme TableCurve™ 2D v. 5.01 (SYSTAT Software Inc., Chicago, USA). One-way analyses of variance (ANOVA) were conducted on optimum temperature and mycelia growth rate data, with means for each species compared with Fisher's least significant difference (LSD) test at the 5 %

Table 1 – Isolates of Botryosphaeriaceae species used in this study.

Taxon	Isolate code[a]	Host	Location	Collector	GenBank Accession No.[b]		
					ITS	EF1-α	β-tubulin
Botryosphaeria dothidea	CMW 8000	*Prunus* sp.	Switzerland	B. Slippers	AY236949	AY236898	
B. dothidea	CBS 110302*	*Vitis vinifera*	Portugal	A. J. L. Phillips	AY259092	AY573218	
Diplodia mutila	CBS 136015	*Populus alba*	Portugal	A. Alves	KJ361838	KJ361830	
D. seriata	CBS 112555*	*Vitis vinifera*	Portugal	A.J.L. Phillips	AY259094	AY573220	
Lasiodiplodia brasiliense	CMM 4011	*Mangifera indica*	Brazil	M.W. Marques	JX464074	JX464037	
L. brasiliense	CMM 4015*	*Mangifera indica*	Brazil	M.W. Marques	JX464063	JX464049	
L. brasiliense	CMM 4469	*Anacardium occidentale*	Brazil	M.S.B. Netto	**KT325574**	**KT325580**	
L. brasiliense	CMM 4470	*Anacardium occidentale*	Brazil	M.S.B. Netto	**KT325575**	**KT325579**	
L. caatinguensis	CMM 1325	*Citrus sinensis*	Brazil	I.B.L. Coutinho & J.S. Lima	KT154760	KT008006	
L. caatinguensis	IBL 40	*Spondias purpurea*	Brazil	J.S. Lima & J.E. Cardoso	KT154762	KT154755	
L. citricola	CBS 124707*	*Citrus* sp.	Iran	J. Abdollahzadeh & A. Javadi	GU945354	GU945340	
L. citricola	CBS 124706	*Citrus* sp.	Iran	J. Abdollahzadeh & A. Javadi	GU945353	GU945339	
L. crassispora	CMW 13448	*Eucalyptus urophylla*	Venezuela	S. Mohali	DQ103552	DQ103559	
L. crassispora	WAC 12533*	*Santalum album*	Australia	T.I. Burgess/B. Dell	DQ103550	DQ103557	
L. egyptiacae	CBS 130992*	*Mangifera indica*	Egypt	A.M. Ismail	JN814397	JN814424	
L. egyptiacae	BOT-29	*Mangifera indica*	Egypt	A.M. Ismail	JN814401	JN814428	
L. euphorbicola	CMM 3651	*Jatropha curcas*	Brazil	A.R. Machado & O.L. Pereira	KF234553	KF226711	
L. euphorbicola	CMM 3609*	*Jatropha curcas*	Brazil	A.R. Machado & O.L. Pereira	KF234543	KF226689	
L. euphorbicola	CMM 3652	*Jatropha curcas*	Brazil	A.R. Machado & O.L. Pereira	KF234554	KF226715	
L. euphorbicola	CMM 4473	*Anacardium humile*	Brazil	M.S.B. Netto	**KT325568**	**KT325581**	
L. exigua	CBS 137785*	*Retama raetam*	Tunisia	B.T. Linaldeddu	KJ638317	KJ638336	
L. exigua	BL 184	*Retama raetam*	Tunisia	B.T. Linaldeddu	KJ638318	KJ638337	
L. gilaniensis	CBS 124704*	Unknown	Iran	J. Abdollahzadeh & A. Javadi	GU945351	GU945342	
L. gilaniensis	CBS 124705	Unknown	Iran	J. Abdollahzadeh & A. Javadi	GU945352	GU945341	
L. gonubiensis	CBS 115812*	*Syzigium cordatum*	South Africa	D. Pavlic	AY639595	DQ103566	
L. gonubiensis	CBS 116355	*Syzigium cordatum*	South Africa	D. Pavlic	AY639594	DQ103567	
L. gonubiensis	CMM 4468	*Anacardium humile*	Brazil	M.S.B. Netto	**KT325571**	**KT325584**	
L. gravistriata	CMM 4564*	*Anacardium humile*	Brazil	M.S.B. Netto	**KT250949**	**KT250950**	
L. gravistriata	CMM 4565	*Anacardium humile*	Brazil	M.S.B. Netto	**KT250947**	**KT266812**	
L. gravistriata	CMM 4566	*Anacardium humile*	Brazil	M.S.B. Netto	**KT250946**	**KT266813**	
L. gravistriata	CMM 4570	*Anacardium humile*	Brazil	M.S.B. Netto	**KT250948**	**KT266814**	
L. hormozganensis	CBS 124709*	*Olea* sp.	Iran	J. Abdollahzadeh & A. Javadi	GU945355	GU945343	
L. hormozganensis	CBS 124708	*Mangifera indica*	Iran	J. Abdollahzadeh & A. Javadi	GU945356	GU945344	
L. iraniensis	CBS 124710*	*Mangifera indica*	Iran	N. Khezrinejad	GU945346	GU945334	
L. iraniensis	CBS 124711	*Juglans* sp.	Iran	A. Javadi	GU943447	GU945335	
L. iraniensis	CMM 4557	*Anacardium occidentale*	Brazil	M.S.B. Netto	**KT325573**	**KT325586**	
L. iraniensis	CMM 4559	*Anacardium occidentale*	Brazil	M.S.B. Netto	**KT325572**	**KT325585**	
L. jatrophicola	CMM 3610*	*Jatropha curcas*	Brazil	A.R. Machado & O.L. Pereira	KF234544	KF226690	
L. jatrophicola	CMM 0247	*V. vinifera*	Brazil	M. A. Silva	KJ417895	KJ417870	
L. jatrophicola	CMM 4471	*Anacardium occidentale*	Brazil	M.S.B. Netto	**KT325569**	**KT325582**	
L. jatrophicola	CMM 4472	*Anacardium occidentale*	Brazil	M.S.B. Netto	**KT325570**	**KT325583**	
L. macrospora	CMM 3833*	*Jatropha curcas*	Brazil	A.R. Machado & O.L. Pereira	KF234557	KF226718	
L. mahajangana	CBS 124927*	*Terminalia catappa*	Madagascar	J. Roux	FJ900597	FJ900643	
L. mahajangana	CBS 124925	*Terminalia catappa*	Madagascar	J. Roux	FJ900595	FJ900641	
L. margaritaceae	CBS 122519*	*Adansonia gibbosa*	Australia	T.I. Burgess	EU144050	EU144065	
L. margaritaceae	CBS 122065	*Adansonia gibbosa*	Australia	T.I. Burgess	EU144051	EU144066	
L. mediterranea	CBS 137783*	*Quercus ilex*	Italy	B.T. Linaldeddu	KJ638312	KJ638331	

Table 1 – (*continued*)

