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Phylogeny and pathogenicity of *Lasiodiplodia* species associated with dieback of mango in Peru

Edgar RODRÍGUEZ-GÁLVEZ[a], *Pakita GUERRERO*[b], *Carla BARRADAS*[c], *Pedro W. CROUS*[d,e], *Artur ALVES*[c,*]

[a]*Departamento de Sanidad Vegetal, Facultad de Agronomía, Universidad Nacional de Piura, Campus Universitario S/N Miraflores, Piura, Peru*
[b]*Agroindustrias Aurora SAC, Chiclayo, Peru*
[c]*Departamento de Biologia, CESAM, Universidade de Aveiro, 3810-193 Aveiro, Portugal*
[d]*CBS-KNAW Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands*
[e]*Department of Microbiology and Plant Pathology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, South Africa*

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ABSTRACT

Mango, which is an important tropical fruit crop in the region of Piura (Peru), is known to be prone to a range of diseases caused by *Lasiodiplodia* spp. The aim of this study was to evaluate the incidence and prevalence of mango dieback in the region of Piura, and to identify the species of *Lasiodiplodia* associated with the disease and evaluate their pathogenicity towards mango. Mango dieback was present in all orchards surveyed but incidence varied with location. Identification of fungal isolates was based on morphological and cultural characteristics as well as sequence data of the rDNA internal transcribed spacer region (ITS) and translation elongation factor 1-alpha gene (*tef1-α*). The following *Lasiodiplodia* species were identified: *Lasiodiplodia brasiliense*, *Lasiodiplodia egyptiacae* (for which the new combination *Lasiodiplodia laeliocattleyae* is introduced), *Lasiodiplodia iraniensis*, *Lasiodiplodia pseudotheobromae*, *Lasiodiplodia theobromae*, and a *Lasiodiplodia* sp. Individual and combined gene genealogies suggest that this *Lasiodiplodia* sp. is possibly a hybrid of *Lasiodiplodia citricola* and *Lasiodiplodia parva*. Apart from *Lasiodiplodia theobromae*, which was the most prevalent species, all other species are newly reported from Peru. Moreover, *L. iraniensis* is reported for the first time on mango. Inoculation trials of mango plants confirmed Koch's postulates, and revealed differences in aggressiveness among species and isolates.

Introduction

Mango (*Mangifera indica*) is an economically relevant fruit crop grown worldwide in tropical climates. In the region of Piura, located in the northwest coast of Peru, it represents one of the main export products. In this region, which has a desert to semi-desert climate without marked seasons, mango fruit (mostly Kent variety) production occupies 17 000 ha, corresponding to 75 % of the national production of mango for export (Webb & Fernández 2013).

* *Corresponding author*. Tel.: +351 234370766; fax: +351 234372587.
E-mail address: artur.alves@ua.pt (A. Alves).
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One of the main diseases affecting mango production is dieback, which in recent years has drawn the attention of producers due to an alarming increase of affected plants in Peru. The symptoms occur in plants of all ages and are characterised by shoot and branch necrosis, defoliation and panicle necrosis. The most typical symptoms of the disease are seen at the foliar level with groups of necrotic leaves dispersed throughout the canopy, covering large sections or even the entire canopy. Sections of affected branches and stems show necrotic wood tissue areas that can spread downwards, towards the root region. With time the disease can progress to cause plant death. The pathogen enters the plant chiefly via pruning wounds and colonizes tissues basipetally, causing dieback.

The impact of mango dieback in Piura was firstly studied in 1998 in an area covering 5118 ha. A prevalence of 100 % was reported but with a low incidence ranging from 0.09 % of affected plants in Alto Piura, to 0.89 % in San Lorenzo (Rodríguez-Gálvez *et al.* 1999). At the time, based on morphological characters, the causal agent of mango dieback was identified as *Lasiodiplodia theobromae* (Rodríguez-Gálvez *et al.* 1999). This species, which is a member of the family *Botryosphaeriaceae*, is a well-known and widespread plant pathogen occurring mostly in tropical and sub-tropical regions, and has been reported on more than 500 host plants (Punithalingam 1980).

Using morphological and phylogenetic data, Alves *et al.* (2008) revealed the existence of cryptic species within what was formerly regarded as *L. theobromae*. Since then a large number of species have been described, and the genus currently comprises 30 species known from culture (Marques *et al.* 2013; Phillips *et al.* 2013; Netto *et al.* 2014; Prasher & Singh 2014; Slippers *et al.* 2014; Chen *et al.* 2015; Trakunyingcharoen *et al.* 2015). Of these 30 species, at least seven including *Lasiodiplodia crassispora*, *Lasiodiplodia egyptiacae*, *Lasiodiplodia hormozganensis*, *Lasiodiplodia iraniensis*, *Lasiodiplodia pseudotheobromae*, *Lasiodiplodia thailandica*, and *Lasiodiplodia theobromae* have been reported from mango (Abdollahzadeh *et al.* 2010; Costa *et al.* 2010; Sakalidis *et al.* 2011; Ismail *et al.* 2012; Marques *et al.* 2013; Phillips *et al.* 2013; Trakunyingcharoen *et al.* 2015). *Lasiodiplodia* species have been associated with several disease symptoms on mango plants including fruit rot, stem-end rot, panicle brown rot, decline, canker and dieback (Costa *et al.* 2010; Sakalidis *et al.* 2011; Ismail *et al.* 2012; Marques *et al.* 2013) but are also known from asymptomatic plants, where they occur as latent endophytes (Trakunyingcharoen *et al.* 2015).

There are no recent data on dieback of mango in Peru, and previous identifications of *L. theobromae* were based solely on morphology. This study was undertaken with the aim of re-evaluating the incidence, severity and prevalence of mango dieback in plantations in the region of Piura, as well as confirming the identification of the causal agents.

Materials and methods

Field survey and sampling

Field surveys were carried out between March and November 2012 in the following regions of Piura (Peru): Valle de San Lorenzo (Hualtaco, Malingas, Partidor, Somate, San Isidro and Valle de los Incas), Valle del Chira (Cieneguillo Norte, Cieneguillo Centro and Cieneguillo Sur) and Valle del Alto Piura (Campanas, La Matanza and Yapatera) (Fig 1). Several plantations covering a total area of 4076 ha were evaluated and in each, which was traversed in a zigzag movement, 10 % of the plants (Table 1) were visually inspected for dieback symptoms.