Taxon	Isolate code[a]	Host	Location	Collector	GenBank Accession No.[b]		
					ITS	EF1-α	β-tubulin
L. mediterranea	CBS 137784	*Vitis vinifera*	Italy	S. Serra	KJ638311	KJ638330	
L. mediterranea	ALG 36	*Citrus sinensis*	Algeria	A. Berraf-Tebbal	KJ638314	KJ638333	
L. missouriana	CBS 128311*	*Vitis* sp.	Missouri, USA	K. Striegler & G.M. Leavitt	HQ288225	HQ288267	
L. missouriana	CBS 128312	*Vitis* sp.	Missouri, USA	K. Striegler & G.M. Leavitt	HQ288226	HQ288268	
L. parva	CBS 45678*	Cassava-field soil	Colombia	O. Rangel	EF622083	EF622063	
L. parva	CBS 49578	Cassava-field soil	Colombia	O. Rangel	EF622084	EF622064	
L. plurivora	CBS 120832*	*Prunus salicina*	South Africa	U. Damm	EF445362	EF445395	
L. pontae	CMM1277	*Spondias purpurea*	Brazil	J.S. Lima & F.C.O. Freire	KT151794	KT151791	
L. pseudotheobromae	CBS 116459*	*Gmelina arborea*	Costa Rica	J. Carranza-Velásquez	EF622077	EF622057	
L. pseudotheobromae	IRAN1518C	*Citrus* sp.	Iran	J. Abdollahzadeh & A. Javadi	GU973874	GU973866	
L. pseudotheobromae	CMM 4474	*Anacardium humile*	Brazil	M.S.B. Netto	**KT728914**	**KT882611**	
L. pseudotheobromae	CMM 4475	*Anacardium humile*	Brazil	M.S.B. Netto	**KT728915**	**KT882612**	
L. pyriformis	CMW 25414*	*Acacia mellifera*	Namibia	F.J.J. van der Walt & J. Roux	EU101307	EU101352	
L. pyriformis	CMW 25415	*Acacia mellifera*	Namibia	F.J.J. van der Walt & J. Roux	EU101308	EU101353	
L. rubropurpurea	CBS 118740*	*Eucalyptus grandis*	Australia	T.I. Burgess/G. Pegg	DQ103553	EU673304	
L. rubropurpurea	WAC 12536	*Eucalyptus grandis*	Australia	T.I. Burgess/G. Pegg	DQ103554	DQ103572	
L. subglobosa	CMM 3872*	*Jatropha curcas*	Brazil	A.R. Machado & O.L. Pereira	KF234558	KF226721	
L. subglobosa	CMM 4046	*Jatropha curcas*	Brazil	A.R. Machado & O.L. Pereira	KF234560	KF226723	
Lasiodiplodia sp.	CPC 22800	*Mangifera indica*	Thailand	T. Trakunyingcharoen	KJ193643	KJ193687	
L. thailandica	CPC 22755	*Phyllanthus acidus*	Thailand	T. Trakunyingcharoen	KM00633	KM006464	
L. thailandica	CPC 22795	*Mangifera indica*	Thailand	T. Trakunyingcharoen	KJ19367	KJ193681	
L. theobromae	CBS 16496*	Fruit along coral reef coast	New Guinea	A. Aptroot	AY64025	AY640258	
L. theobromae	CMM 0310	*Vitis vinifera*	Brazil	M. A. Silva	KJ41790	KJ417880	
L. theobromae	CMM 0384	*Vitis vinifera*	Brazil	M. A. Silva	KJ417904	KJ417876	
L. theobromae	CMM 2269	*Carica papaya*	Brazil	J.H.A. Monteiro	KC484821	KC481585	
L. theobromae	CMM 4499	*Anacardium occidentale*	Brazil	M.S.B. Netto	**KT325578**	**KT325587**	
L. theobromae	CMM 4508	*Anacardium occidentale*	Brazil	M.S.B. Netto	**KT325576**	**KT325588**	
L. theobromae	CMM 4513	*Anacardium occidentale*	Brazil	M.S.B. Netto	**KT325577**	**KT325589**	
L. venezuelensis	CBS 118739*	*Acacia mangium*	Venezuela	S. Mohali	DQ103547	DQ103568	
L. venezuelensis	WAC 12540	*Acacia mangium*	Venezuela	S. Mohali	DQ103548	DQ103569	
L. viticola	CBS 128313	*Vitis vinifera*	USA	R.D. Cartwright & W.D. Gubler	HQ288227	HQ288269	
L. viticola	UCD 2604MO	*Vitis vinifera*	USA	K. Striegler & W.D. Gubler	HQ288228	HQ288270	
Neofusicoccum batangarum	CBS 124924*	*Terminalia catappa*	Africa	D. Begoude/J. Roux	FJ900607	FJ900653	FJ900615
N. batangarum	CBS 124923	*Terminalia catappa*	Africa	D. Begoude/J. Roux	FJ900608	FJ800654	FJ900616
N. batangarum	CMM 4547	*Anacardium othonianum*	Brazil	M.S.B. Netto	**KT728916**	**KT728920**	**KT728912**
N. batangarum	CMM 4553	*Anacardium othonianum*	Brazil	M.S.B. Netto	**KT728917**	**KT728921**	**KT728913**
N. brasiliense	CMM 1269*	*Mangifera indica*	Brazil	M.W. Marques	JX513629	JX513609	KC794932
N. brasiliense	CMM 1285	*Mangifera indica*	Brazil	M. W. Marques	JX513628	JX513608	KC794030
N. cordaticola	CBS 123634*	*Syzigium cordatum*	South Africa	D. Pavlic	EU821898	EU821868	EU821838
N. cordaticola	CBS 123635	*Syzigium cordatum*	South Africa	D. Pavlic	EU821903	EU821843	EU821843
N. kwambonambiense	CBS 123639*	*Eucalyptus grandis*	South Africa	D. Pavlic	EU821900	EU821870	EU821840
N. kwambonambiense	CBS 123641	*Eucalyptus grandis*	South Africa	D. Pavlic	EU821949	EU821889	EU821859
N. macroclavatum	WAC 12445*	*Eucalyptus globulus*	Australia	T.I. Burgess	DQ093197	DQ093218	DQ093207
N. macroclavatum	WAC 12446	*Eucalyptus globulus*	Australia	T.I. Burgess	DQ093219	DQ093219	DQ093208
N. occulatum	CBS 128008*	*Eucalyptus grandis* hybrid	Australia	T.I. Burgess	EU730103	EU339509	EU339472
N. occulatum	MUCC 296	*Eucalyptus pellita*	Australia	T.I. Burgess	EU301034	EU339512	EU339475
N. parvum	PD 106	*Prunus dulcis*	USA	T.J. Michailides	GU251139	GU251271	KC794029
N. parvum	ATCC 58189*	*Malus sylvestris*	New Zealand	G.J. Samuels	AF243395	AY236883	AY236912
N. ribis	CBS 12126*	*Ribis rubrum*	USA	B. Slippers	AF241177	AY236879	AY236908
N. ribis	CMW 7772	*Ribis* sp.	USA	B. Slippers & G. Hudler	AY236925	AY236877	AY236906
N. umdonicola	CMW 14058*	*Syzigium cordatum*	South Africa	D. Pavlic	EU821934	EU821874	EU821844
N. umdonicola	CMW 14060	*Syzigium cordatum*	South Africa	D. Pavlic	EU821935	EU821875	EU821845

(*continued on next page*)

Table 1 – (continued)

Taxon	Isolate code[a]	Host	Location	Collector	GenBank Accession No.[b]		
					ITS	EF1-α	β-tubulin
Pseudofusicoccum adansoniae	CBS 122054*	*Eucalyptus* sp.	Australia	D. Pavlic	EF585532	EF585570	
P. adansoniae	WAC 13299	*Mangifera indica*	Australia	J. Ray	GU172404	GU172436	
P. ardesiacum	CBS 122062*	*Adansonia gibbosa*	Australia	D. Pavlic	EU144060	EU144075	
P. ardesiacum	WAC 13294	*Mangifera indica*	Australia	J. Ray	GU172405	GU172437	
P. artocarpi	CPC 22796	*Artocarpus heterophyllus*	Thailand	T. Trakunyingcharoen	KM006452	KM006483	
P. kimberleyense	CBS 122061*	*Ficus opposita*	Australia	D. Pavlic	EU144059	EU144074	
P. kimberleyense	WAC 13293	*Mangifera indica*	Australia	J. Ray	GU172406	GU172438	
P. olivaceum	CBS 124939*	*Pterocarpus angolensis*	Africa	J. Roux	FJ888459	FJ888437	
P. olivaceum	CBS 124940	*Pterocarpus angolensis*	Africa	J. Mehl & J Roux	FJ888462	FJ888438	
P. stromaticum	CMW 13435	*Eucalyptus hybrid*	Venezuela	S. Mohali	DQ436935	DQ436936	
P. stromaticum	CMW 13434*	*Eucalyptus hybrid*	Venezuela	S. Mohali	AY693974	AY693975	
P. stromaticum	CMM 4541	*Anacardium othonianum*	Brazil	M.S.B. Netto	**KT728918**	**KT728922**	
P. stromaticum	CMM 4544	*Anacardium othonianum*	Brazil	M.S.B. Netto	**KT728919**	**KT728923**	
P. violaceum	CBS 124936*	*Pterocarpus angolensis*	Africa	J. Mehl & J Roux	FJ888474	FJ888442	
P. violaceum	CBS 124937	*Pterocarpus angolensis*	Africa	J. Roux	FJ888458	FJ888440	

a *ALG* = A. Berraf-Tebbal, Université Saad Dahleb, Blida, Algeria; *ATCC* = American Type Culture Collection, Manassas, USA; *BL* = B.T. Linaldeddu, Università degli Studi di Sassari, Sassari, Italy; *BOT* = A. M. Ismail, Plant Pathology Research Institute, Giza, Egypt; *CBS* = Centraalbureau voor Schimmelcultures Utrecht, Netherlands; *CMM* = Culture Collection of Phytopathogenic Fungi 'Prof. Maria Menezes', Universidade Federal Rural de Pernambuco, Recife, Brazil; *CMW* = Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa; *CPC* = Culture Collection of P.W. Crous, housed at CBS; *MUCC* = Murdoch University Culture Collection, Perth, Australia; *IRAN* = Culture Collection of the Iranian Research Institute of Plant Protection, Tehran, Iran; *PD* = Culture Collection, University of California, Davis, USA; *STE-U* = Culture Collection of the Department of Plant Pathology, University of Stellenbosch, South Africa; *UCD* = Phaff Yeast Culture Collection, Department of Food Science and Technology, University of California, Davis, USA; *WAC* = Department of Agriculture Western Australia Plant Pathogen Collection, Perth, Australia. * ex-type or ex-epitype. b Sequences derived in this study are emphasized in bold.

significance level using STATISTIX v. 9.0 (Analytical Software, Tallahassee, USA).

Pathogenicity and aggressiveness assays

Pathogenicity and aggressiveness of all Botryosphaeriaceae isolates characterized morphologically were examined using detached green shoots of *Anacardium occidentale* (cv. BRS 274) (Amponsah *et al.* 2011; Correia *et al.* 2016). Healthy 30 cm sections of soft green shoots were obtained from cashew trees (cv. BRS 274) from a non-commercial orchard at the Universidade Federal Rural de Pernambuco where Botryosphaeriaceae species were considered absent, based upon extensive sampling. The cut ends were firstly dipped in wax then cut in the centre of each shoot using a sterilized scalpel. Each superficial wound (~4-mm length, 2-mm deep) was inoculated with a 4 mm diameter mycelial plug taken from the growing margin of a 5-day-old PDA culture of each isolate. As negative control checks, non-colonized PDA plugs were used for inoculation of shoots. In order to prevent drying, all inoculated areas were covered with Parafilm (Pechiney Co., Chicago, USA). Shoots were then incubated in a growth chamber for a 10 d period at 25 °C and 12-h photoperiod. Following incubation, Parafilm was removed and shoots were sliced lengthwise to enable visual observation of internal lesions. The presence of lesions advancing beyond the original 4-mm diameter inoculation point was considered indicative of pathogenicity. Isolate virulence was evaluated through accurate digital calliper-based (Mitutoyo Co., Kanagawa, Japan) measurement of lesions dimensions. The entire experiment was arranged in a completely randomized design, with four replicates employed per treatment (isolate) and one shoot per replicate. The entire experiment was conducted in duplicate. Differences in virulence were determined by analysis of data with a one-way ANOVA, with means compared by LSD test at the 5 % significance level using the program STATISTIX.

Results

Phylogenetic analyses

Sampling from *Anacardium* spp. from numerous growing regions in Brazil (Fig 1) resulted in isolation of 138 isolates of Botryosphaeriaceae. Phylogenetic analysis of the EF1-α gene was employed for identification of all isolates, with rDNA ITS sequences analysed for 17 isolates that represented *EF1-α* haplotypes, and partial *TUB* gene sequences for six fusicoccum-like isolates. The GenBank accession numbers are listed in Table 1. Analysed *EF1-α* and *TUB* sequences were approximately 450 bp in size, while rDNA ITS sequences were approximately 580 bp in size. The *EF1-α* and rDNA ITS sequences were combined in separate datasets, which corresponded to *Lasiodiplodia* species and *Pseudofusicoccum* species. The ITS, *EF1-α*, and *TUB* sequences were combined in a third dataset corresponding to *Neofusicoccum* species. Datasets were analysed separately, resulting in three phylogenetic trees, one for each genus (Figs 2–4). The isolates obtained in this study grouped into 10 distinct clades. The combined *EF1-α* and rDNA ITS sequences for *Lasiodiplodia* contained data for 78 isolates, including two outgroup taxa. Out of a total of 1393 characters, 1136