Disease incidence (I) was determined following the approach of Teng & James (2001) using the formula: I (%) = (ni/N) × 100 (ni: total number of affected plants, N: total number of evaluated plants). Disease prevalence in a given geographic area was also determined and expressed as a percentage (Teng & James 2001). Disease severity was determined according to the formula proposed by French and Hebert (1982): Severity = n(L0) + n(L1) + n(L2) + n(L3) + n(L4) + n(L5)/∑n, where n = number of diseased plants in each corresponding damage level (L). For this purpose six damage levels were considered: L0: healthy plant; L1: plants with shoot necrosis; L2: plants with defoliation in 25 % of the canopy; L3: plants with defoliation in 50 % of the canopy; L4: plants with defoliation in 75 % of the canopy and L5: dead plant.

Fungal isolation and morphology

Samples collected from symptomatic plants (one per plant) were washed under tap water, dried and briefly flamed. Small pieces of wood were taken from the interface between healthy and diseased plant tissue, submerged in 2.5 % sodium hypochlorite for 2 min, and washed twice in sterile distilled water. Wood pieces were plated on potato dextrose agar medium (PDA, Merck, Darmstadt, Germany) and incubated at 28 °C for 5 d. Fungal colonies were transferred to fresh PDA plates and after sporulation single spore cultures were obtained. In order to identify botryosphaeriacous isolates, micromorphological characteristics (e.g. conidial size, shape, colour, striation, septation, conidiogenous cells, presence of paraphyses) of the isolates were observed with a Nikon 80i microscope and pictures captured with a Nikon DS-Ri1 camera.

Molecular identification of isolates

Genomic DNA was extracted from mycelium as described by Alves *et al.* (2004). PCR reactions were carried out with NZYTaq 2 × Green Master Mix (NZYTech, Lisboa, Portugal).

The internal transcribed spacer (ITS) region of the ribosomal DNA cluster and part of the translation elongation factor 1-alpha (*tef1-α*) were sequenced for the 32 selected isolates as described previously (Alves *et al.* 2004, 2008), and the sequences deposited in GenBank (Table 2). The amplified PCR fragments were purified with the DNA Clean and Concentrator™-5 kit (Zymo Research, California, USA). Both strands of the PCR products were sequenced at GATC Biotech (Germany). The nucleotide sequences were read and edited with FinchTV v. 1.4.0 (Geospiza Inc. http://www.geospiza.com/finchtv).

The ITS and *tef1-α* sequences of the isolates from mango were combined and aligned with sequences retrieved from GenBank, representing 28 species of the genus *Lasiodiplodia*.

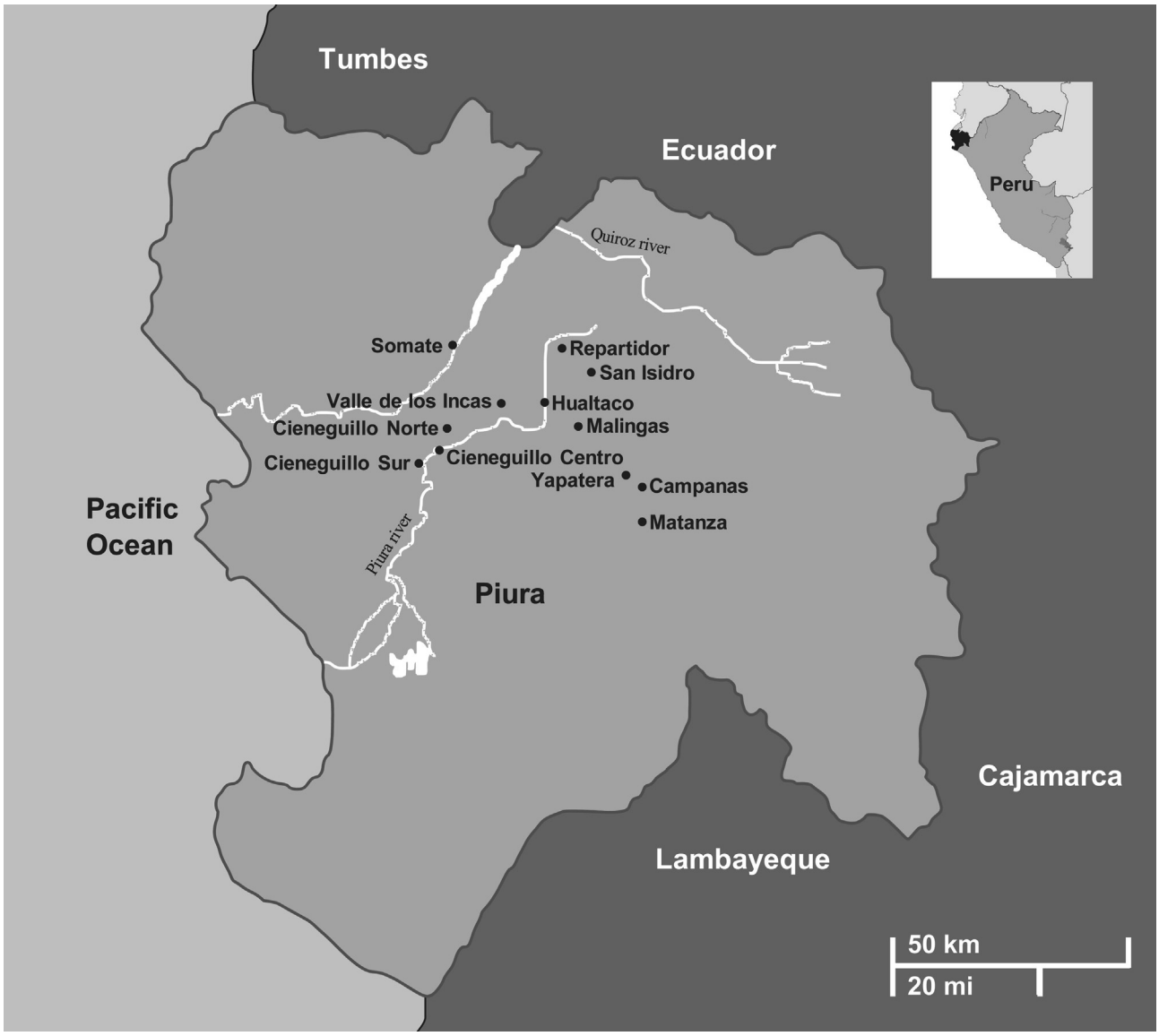


Fig 1 – Map of Piura (Peru) indicating the regions where field surveys and sampling were conducted.