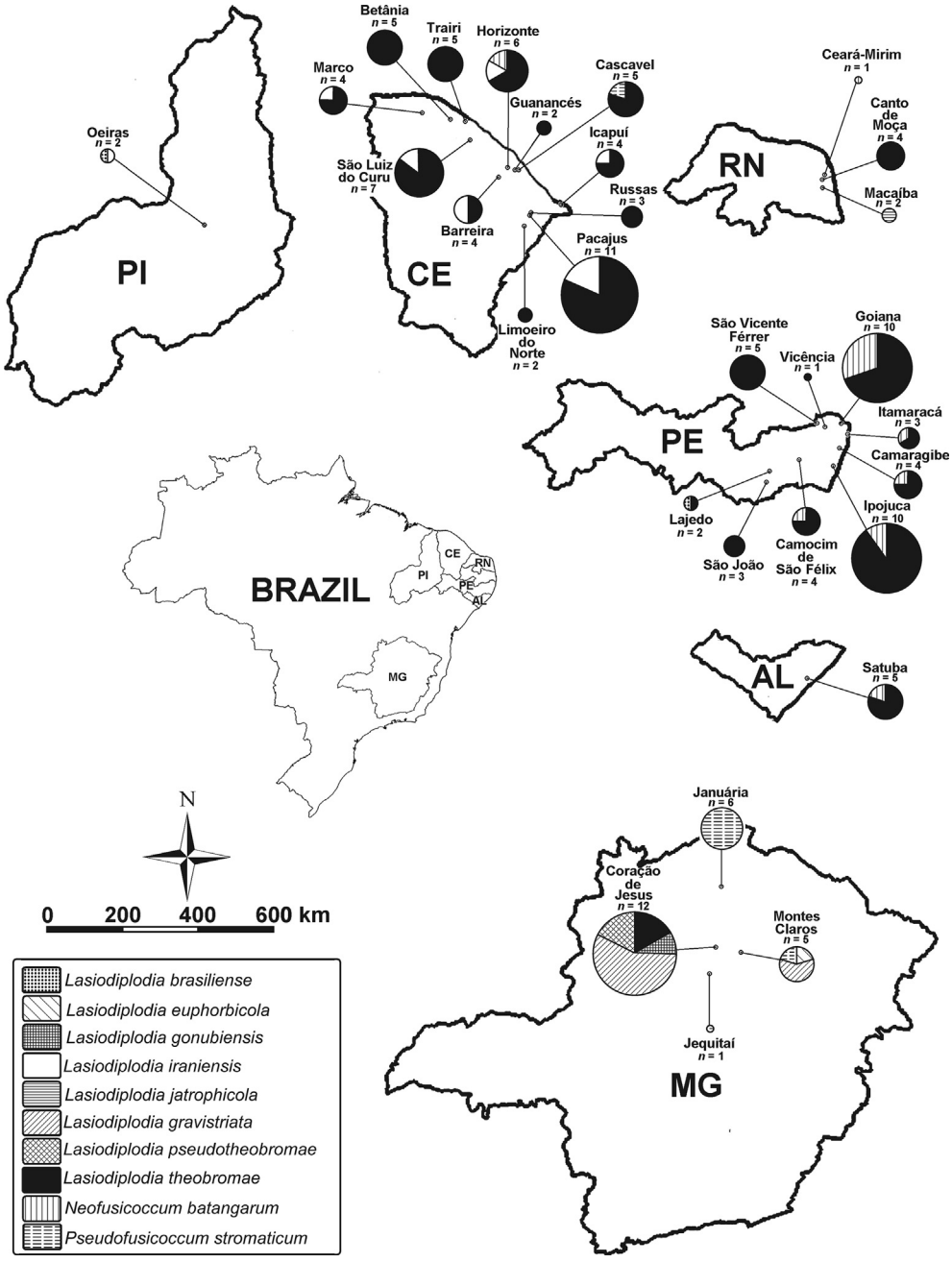

Fig 1 – Collection sites of Botryosphaeriaceae isolates associated with gummosis of *Anacardium* in seven different states of Brazil. Circles represent association frequency of each species with plants exhibiting symptoms of gummosis in each orchard sampled, *n* is the number of isolates analysed in each orchard.

were constant, 231 were variable and parsimony uninformative, and 163 were parsimony informative. The Maximum Likelihood (ML) and Bayesian methods (BM) for phylogenetic analyses produced trees with nearly identical topologies (Bayesian tree not shown). The majority (76 isolates) grouped together in a large clade containing *Lasiodiplodia theobromae* (CBS 16496). Nine isolates grouped with *Lasiodiplodia iraniensis* (CBS 124710). Four isolates grouped with *Lasiodiplodia brasiliense* (CMM 4015) and *Lasiodiplodia jatrophicola* (CMM 3610). Three isolates grouped with *Lasiodiplodia pseudotheobromae* (CBS 116459). Two isolates grouped with *Lasiodiplodia euphorbicola* (CMM 3609) and *Lasiodiplodia gonubiensis* (CBS 115812), respectively. Ten isolates did not cluster with any known *Lasiodiplodia* species (Fig 2). The *Neofusicoccum* combined ITS, *EF1-α*, and *TUB* dataset (which comprised two isolates from this study and 18 sequences originating from GenBank) comprised 1830 characters, with 1370 constant, 427 variable and parsimony uninformative, and 366 parsimony informative. The two isolates clustered with *Neofusicoccum batangarum* (CBS 124924). The dataset of combined rDNA ITS and *EF1-α* sequence data for *Pseudofusicoccum* species comprised 17 isolates including the outgroup, with comprised total of 1411 characters, of which

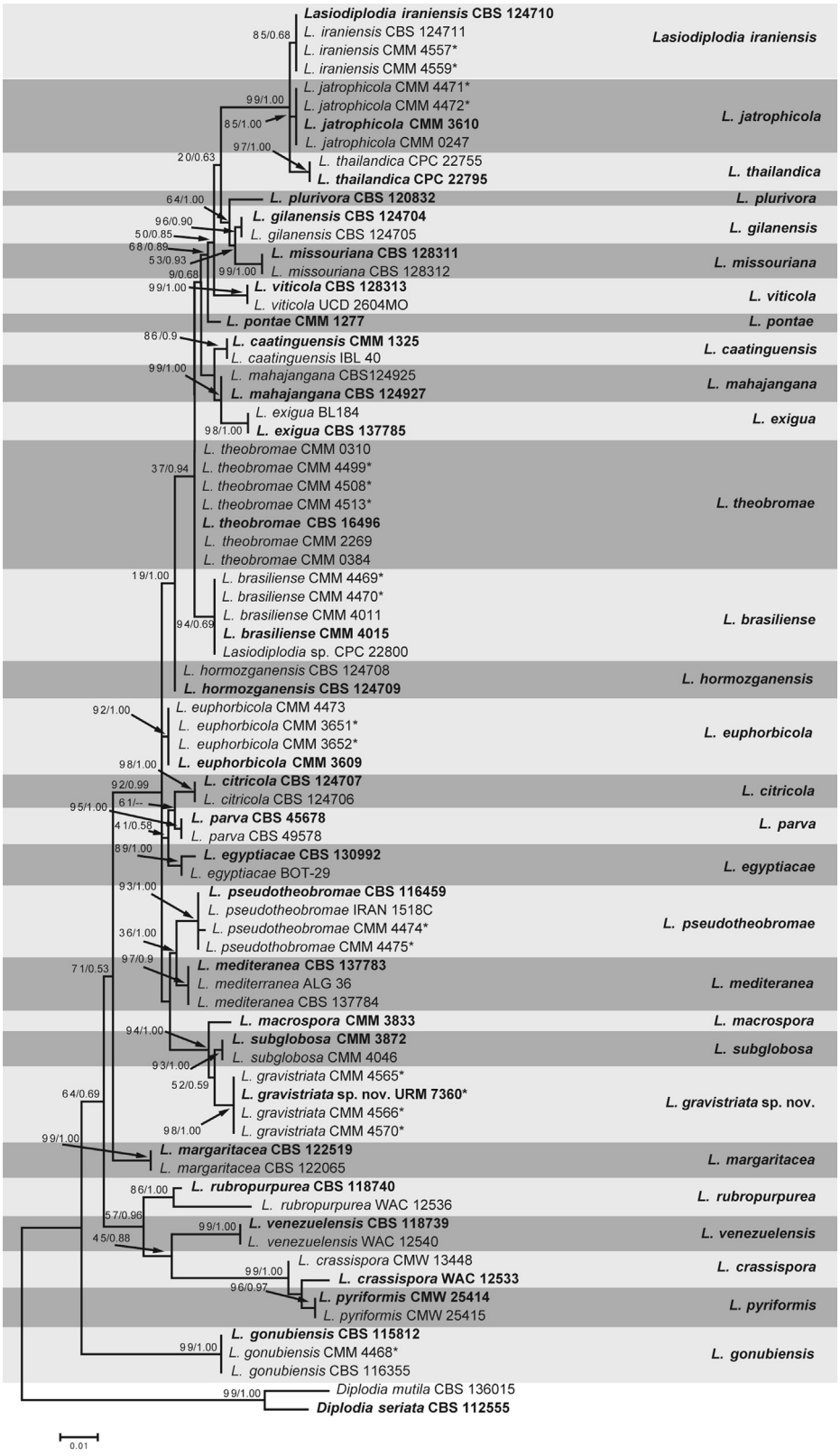

Fig 2 – Maximum likelihood tree resulting from the combined analysis of ITS and EF1-α sequence data. ML Bootstrap support values and Bayesian posterior probability scores are given at the nodes. The tree was rooted to *Diplodia mutila* and *Diplodia seriata*. Ex-type isolates are in bold. The scale bar represents the number of substitutions per site.

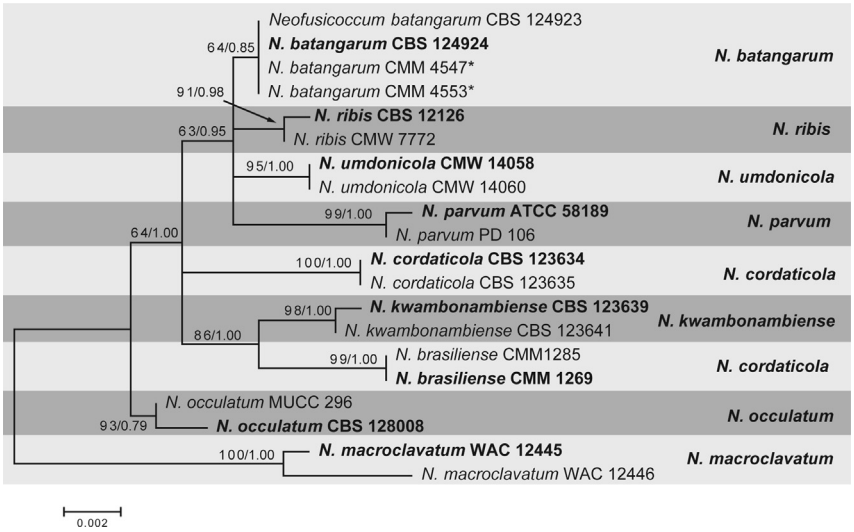

Fig 3 – Maximum likelihood tree resulting from the combined analysis of ITS, EF1-α, and β-tubulin sequence data. ML Bootstrap support values and Bayesian posterior probability scores are given at the nodes. The tree was rooted to *Neofusicoccum macroclavatum*. Ex-type isolates are in bold. The scale bar represents the number of substitutions per site.

1187 were constant, 162 were variable and parsimony uninformative, and 137 parsimony informative. All 16 isolates clustered with *Pseudofusicoccum stromaticum* (CMW 13434).

Morphology and cultural characteristics

All the isolates that were identified based on the phylogenetic analyses using the combined data, comprising 23 *Lasiodiplodia* isolates [*Lasiodiplodia brasiliense* (2), *L. euphorbicola* (1), *L. gonubiensis* (1), *L. iraniensis* (5), *L. jatrophicola* (2), *L. gravistriata* (5), *L. pseudotheobromae* (2), and *L. theobromae* (5)] and the 10 fusicoccum-like isolates [*Neofusicoccum batangarum* (5) and *Pseudofusicoccum stromaticum* (5)] were characterized on the basis of colony morphology and conidial characteristics. Growth was rapid for all isolates on PDA, with mycelia covering the entire surface of the Petri dishes. Aerial mycelium was initially white, then turning dark greenish grey or greyish after 4–5 d incubation at 25 °C in the dark. For all isolates, structures of the asexual morph appeared within 2–4 weeks colonization of PNA. Sexual structures were absent throughout the growth period. All species showed morphological features typical of the genus (Punithalingam 1976, 1980). The new species of *Lasiodiplodia* described here showed differences in conidial size to previously described species. The conidial dimensions of *L. gravistriata* were also outside the range previously documented in the literature for this species (Table 2).

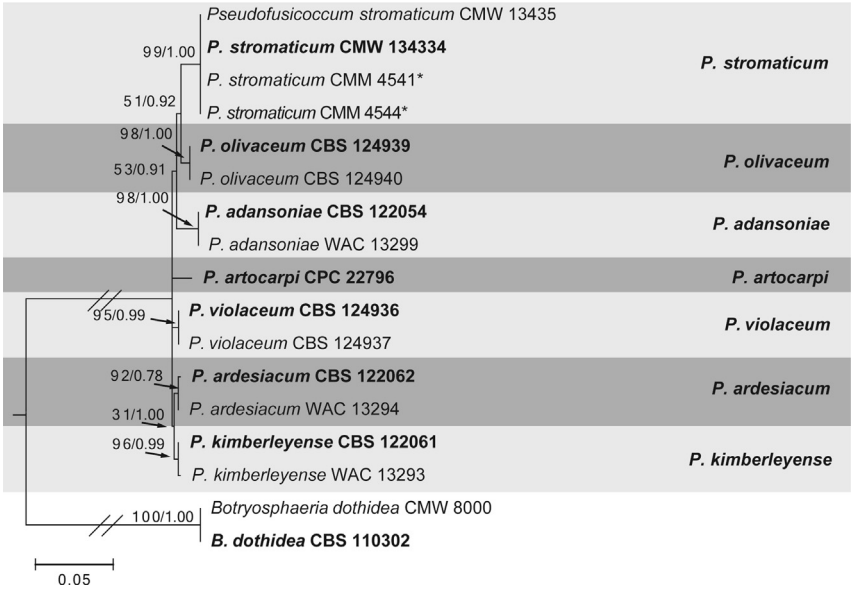

Fig 4 – Maximum likelihood tree resulting from the combined analysis of ITS and EF1-α sequence data. ML Bootstrap support values and Bayesian posterior probability scores are given at the nodes. The tree was rooted to *Botryosphaeria dothidea*. Ex-type isolates are in bold. The scale bar represents the number of substitutions per site.

Only *L. gravistriata* and *L. pseudotheobromae* grew at 5 °C and 10 °C. The optimum temperature for mycelial growth and growth rate differed significantly ($P \leq 0.05$) among the Botryosphaeriaceae species (Table 3). The optimum growth temperature for *N. batangarum* (27.9 °C) was significantly lower than observed for *P. stromaticum* (32.3 °C), *L. brasiliense* (31.2 °C), *L. jatrophicola* (31.0 °C), and five additional species (*L. gravistriata*, *L. gonubiensis*, *L. theobromae*, *L. pseudotheobromae*, and *L. iraniensis*) where temperatures varied from 30.1 °C to 30.7 °C (Table 3). The mycelial growth rates of *L. gravistriata* (69.6 mm d^{-1}) and *L. iraniensis* (64.0 mm d^{-1}) were significantly higher than those the other seven species, which varied from 24.8 mm d^{-1} (*L. pseudotheobromae*) to 53.7 mm d^{-1} (*L. theobromae*).

Taxonomy

Lasiodiplodia gravistriata M.S.B. Netto & M.P.S. Câmara, **sp. nov.** (Fig 5)

MycoBank No.: MB816925

Etymology: In reference to the pronounced longitudinal striations compared to most species of *Lasiodiplodia*.

Mycelium immersed or superficial, branched, septate, dark brown. Aerial mycelia becoming olivaceous grey to violaceous black at the surface and dark mouse grey to olivaceous black. *Colonies* reaching 60 mm on MEA after 2 d in the dark at 25 °C. Optimum temperature for mycelia growth at 31.2 °C. *Ascomata* not seen. *Conidiomata* stromatic, pycnidial, produced on pine needles on WA within 2–4 weeks, immersed or superficial, dark brown to black, covered with mycelium, mostly uniloculate, solitary, globose, thick-walled, non-papillate with a central ostiole. *Paraphyses* hyaline, cylindrical, aseptate, rounded at apex. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* holoblastic, not proliferating, discrete, hyaline, smooth, thin-walled, cylindrical, 9–14 × 3–5 μm. *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, 24.5–28.5 × 10.5–16 μm (av. = 26.2 × 13.8, n = 50).

Habitat: On *Anacardium humile*.