Table 1 – List of regions surveyed in this study with corresponding areas, number of plantations evaluated, disease incidence and severity and number of isolates obtained.

Valley	Region	Area (ha)	No plantations	Incidence (%)	Severity	No isolates
Alto Piura	Campanas	146.6	36	3.71	2.45	72
	La Matanza	92.5	19	29.71	2.56	48
	Yapatera	403.5	52	8.09	2.23	104
		642	**107**	**10.20**	**2.36**	**224**
Chira	Cieneguillo Norte	219	37	10.92	2.30	74
	Cieneguillo Sur	549	9	6.65	2.29	58
	Cieneguillo Centro	19	4	6.69	2.40	8
		787	**50**	**9.93**	**2.31**	**140**
San Lorenzo	Hualtaco	478	28	6.83	1.93	56
	Malingas	1062	83	4.70	2.19	166
	Repartidor	476	14	9.52	2.12	28
	San Isidro	240	16	10.62	2.25	38
	Somate	255	6	7.23	1.82	24
	Valle de los Incas	136.5	41	4.95	2.36	82
		2647.5	**188**	**6.74**	**2.18**	**394**

Table 2 – List of isolates used in this study.

Species	Isolate	Host	Locality	Collector	GenBank	
					ITS	*tef1-α*
L. brasiliense	LAYAP1	*M. indica*	Yapatera, Peru	P. Guerrero	KU507473	KU507440
L. iraniensis	LASID3	*M. indica*	San Isidro, Peru	P. Guerrero	KU507480	KU507447
L. laeliocattleyae	LACIC1	*M. indica*	Cieneguillo Centro, Peru	P. Guerrero	KU507462	KU507429
	LAREP1	*M. indica*	Repartidor, Peru	P. Guerrero	KU507484	KU507451
	CBS 167.28	*Laeliocattleya*	Italy	C. Sibilia	KU507487	KU507454
L. pseudotheobromae	LASOM2	*M. indica*	Somate, Peru	P. Guerrero	KU507476	KU507443
L. theobromae	LAMAL1	*M. indica*	Malingas, Peru	P. Guerrero	KU507455	KU507422
	LAMAL2	*M. indica*	Malingas, Peru	P. Guerrero	KU507456	KU507423
	LAMAL3	*M. indica*	Malingas, Peru	P. Guerrero	KU507457	KU507424
	LACIS1	*M. indica*	Cieneguillo Sur, Peru	P. Guerrero	KU507458	KU507425
	LACIS2	*M. indica*	Cieneguillo Sur, Peru	P. Guerrero	KU507459	KU507426
	LACIS3	*M. indica*	Cieneguillo Sur, Peru	P. Guerrero	KU507460	KU507427
	LACIC2	*M. indica*	Cieneguillo Centro, Peru	P. Guerrero	KU507462	KU507429
	LACIN1	*M. indica*	Cieneguillo Norte, Peru	P. Guerrero	KU507463	KU507430
	LACIN2	*M. indica*	Cieneguillo Norte, Peru	P. Guerrero	KU507464	KU507431
	LACIN3	*M. indica*	Cieneguillo Norte, Peru	P. Guerrero	KU507465	KU507432
	LAVIN1	*M. indica*	Valle de los Incas, Peru	P. Guerrero	KU507466	KU507433
	LAVIN2	*M. indica*	Valle de los Incas, Peru	P. Guerrero	KU507467	KU507434
	LAVIN3	*M. indica*	Valle de los Incas, Peru	P. Guerrero	KU507468	KU507435
	LAMAT1	*M. indica*	La Matanza, Peru	P. Guerrero	KU507470	KU507437
	LAMAT2	*M. indica*	La Matanza, Peru	P. Guerrero	KU507471	KU507438
	LAMAT3	*M. indica*	La Matanza, Peru	P. Guerrero	KU507472	KU507439
	LAYAP2	*M. indica*	Yapatera, Peru	P. Guerrero	KU507474	KU507441
	LASOM1	*M. indica*	Somate, Peru	P. Guerrero	KU507475	KU507442
	LASOM3	*M. indica*	Somate, Peru	P. Guerrero	KU507477	KU507444
	LASID1	*M. indica*	San Isidro, Peru	P. Guerrero	KU507478	KU507445
	LASID2	*M. indica*	San Isidro, Peru	P. Guerrero	KU507479	KU507446
	LAHUAL1	*M. indica*	Hualtaco, Peru	P. Guerrero	KU507481	KU507448
	LAHUAL2	*M. indica*	Hualtaco, Peru	P. Guerrero	KU507482	KU507449
	LAHUAL3	*M. indica*	Hualtaco, Peru	P. Guerrero	KU507483	KU507450
	LAREP2	*M. indica*	Repartidor, Peru	P. Guerrero	KU507485	KU507452
	LAREP3	*M. indica*	Repartidor, Peru	P. Guerrero	KU507486	KU507453
Lasiodiplodia sp.	LACAM1	*M. indica*	Campanas, Peru	P. Guerrero	KU507469	KU507436

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 made if necessary using BioEdit v. 7.2.5 (Hall 1999).

Maximum parsimony (MP) analyses were performed with PAUP v. 4.0b10 (Swofford 2003). All characters were unordered and of equal weight, and gaps were treated as missing data. The heuristic search option with 100 random taxon additions and tree bisection and reconnection as the branch-swapping algorithm were applied. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved.

Maximum likelihood (ML) analyses were done using MEGA6 (Tamura *et al.* 2013). MEGA6 was also used to determine the best fitting DNA evolution model to be used for ML analysis. ML analyses were performed on a Neighbour-Joining starting tree automatically generated by the software. Nearest-Neighbour-Interchange (NNI) was used as the heuristic method for tree inference and 1000 bootstrap replicates were performed. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The robustness of the trees was evaluated by 1000 bootstrap replications. Trees were rooted to an outgroup and visualised with TreeView v. 1.6.6 (Page 1996).

Pathogenicity trials

Pathogenicity of all isolates was tested on potted 8-mo-old mango plants of the cultivar Kent obtained from a commercial nursery. The fungal isolates were grown on PDA at 28 °C for 5 d prior to inoculation. For inoculation, the apex of an actively growing shoot was cut and a colonized agar disc (5 mm diam) was placed on top of the damaged area, which was then covered with sterilized cotton and sealed with Parafilm. Finally, sterile water was injected with a hypodermic needle into the inoculated area to moisten the cotton. Controls were inoculated with pieces of non-colonized PDA. Five replicates were used for inoculated and control plants. The plants were maintained in a non-controlled greenhouse at room temperature (approx. 26 °C) for 14 d after which the aggressiveness of the isolates was determined by assessing the length of the necrotic lesion. Statistical analyses were carried out using STATGRAPHICS Centurion XV. The Tukey's test was used for comparison of treatment (isolate) means at P = 0.05.

Results

Field survey and sampling

A total of 345 plantations corresponding to an area of 4076 Ha were evaluated in three main valleys of Piura (Table 1). Mango dieback was detected in all plantations and regions surveyed. The Alto Piura valley had the highest incidence of mango dieback, with 10.2 % of the plants affected, followed by Chira with 9.9 %, and San Lorenzo with 6.7 %. Among the regions La Matanza had the highest incidence value (29.7 %), followed by Cieneguillo Norte (10.9 %) and San Isidro (10.6 %). Nevertheless, there where plantations reaching incidence values up to 60 % (Cieneguillo Norte), and severity of 4.5 (Campanas). In terms of severity of the disease it did not vary significantly among valleys with Alto Piura having a value of 2.36 followed by Chira with 2.31, and San Lorenzo with 2.18.

The first symptom shown by affected plants was leaf chlorosis that lead to leaf necrosis, and groups of necrotic leaves that could be seen dispersed throughout the canopy (Fig 2A) as a consequence of pathogen infection through pruning wounds (Fig 2A and C). This was followed by leaf fall, giving rise to defoliation starting at the apex and moving downwards to the base of the plant (Fig 2D). In branches and stems internal wood necrosis could be observed (Fig 2E–H). Defoliation

Fig 2 – Dieback symptoms on *Mangifera indica* in Peru associated to *Lasiodiplodia* spp. (A) Leaf necrosis. (B) Starting of necrosis after pruning and leaf necrosis. (C) Magnification of B where it is possible to observe internal tissue necrosis. (D) Branch defoliation. (E, F) Branch internal necrosis. (G) Branch with advanced necrosis. (H) Same branch with internal tissue necrosis.

occurred with various degrees of intensity, with the few remaining leaves showing chlorosis, conferring an aspect of decline. In more advanced states, the disease gave rise to severe defoliation and even plant death.

Fungal isolation and morphology

All samples collected from symptomatic mango plants yielded typical botryosphaeriaceous fungi, leading to a collection of 758 isolates. All isolates had morphological characteristics typical of the genus *Lasiodiplodia* (Phillips *et al.* 2013). No other botryosphaeriaceous fungi apart from *Lasiodiplodia* spp. were isolated from symptomatic plants. A subsample of 32 isolates representative of the overall morphological diversity (e.g. cultural characteristics, colony colour) and the different regions sampled was selected for further molecular identification.

Molecular identification of isolates

The combined ITS and *tef1-α* dataset consisted of 86 ingroup and one outgroup taxa (32 sequences obtained in this study and 55 from GenBank) and contained 814 characters (515 from ITS and 299 from *tef1-α*). MP and ML analyses generated trees with identical topologies. The resulting ML tree is presented in Fig 3 with bootstrap support values above the branches. Within the ingroup 28 clades corresponding to *Lasiodiplodia* species and with moderate to high bootstrap support were identified.

The sequences of the isolates obtained in this study clustered within five of the above-mentioned clades. The vast majority of the isolates (26) clustered within the clade corresponding to *Lasiodiplodia theobromae*. One isolate each clustered within the clades corresponding to the species *Lasiodiplodia brasiliense*, *Lasiodiplodia iraniensis*, and *Lasiodiplodia pseudotheobromae*. Two isolates clustered in a clade containing *Lasiodiplodia egyptiacae,* as well as the ex-type strain (CBS 167.28) of *Diplodia laeliocattleyae*. The remaining isolate (LACAM1) formed a separate clade clustering between *Lasiodiplodia citricola* and *Lasiodiplodia parva*. The molecular identification of this isolate was uncertain given its position in the phylogenetic tree. A comparison of the ITS and *tef1-α* sequences of *Lasiodiplodia* sp. LACAM1 with those of *L. citricola* and *L. parva* showed that there are no unique polymorphisms in the sequences of both loci from *Lasiodiplodia* sp. (Table 3). Besides, the ITS sequence had 99 % similarity with several *Lasiodiplodia* species including *L. citricola* and *L. parva* while the *tef1-α* sequence was 100 % identical with that of *L. parva*. Furthermore, in individual gene genealogies (Fig S1) *Lasiodiplodia* sp. LACAM1 grouped with either *L. citricola* (in ITS only) or *L. parva* (in *tef1-α* only).

The phylogenetic analyses revealed that *Lasiodiplodia euphorbiicola* and *Lasiodiplodia marypalme* resided in the same clade with high bootstrap support and are indistinguishable. The same also occurred with *Lasiodiplodia exigua* and *Lasiodiplodia americana* that formed a single and highly supported clade as well as *L. iraniensis* and *Lasiodiplodia jatrophicola* that formed also a single and highly supported clade. Thus, *L. exigua*/*L. americana* and *L. iraniensis*/*L. jatrophicola* could not be distinguished in the phylogenetic analyses.