Known distribution: Minas Gerais state, Brazil.

Type: Brazil, Minas Gerais, Coração de Jesus, on *Anacardium humile* stems, 2013, coll. M. S. B. Netto (holotype URM 89942 dry culture and dry pycnidium produced on pine needles, ex-type culture URM 7360 = CMM 4564).

Notes: *Lasiodiplodia gravistriata* is closely related to *L. subglobosa*, although conidia of *L. gravistriata*, are longer and narrower than those of *L. subglobosa* (Table 2). *Lasiodiplodia gravistriata* differs from its closest phylogenetic neighbour, *L. subglobosa*, by unique fixed alleles in two genomic DNA loci, based on alignments of the separate loci deposited in TreeBASE as study S19242: rDNA ITS position 23(T); EF-α positions 50(T), 56(A), 167(GAP), 187(T), and 227(C). *L. gravistriata* is also distinguished from *L. subglobosa* on the basis of conidial size and the prominent longitudinal striations in conidia of *L. gravistriata*.

Table 2 – Comparison of conidial size of *Lasiodiplodia* species examined in this study and previous studies.

Species	Conidia (μm)	L/W ratio	Reference
Lasidodiplodia brasiliense	22.7–29.2 × 11.7–17.0	1.8	Netto *et al.* (2014)
L. citricola	(20–)24.5 (–31) × (10.9–)15.4 (–19)	1.6	Abdollahzadeh *et al.* (2010)
L. crassispora	(27–)28.8 (–33) × (14–)16 (–17)	1.8	Burgess *et al.* (2006)
L. egyptiacae	(17–)22 (–27) × (11–)12 (–13)	2	Ismail *et al.* (2012)
L. euphorbicola	15–23 × 9–12	–	Machado *et al.* (2014)
L. exigua	(19.6–)21.8 (–24.3) × (10.8–)12.3 (–13.3)	1.8	Linaldeddu *et al.* (2015)
L. gilaniensis	(25.2–)31 (–38.8) × (14.4–)16.6 (–19)	1.9	Abdollahzadeh *et al.* (2010)
L. gonubiensis	(28–)33.8 (–39) × (14–)17.3 (–21)	1.9	Pavlic *et al.* (2004)
L. gravistriata	(24.7–)26.2 (–28.7) × (10.6–)13.8 (–16.1)	1.9	Present study
L. hormozganensis	(15.3–)21.5 (–25.2) × (11–)12.5 (14)	1.7	Abdollahzadeh *et al.* (2010)
L. iraniensis	(15.3–)20.7 (–29.7) × (11–)13 (–14)	1.6	Abdollahzadeh *et al.* (2010)
L. jatrophicola	22–26 × 14–17	–	Machado *et al.* (2014)
L. lignicola	(15–)16 (–17.5) × (8–)8.5–10.5 (–11)	1.7	Phillips *et al.* (2013)
L. macrospora	28–35 × 15–17	–	Machado *et al.* (2014)
L. mahajangana	(13.5–)17.5 (–21.5) × (10–)11.5 (–14)	1.4	Begoude *et al.* (2010)
L. margaritacea	(12–)15.3 (–19) × (10–)11.4 (–12.5)	1.3	Pavlic *et al.* (2008)
L. mediterranea	(26.3–)30.6 (–37) × (13.5–)16.1 (–18)	1.9	Linaldeddu *et al.* (2015)
L. missouriana	(16.1–)18.5 (–21) × (8.1–)9.8 (–11.8)	1.9	Úrbez-Torres *et al.* (2012)
L. parva	(15.5–)20.2 (–24.5) × (10–)11.5 (–14.5)	1.8	Alves *et al.* (2008)
L. plurivora	(22–)29.6 (–35) × (13–)15.6 (–18.5)	1.9	Damm *et al.* (2007)
L. pseudotheobromae	(22.5–)28 (–33) × (13.5–)16 (–20)	1.7	Alves *et al.* (2008)
L. pyriformis	(19–)21.5–25 (28–) × (13.5)–15.5–19.5 (–21.5)	1.3	Slippers *et al.* (2014)
L. rubropurpurea	(24–)28.2 (–33) × (13–)14.6 (–17)	1.9	Burgess *et al.* (2006)
L. subglobosa	16–23 × 11–17	–	Machado *et al.* (2014)
L. thailandica	(20–)22–25 (–26) × (12–)13–15 (–16)	–	Trakunyingcharoen *et al.* (2015)
L. theobromae	(19–)26.2 (–32.5) × (12–)14.2 (–18.5)	1.9	Phillips *et al.* (2013)
L. venezuelensis	(26–)28.4 (–33) × (12–)13.5 (–15)	2.1	Burgess *et al.* (2006)
L. viticola	(16.8–)19.5 (–22.9) × (7.9–)9.5 (–10.7)	2.1	Úrbez-Torres *et al.* (2012)

Table 3 – Optimum temperature for mycelial growth and mycelial growth rate at 30 °C of *Lasiodiplodia* species associated with gummosis of *Anacardium* in Brazil.

Species	n	Optimum temperature (°C) ± SE	Mycelial growth rate (mm d^{-1}) ± SE
Lasiodiplodia brasiliense	2	31.2 ± 0.64 ab	42.0 ± 3.11 bc
L. euphorbicola	1	30.6 ± 0.42 b	56.8 ± 4.20 ab
L. gonubiensis	1	28.4 ± 0.81 cd	44.1 ± 5.18 bc
L. gravistriata	5	30.7 ± 0.40 b	69.6 ± 3.22 a
L. iraniensis	5	30.1 ± 0.41 bc	64.0 ± 3.22 a
L. jatrophicola	2	31.0 ± 0.60 ab	31.2 ± 5.57 cd
L. pseudotheobromae	2	30.1 ± 0.59 bc	24.8 ± 3.15 d
L. theobromae	5	30.5 ± 0.36 b	53.7 ± 3.19 b
Neofusicoccum batangarum	5	27.9 ± 0.52 d	32.0 ± 3.12 cd
Pseudofusicoccum stromaticum	5	32.3 ± 0.34 a	33.1 ± 2.76 cd

Mean ± standard error. Values within columns followed by the same letter do not differ significantly according to Fisher's LSD test ($P \leq 0.05$).

Distribution of Botryosphaeriaceae species

Lasiodiplodia theobromae was the predominant species observed on *Anacardium* spp. (66.7 %), followed by *Lasiodiplodia gravistriata* and *Neofusicoccum batangarum* (7.2 %), *Pseudofusicoccum stromaticum* and *Lasiodiplodia iraniensis* (6.5 %), *Lasiodiplodia brasiliense*, *L. jathrophicola* and *L. pseudotheobromae* (1.4 %), *Lasiodiplodia euphorbicola* and *Lasiodiplodia gonubiensis* (0.7 %). The overall distribution of these *Botryosphaeria* species differed among the Brazilian states sampled. *Lasiodiplodia theobromae* was found in five Brazilian states (Alagoas, Ceará, Minas Gerais, Pernambuco, and Rio Grande do Norte). *Neofusicoccum batangarum* was found in four Brazilian states (Alagoas, Ceará, Pernambuco, and Rio Grande do Norte). The new species *L. gravistriata*, together with *L. euphorbicola* and *L. gonubiensis*, were found only in the state of Minas Gerais (Fig 1).

Pathogenicity and virulence on detached green shoots

All isolates of Botryosphaeriaceae were found to be pathogenic on *Anacardium occidentale* (cv. BRS 274), with inoculated detached green shoots showing visible lesions 10 d after inoculation. Dark brown necrotic lesions were observed both on the tissue surface and internally, advancing upwards and downwards from the point of inoculation. Significant differences ($P \leq 0.05$) in internal lesion lengths were apparent between the examined isolates for the different Botryosphaeriaceae species.