Pathogenicity trials

The inoculated plants developed typical dieback symptoms showing necrotic leaves accompanied by internal tissue necrosis (Fig 4A) starting from the point of inoculation. Defoliation was observed later on inoculated plants. The development of conidiomata in inoculated plants was also observed (Fig 4B). Control plants did not develop any symptoms. The size of the necrotic lesion varied significantly among isolates (Table 4). Thus, isolates LACIN2, LASID1, LASOM1 (*Lasiodiplodia theobromae*) and LASID3 (*Lasiodiplodia iraniensis*) were the most aggressive ones while isolates LACIS1, LAMAT1, LAMAT3, and LAREP3 (*Lasiodiplodia theobromae*) were the least aggressive (Table 4). The pathogens were consistently re-isolated from all inoculated plants and identified by means of DNA sequencing, thus confirming Koch's postulates. No isolates were obtained from the negative controls.

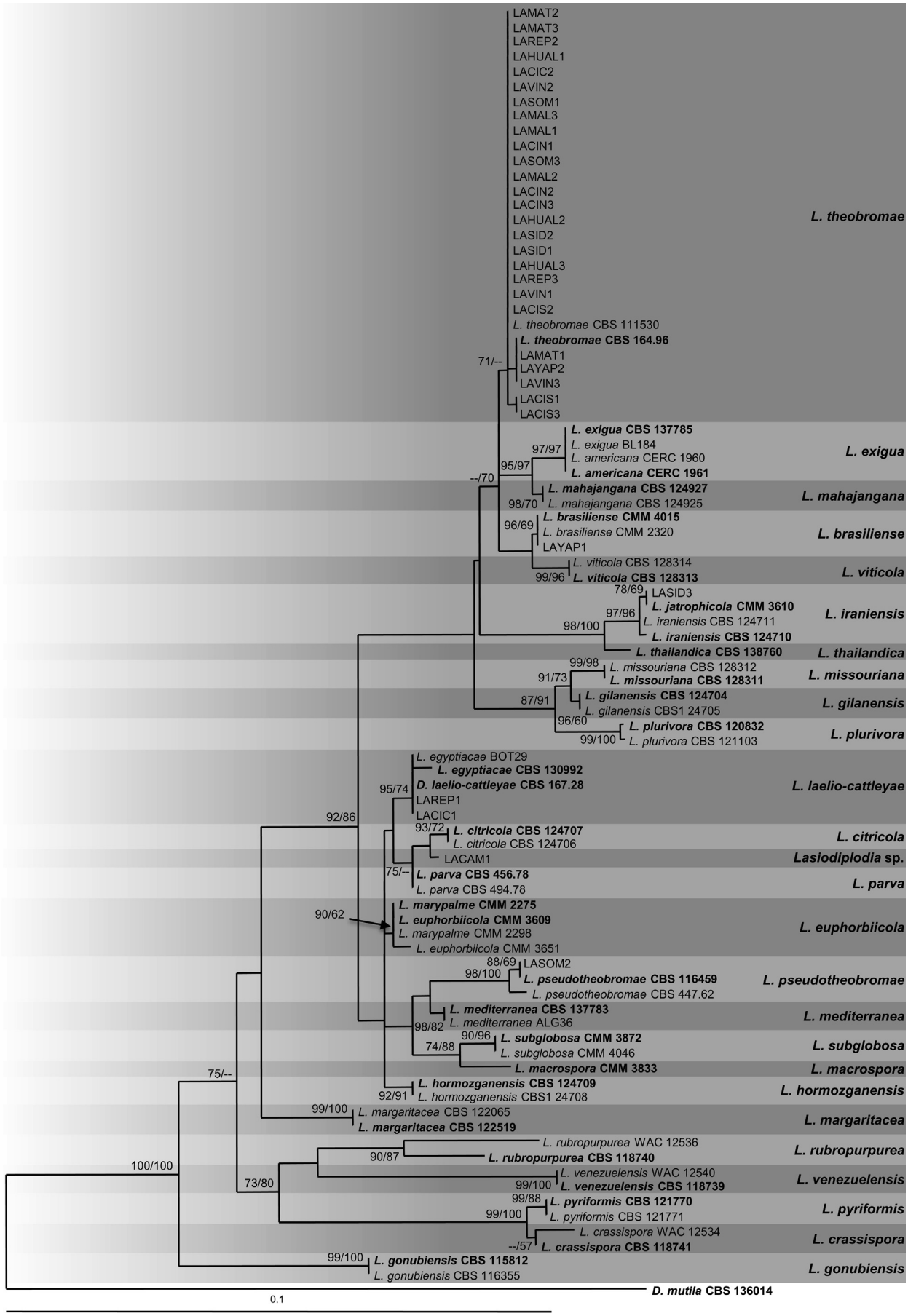
Taxonomy

Lasiodiplodia laeliocattleyae (Sibilia) A. Alves, **comb. nov.** MycoBank MB815697 Fig 5.

Basionym: *Diplodia laeliocattleyae* Sibilia, *Boll. R. Staz. Patalog. Veget. Roma, N.S.* **7**: 433 (1927). MycoBank MB268837
Synonym: *Lasiodiplodia egyptiacae* A.M. Ismail *et al.*, *Australas. Plant Path.* **41**: 655 (2012). MycoBank MB564516.

Ascomata not observed. *Conidiomata* stromatic, produced on pine needles or poplar twigs on ¼ strength PDA within 1–2 wks, mostly solitary, black, globose to subglobose. *Paraphyses* hyaline, subcylindrical, aseptate, up to 95 μm long, 2–3 μm wide. *Conidiophores* absent. *Conidiogenous cells* holoblastic, hyaline, smooth, thin-walled, cylindrical, 11–14 × 3–4 μm. *Conidia* thick-walled, initially hyaline, aseptate, obovoid to ellipsoid, with granular content, rounded at apex and occasionally tapered at the base, becoming dark brown, verruculose, 1-septate, with longitudinal striations, (18–)22.8(–27.4) × (11.7–)14.6(–17.2) μm (av. of 250 conidia ± SD = 22.8 ± 1.4 × 14.6 ± 1.1 μm, L/W ratio = 1.6).