The longest lesions were produced by *Neofusicoccum batangarum* (27.0 mm) and *Lasiodiplodia iraniensis* (26.2 mm), which were thus considered to be the most aggressive species in this study. By contrast, the shortest lesions were observed for the least aggressive species, *Lasiodiplodia euphorbicola* and *Lasiodiplodia pseudotheobromae* (<12 mm), with lesion size differing significantly from *N. batangarum* and *L. iraniensis*. The other species (*Lasiodiplodia brasiliense*, *L. gonubiensis*, *L. jathrophicola*, *L. gravistriata*, *L. theobromae*, and *Pseudofusicoccum*

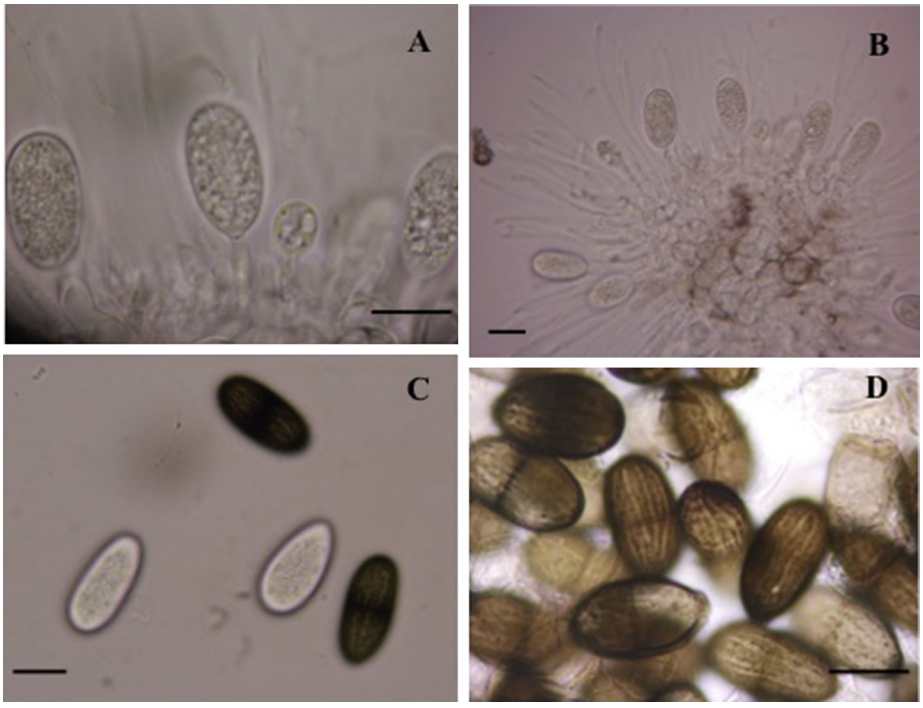

Fig 5 – *Lasiodiplodia gravistriata* (CMM4564) (A–B). Conidiogenous cells giving rise to conidia; (C). mature conidia in two different focal planes to show the longitudinal striations; (D). brown, 1-septate conidia. Scale bars: (A–D) = 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

stromaticum) displayed intermediate aggressiveness, with lesions varying in length from 15.5 mm to 22.2 mm (Fig 6).

Discussion

In this study, we describe the species of Botryosphaeriaceae which are associated with gummosis of *Anacardium* in Brazil. Data were based on morphological, molecular, and pathogenicity testing for a large set of isolates from different growing regions across the country. Ten species of Botryosphaeriaceae were identified associated with gummosis on *Anacardium* spp.: *Lasiodiplodia brasiliense*, *L. euphorbicola*, *L. gonubiensis*, *L. iraniensis*, *L. jatrophicola*, *L. gravistriata*, *L. pseudotheobromae*, *L. theobromae*, *Neofusicoccum batangarum*, and *Pseudofusicoccum stromaticum*. With the exception of *L. theobromae*, all the other species described represent first reports on *Anacardium*.

Following identification, *L. theobromae* was concluded to be both the most frequent species associated with gummosis of *Anacardium*, as well as was the most widespread of all the Botryosphaeriaceae species (Fig 1). Similar findings were observed for this species when associated with dieback and stem-end rot of mango (Marques *et al.* 2013a), stem-end rot of papaya (Netto *et al.* 2014) and grapevine dieback (Correia *et al.* 2016) across the semi-arid region of north-eastern Brazil. Such data supports previous descriptions of this species as a pantropical pathogen occurring on a diverse range of hosts plants (Punithalingam 1980; Burgess *et al.* 2006). In recent years, a number of species have been described in the *L. theobromae* complex globally, which likely reflects the increased employment of DNA sequence data, as well as sampling of relatively unexplored areas, including Australia (Pavlic *et al.* 2008), Brazil (Marques *et al.* 2013a; Machado *et al.* 2014; Netto *et al.* 2014; Correia *et al.* 2016; Coutinho *et al.* 2016), Egypt

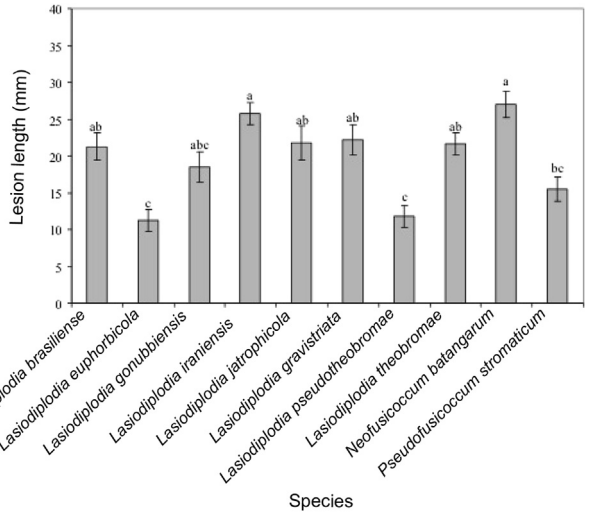

Fig 6 – Mean internal lesion lengths (mm) caused by 10 Botryosphaeriaceae species associated with cashew gummosis in Brazil, 10 d after inoculation with mycelium colonized agar plugs onto detached green shoots of *Anacardium occidentale* (cv. BRS 274). Bars above columns are the standard error of the mean. Columns with same letter do not differ significantly according to Fisher's LSD test ($P \leq 0.05$).

(Ismail *et al.* 2012), Iran (Abdollahzadeh *et al.* 2010), Italy, Alergia and Tunisia (Linaldeddu *et al.* 2015), Oman and The United Arab Emirates (Al-Sadi *et al.* 2013), Thailand (Trakunyingcharoen *et al.* 2015), and Venezuela (Burgess *et al.* 2006).

Lasiodiplodia gravistriata is recognized as a new species in the genus *Lasiodiplodia*, which is phylogenetically closely related to *Lasiodiplodia subglobosa*. However, five nucleotides in the *EF1-α* gene distinguish *L. gravistriata* from *L. subglobosa*. The cashew derived isolates of *L. gravistriata* formed a clade strongly supported in both the Bayesian (1.00) and in the ML (98 %) analyses. *Lasiodiplodia gravistriata* can also be distinguished from *L. subglobosa* on the basis of both conidial size, which are longer and narrower than those typical of *L. subglobosa* (Machado *et al.* 2014), and the prominent longitudinal striations in which occur in the conidia of this species. This new species was also one of the most frequently occurring as pathogen of *Anacardium humile* in Brazil (Fig 1), and did not differ in virulence from *L. brasiliense*, *L. iraniensis*, *L. gonubiensis*, *L. jatrophicola*, *L. theobromae*, and *N. batangarum*.