Culture characteristics: Colonies on PDA at 25 °C with abundant aerial mycelium, initially white to smoke-grey, turning greenish grey on the surface and reverse, becoming dark slate blue with age.
Cultures examined: **Italy**, from living leaves and pseudobulbs of the cultivated orchid *Laeliocattleya*, 1927, C. Sibilia (CBS 167.28 – ex-holotype of *Diplodia laeliocattleyae*), **Peru**: Piura, Cieneguillo Centro, from *M. indica* branch with necrosis, June 2012, E. Rodríguez-Gálvez (LACIC1 and LAREP1).
Hosts: *Laeliocattleya*, on living leaves and pseudobulbs (Sibilia 1927); *Mangifera indica* (Ismail *et al.* 2012; Marques *et al.* 2013; present study); *Cocos nucifera* (Rosado *et al.* 2016).
Known distribution: Italy (Sibilia 1927), Brazil (Marques *et al.* 2013; Rosado *et al.* 2016), Egypt (Ismail *et al.* 2012) and Peru (current study).
Notes: Ismail *et al.* (2012) reported shorter paraphyses (up to 57 μm long) for *L. egyptiacae*. Moreover, in their study conidia


LAMAT2
LAMAT3
LAREP2
LAHUAL1
LACIC2
LAVIN2
LASOM1
LAMAL3
LAMAL1
LACIN1
LASOM3
LAMAL2
LACIN2
LACIN3
LAHUAL2
LASID2
LASID1
LAHUAL3
LAREP3
LAVIN1
LACIS2
L. theobromae CBS 111530
L. theobromae CBS 164.96
LAMAT1
LAYAP2
LAVIN3
LACIS1
LACIS3
L. theobromae
L. exigua CBS 137785
L. exigua BL184
L. americana CERC 1960
L. americana CERC 1961
L. exigua
L. mahajangana CBS 124927
L. mahajangana CBS 124925
L. mahajangana
L. brasiliense CMM 4015
L. brasiliense CMM 2320
LAYAP1
L. brasiliense
L. viticola CBS 128314
L. viticola CBS 128313
L. viticola
LASID3
L. jatrophicola CMM 3610
L. iraniensis CBS 124711
L. iraniensis CBS 124710
L. iraniensis
L. thailandica CBS 138760
L. thailandica
L. missouriana CBS 128312
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L. missouriana
L. gilanensis CBS 124704
L. gilanensis CBS1 24705
L. gilanensis
L. plurivora CBS 120832
L. plurivora CBS 121103
L. plurivora
L. egyptiacae BOT29
L. egyptiacae CBS 130992
D. laelio-cattleyae CBS 167.28
LAREP1
LACIC1
L. laelio-cattleyae
L. citricola CBS 124707
L. citricola CBS 124706
L. citricola
LACAM1
Lasiodiplodia sp.
L. parva CBS 456.78
L. parva CBS 494.78
L. parva
L. marypalme CMM 2275
L. euphorbiicola CMM 3609
L. marypalme CMM 2298
L. euphorbiicola CMM 3651
L. euphorbiicola
LASOM2
L. pseudotheobromae CBS 116459
L. pseudotheobromae CBS 447.62
L. pseudotheobromae
L. mediterranea CBS 137783
L. mediterranea ALG36
L. mediterranea
L. subglobosa CMM 3872
L. subglobosa CMM 4046
L. subglobosa
L. macrospora CMM 3833
L. macrospora
L. hormozganensis CBS 124709
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L. hormozganensis
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L. venezuelensis WAC 12540
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L. venezuelensis
L. pyriformis CBS 121770
L. pyriformis CBS 121771
L. pyriformis
L. crassispora WAC 12534
L. crassispora CBS 118741
L. crassispora
L. gonubiensis CBS 115812
L. gonubiensis CBS 116355
L. gonubiensis
D. mutila CBS 136014
0.1

Table 3 – Polymorphisms in the nucleotide sequences of ITS and *tef1-α* loci showing the relationship between *Lasiodiplodia* sp. LACAM1 and its closest phylogenetic relatives *L. parva* and *L. citricola*. Shared polymorphisms with either *L. parva* or *L. citricola* are highlighted in grey.

Species	ITS			*tef1-α*		
	42	98	157	46	79	164
L. citricola	G	T	C	T	C	G
Lasiodiplodia sp.	G	T	T	C	T	–
L. parva	A	C	T	C	T	–

were narrower (17–)20–24(–27) × (11–)11–12(–13) μm (av. ± SD = 22 ± 2 μm, 12 ± 1 μm) than the ones reported here and had a higher L/W ratio = 2. However, as shown here, our isolates are phylogenetically indistinguishable from the ones studied by Ismail *et al.* (2012) and therefore correspond to the same species.

***Lasiodiplodia* sp.** Fig 6.

Conidiomata stromatic, produced on pine needles or poplar twigs on ¼ strength PDA within 1–2 wks, mostly solitary, black, globose to subglobose. *Paraphyses* hyaline, subcylindrical, rarely septate and branched, up to 61 μm long, 2–3 μm wide. *Conidiophores* absent. *Conidiogenous cells* holoblastic, hyaline, smooth, thin-walled, cylindrical, 11–15 × 3–4 μm. *Conidia* thick-walled, initially hyaline, aseptate, ranging from subglobose to obovoid and ellipsoid, with granular content, rounded at apex, and occasionally truncate at base, becoming brown soon after being formed, verruculose, 1-septate, with longitudinal striations, (16–)20.2(–25.7) × (9.1–)12(–15.9) μm (av. of 325 conidia ± SD = 20.2 ± 1.7 × 12 ± 1.2 μm, L/W ratio = 1.7).

Culture characteristics: Colonies on PDA with moderate aerial mycelium, initially white to smoke-grey, turning greenish grey on the surface and reverse, becoming dark slate blue with age.

Cultures examined: **Peru**: Piura, Campanas, from *M. indica* branch with necrosis, June 2012, E. Rodríguez-Gálvez (LACAM1).

Host: Mangifera indica.

Known distribution: Peru.

Notes: Phylogenetically this taxon clusters between *L. parva* and *L. citricola*. Conidia are smaller than those of *L. citricola* but conidial sizes are virtually indistinguishable from those of *L. parva*. *Lasiodiplodia* sp. shares polymorphisms in the ITS and *tef1-α* loci with either *L. parva* or *L. citricola* (Table 3).

Lasiodiplodia exigua Linaldeddu *et al.*, *Fungal Divers.* **71**: 207 (2015).

MycoBank MB808355

Synonym: *Lasiodiplodia americana* S.F. Chen *et al.*, *Mycologia* **107**: 785 (2015). MycoBank MB810934.