Lasiodiplodia iraniensis was described from Iran on the susceptible hosts *Mangifera indica* and *Juglans* sp. (Abdollahzadeh *et al.* 2010), then subsequently reported in Brazil associated with mango (Marques *et al.* 2013a). This current work represents the first report of this species as causing gummosis in *Anacardium* spp. Although *L. iraniensis* was only moderately prevalent, it was one of the most aggressive species observed following inoculation of detached green cashew shoots, and therefore *L. iraniensis* should be regarded as a potential threat to this crop. These findings contrast those reported by Marques *et al.* (2013a), where *L. iraniensis* isolates produced smaller lesions on mango fruits than other species.

Lasiodiplodia brasiliense was first described in Brazil in 2014 causing stem-end rot of papaya (Netto *et al.* 2014). Its identification in the present study represents the first report of this species causing gummosis on *Anacardium*. Although most closely related to *Lasiodiplodia viticola* based on phylogenetic analyses, conidia in *L. brasiliense* are longer and wider than those typical of *L. viticola*. Genomic DNA for this species also differed from *L. viticola*, with specific alleles at ITS nucleotide positions: 2(C), 12(G), 42(T), 46(A), 50(C), 56(GAP), 62(GAP), 75(GAP), 123(T), and 370(A). *Lasiodiplodia brasiliense* was pathogenic on detached green cashew shoots and one of the least prevalent species associated with *Anacardium*. This contrasts to reports for this species as being a prevalent species associated with stem-end rot of papaya (Netto *et al.* 2014) and grapevine dieback (Correia *et al.* 2016) in the Brazilian São Francisco Valley region.

Prior to this study, *L. jatrophicola* and *L. euphorbicola* were described on physic nut in Brazil (Machado *et al.* 2014). *L. jatrophicola* is phylogenetically closely related, yet clearly distinct from *L. iraniensis*, with larger conidia and shorter paraphyses typical of this species. *Lasiodiplodia euphorbicola* is phylogenetically closely related to *Lasiodiplodia parva*. These two taxa share morphological characteristics, although paraphyses are smaller in *L. euphorbicola* (Machado *et al.* 2014). In this study, *L. jatrophicola* was the one of least prevalent species (0.7 %), and only moderately aggressiveness on *Anacardium occidentale*. Similarly *L. euphorbicola* was rarely encountered and showed only low levels of aggressiveness. A similar result

was found by Correia *et al.* (2016), where *L. jatrophicola* and *L. euphorbicola* isolates displayed moderate and low levels of aggressiveness, respectively, on grapevines. This study report for the first time *L. jatrophicola* causing gummosis in *Anacardium* anywhere in the world, and identification of a third host of this species in Brazil.

Lasiodiplodia pseudotheobromae was also identified on *A. occidentale* in Brazil (Coutinho *et al.* 2016). Globally, this species, like *L. theobromae*, has a wide distribution and a wide host range, and has been reported on hosts that include *Acacia*, *Citrus*, *Coffea*, *Gmelina*, and *Rosa* species (Alves *et al.* 2008; Phillips *et al.* 2008; Abdollahzadeh *et al.* 2010; Perez *et al.* 2010; Sakalidis *et al.* 2011; Ismail *et al.* 2012; Slippers *et al.* 2014; Trakunyingcharoen *et al.* 2015). In Brazil, *L. pseudotheobromae* has so far been reported on mango (Marques *et al.* 2013a), physic nut (Machado *et al.* 2014), papaya (Netto *et al.* 2014), and grapevine (Correia *et al.* 2016). Morphologically, this species differs from *L. theobromae* in terms of conidial dimension and form, with conidia generally being larger, more ellipsoid and with less pronounced tapering towards the base (Alves *et al.* 2008). In terms of pathogenicity, *L. pseudotheobromae* was the most aggressive species on mango in Australia (Sakalidis *et al.* 2011), and Egypt (Ismail *et al.* 2012) as well as on *Terminalia catappa* (Combretaceae) in Cameroon (Begoude *et al.* 2010). Here, by contrast, *L. pseudotheobromae* was only moderately aggressiveness on cashew shoots, and was reported on mango (Marques *et al.* 2013a), papaya (Netto *et al.* 2014), grape (Correia *et al.* 2016), and cashew (Coutinho *et al.* 2016) in Brazil.

Lasiodiplodia gonubiensis was the first species for the genus to be reported on native trees in South Africa, where it was encountered as an endophytic fungus of *Syzygium cordatum* (Pavlic *et al.* 2004). The present study represents the first report of *L. gonubiensis* in Brazil and causing gummosis on *Anacardium*. Here, this species was isolated infrequently on *A. occidentale*, with aggressiveness on this host similar to levels observed for *L. brasiliense*, *L. jatrophicola*, *L. theobromae*, and *P. stromaticum*.

The application of molecular tools has facilitated the recognition of species in the Botryosphaeriaceae, with numerous species recently described on native vegetation, and economically important crops (Liu *et al.* 2012; Phillips *et al.* 2013). In this work, two additional genera were identified as associated with gummosis on *Anacardium*: *N. batangarum* and *P. stromaticum*. Information on *N. batangarum* is scarce given it was only recently described (Begoude *et al.* 2010). This fungus was reported as an endophytic fungus on *T. catappa* in Cameroon (Shetty *et al.* 2011). As this fungus can cause pathogenic reactions on *T. catappa* under greenhouse conditions (Begoude *et al.* 2010), however, the fungus may therefore switch from an endophytic life style in plant organs to aggressive pathogen, when environmental conditions become unfavourable for the tree host. In this study, this species was frequently isolated from *A. occidentale* and produced the largest lesions on detached cashew green shoots.

Pseudofusicoccum stromaticum was also an abundant species, indicating this genus to be more widely distributed than earlier considered. Previously, this species has been found on *Eucalyptus* and *Acacia* spp. in Venezuela (Mohali *et al.* 2006; 2007) and on mango in Brazil (Marques *et al.* 2012). Pavlic *et al.* (2008) also reported *Pseudofusicoccum* spp. on native hosts plants in undisturbed areas in Australia, providing evidence for these species to be native to the country. Our study contradicts this suggestion, with *P. stromaticum* found in Brazil on native cashew (*Anacardium othonianum*).

Optimum growth temperatures of Botryosphaeriaceae species varied from 27.9 °C to 32.3 °C. *Lasiodiplodia gravistriata* and *L. pseudotheobromae* also grew at as low as 5 °C and 10 °C. Such growth of *L. pseudotheobromae* at low temperatures contradicts a number of previous studies (Abdollahzadeh *et al.* 2010; Marques *et al.* 2013a; Netto *et al.* 2014), although other studies (Alves *et al.* 2008; Ismail *et al.* 2012) clearly provide data showing that these *Lasiodiplodia* species have a much wider temperature range than was previously assumed.

In summary, this paper reports 10 species of Botryosphaeriaceae associated with *Anacardium* in Brazil. *L. theobromae*, although the species most frequently observed on this host, is neither the exclusive etiologic agent nor the most aggressive species. All species showed potential to cause cashew gummosis, with the species *N. batangarum* and *L. iraniensis* identified as the most aggressive species. Continued investigation of the epidemiology and impact of gummosis on cashew production is necessary, together with improved understanding of the ecology, distribution, host range, and fungicide sensitivity of all Botryosphaeriaceae species reported on *Anacardium*. Such information will be crucial for the development of novel gummosis control strategies and genetic improvement of cashew for resistance to biotic stress.

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