Notes: Chen *et al.* (2015) described *L. americana* as a distinct species based on morphological and DNA sequence data. However, we clearly show that it is phylogenetically indistinguishable from *L. exigua*. The name *L. exigua* was published online on 29 August 2014, and therefore takes precedence over *L. americana* which was published online on 14 May 2015.

Lasiodiplodia iraniensis Abdollahz. *et al.*, *Persoonia* **25**: 8 (2010). MycoBank MB16780

Synonym: *Lasiodiplodia jatrophicola* A.R. Machado & O.L. Pereira, *Fungal Divers.* **67**: 239 (2014). MycoBank MB804869.

Notes: Machado *et al.* (2014) recognised *L. jatrophicola* as distinct from *L. iraniensis* (Abdollahzadeh *et al.* 2010) based on phylogenetic analysis and due to its larger conidia and smaller paraphyses. However, morphology is of poor value for species discrimination and we show here that both species cannot be separated on the basis of ITS and *tef1-α* sequence data.

Discussion

Mango dieback was detected in all regions surveyed but with different levels of incidence. In comparison to a previous survey carried out in 1998 (Rodríguez-Gálvez *et al.* 1999), the incidence values in 2012 were much higher. In some cases, the increase was remarkable. For example, in Alto Piura the incidence increased from 0.09 % to 10.20 % (more than 100 times higher) while in San Lorenzo it increased from 0.89 % to 6.74 %.

Six species of *Lasiodiplodia* were identified from symptomatic mango plants: *Lasiodiplodia brasiliense*, *Lasiodiplodia iraniensis*, *Lasiodiplodia laeliocattleyae*, *Lasiodiplodia pseudotheobromae*, *Lasiodiplodia theobromae*, and a *Lasiodiplodia* sp. Apart from *L. theobromae*, all other species are reported for the first time in the country. A drawback of this study is the fact that only a small percentage of the isolates obtained were identified molecularly. In the future the entire collection should be identified by DNA sequence data in order to obtain a full representation of the species identity, their incidence and distribution among orchards.

In *Lasiodiplodia* species identification using only morphology is virtually impossible and it is crucial to use DNA sequence data, preferably combining sequences from multiple loci (Phillips *et al.* 2013; Slippers *et al.* 2014). For *Lasiodiplodia*, ITS and *tef1-α* sequences have been widely used to discriminate between species, especially cryptic species which seem frequent in the genus (Burgess *et al.* 2006; Alves *et al.* 2008; Ismail *et al.* 2012; Marques *et al.* 2013; Netto *et al.* 2014; Slippers *et al.* 2014; Linaldeddu *et al.* 2015; Trakunyingcharoen *et al.* 2015). The present study provides a clear illustration of the problems associated with morphology-based identification. *Lasiodiplodia theobromae* has

Fig 3 – Phylogenetic relationships of *Lasiodiplodia* species inferred using the Maximum Likelihood method based on the Tamura-Nei model. ML/MP bootstrap values (>70 %) are given at the nodes. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site and rooted to *D. mutila*. Ex-type strains are in bold face.

Fig 4 – Dieback symptoms developed by *M. indica* plants cv Kent artificially inoculated with *Lasiodiplodia*. (A) – basipetal necrosis of the stem and leaf necrosis, (B) – pycnidia development in the infected stem.

Table 4 – Mean lesion length ± standard deviations (SD) caused by each *Lasiodiplodia* spp. isolate on *M. indica*. Means with the same letter are not significantly different at P = 0.05.

Species	Isolate	Mean lesion length (cm) ± SD
L. theobromae	LACIN-2	11.04 ± 2.54 a
L. iraniensis	LASID-3	9.76 ± 1.50 ab
L. theobromae	LASID-1	9.26 ± 1.21 abc
L. theobromae	LASOM-1	9.14 ± 1.65 abc
L. theobromae	LASID-2	8.86 ± 1.35 abcd
L. theobromae	LAHUAL-2	8.00 ± 0.95 abcde
L. theobromae	LAVIN-2	7.76 ± 2.70 abcdef
L. theobromae	LACIN-1	7.26 ± 1.20 bcdefg
L. theobromae	LAHUAL-3	7.20 ± 1.48 bcdefg
L. laeliocattleyae	LAREP-1	7.20 ± 1.79 bcdefg
L. pseudotheobromae	LASOM-2	6.96 ± 1.42 bcdefg
L. theobromae	LACIS-2	6.80 ± 1.42 bcdefg
L. theobromae	LAVIN-3	6.78 ± 1.75 bcdefg
L. laeliocattleyae	LACIC-1	6.50 ± 0.65 bcdefg
L. theobromae	LACIS-3	6.44 ± 1.34 bcdefg
L. theobromae	LACIN-3	6.42 ± 1.84 bcdefg
L. theobromae	LAMAT-2	6.24 ± 0.97 bcdefg
L. theobromae	LAVI-1	6.18 ± 2.04 cdefg
L. theobromae	LAMAL-2	6.14 ± 1.50 cdefg
L. theobromae	LAMAL-3	6.06 ± 1.22 cdefg
L. theobromae	LAYAP-2	5.40 ± 1.09 efg
L. theobromae	LASOM-3	5.18 ± 1.60 efg
L. brasiliense	LAYAP-1	5.10 ± 0.66 efg
L. theobromae	LACIC-2	5.04 ± 1.20 efg
L. theobromae	LAREP-2	4.66 ± 1.62 efg
Lasiodiplodia sp.	LACAM-1	4.62 ± 1.51 efgh
L. theobromae	LAHUAL-1	4.60 ± 0.89 efgh
L. theobromae	LAMAL-1	4.46 ± 0.78 fgh
L. theobromae	LAREP-3	4.20 ± 1.15 gh
L. theobromae	LAMAT-3	3.96 ± 0.86 gh
L. theobromae	LAMAT-1	3.88 ± 0.75 gh
L. theobromae	LACIS-1	1.30 ± 0.57 h

previously been reported as the causal agent of mango dieback in Peru (Rodríguez-Gálvez *et al.* 1999), but in this study using ITS and *tef1-α* sequence data we show that four other species of *Lasiodiplodia* are also involved in causing this disease.

Lasiodiplodia theobromae was the most frequently isolated species among the selected isolates (approx. 81 %) obtained from mango plants with dieback symptoms. These results are not surprising, giving that this species is known to be widespread, particularly in tropical regions, and is commonly reported as a pathogen of mango trees (Abdollahzadeh *et al.* 2010; Costa *et al.* 2010; Ismail *et al.* 2012; Marques *et al.* 2013). Moreover, Marques *et al.* (2013) in their study of *Lasiodiplodia* species associated with mango in Brazil reported similar results with *L. theobromae* being the most frequent among the seven species found. Likewise, Netto *et al.* (2014) reported *L. theobromae* to be the predominant species among five *Lasiodiplodia* species associated with papaya (*Carica papaya*) stem-end rot in Brazil. Furthermore, Rodríguez-Gálvez *et al.* (2015) reported *L. theobromae* as the only species associated with Botryosphaeria dieback and canker of table grapes in the region of Piura (Peru).

Lasiodiplodia pseudotheobromae appears to also have a wide host range and geographic distribution (Alves *et al.* 2008; Abdollahzadeh *et al.* 2010; Phillips *et al.* 2013). It has been associated with cankers, dieback and stem-end rot of mango in Egypt (Ismail *et al.* 2012), Brazil (Marques *et al.* 2013), and Western Australia (Sakalidis *et al.* 2011). In this study we show it to be associated with dieback of mango in Peru.

Lasiodiplodia brasiliense was described for the first time on papaya in Brazil associated with stem-end rot (Netto *et al.* 2014). More recently, it was also associated with dieback of table grape (Correia *et al.* 2016) and with postharvest stem-end rot of coconut (*Cocos nucifera*) (Rosado *et al.* 2016) both in Brazil. A *Lasiodiplodia* sp. reported by Marques *et al.*

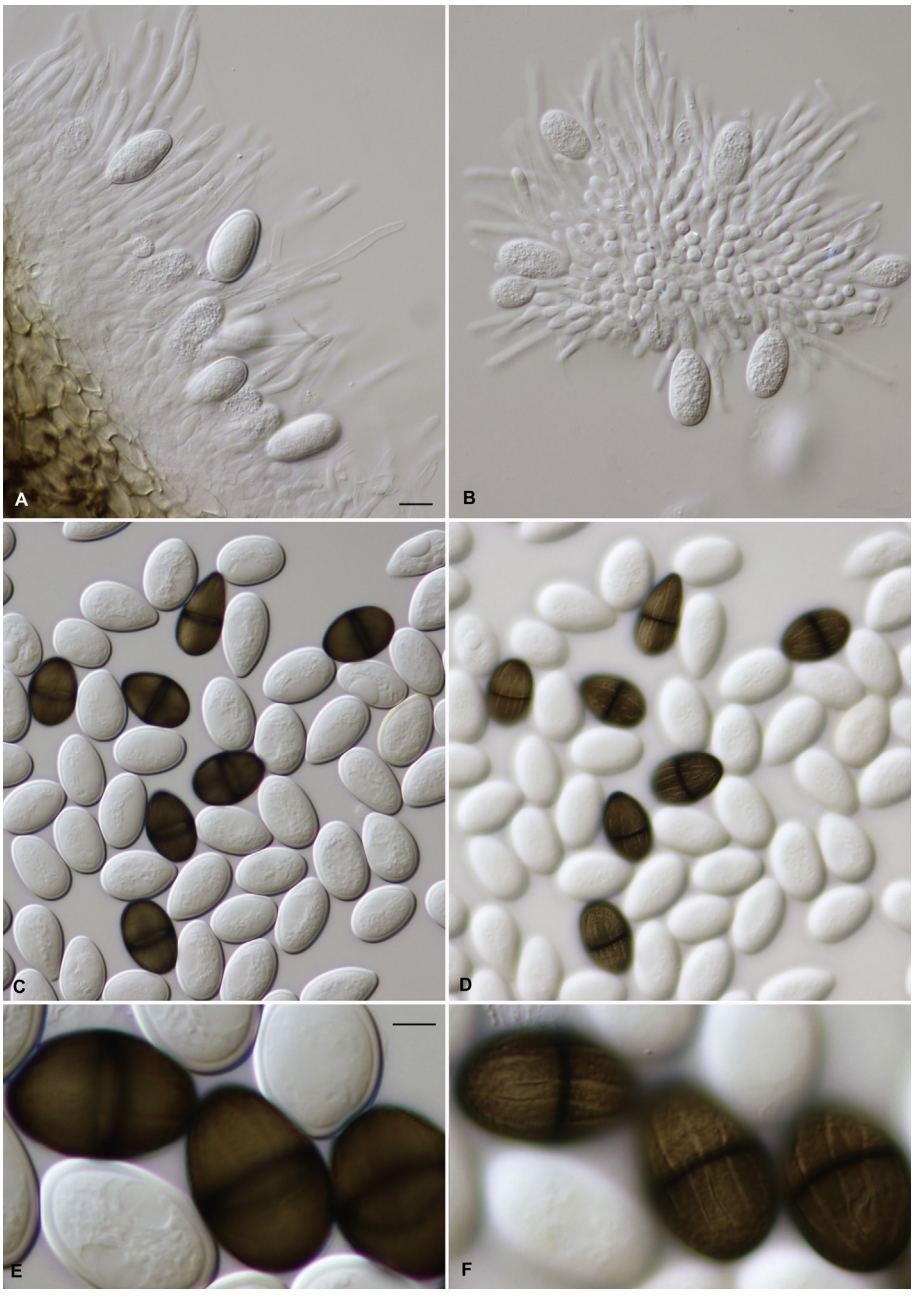

Fig 5 – *Lasiodiplodia laeliocattleyae* LACIC1. (A). Conidiogenous layer with conidia developing on conidiogenous cells. (B). Conidia developing on conidiogenous cells and paraphyses. (C, D). Hyaline aseptate and brown 1-septate conidia in two focal planes to show the striations in the inner surface of the wall. (E, F). Detail of brown 1-septate conidia in two focal planes to show the striations in the inner surface of the wall. Scale bars: A–D = 10 μm; E–F = 5 μm.

(2013) on mango in Brazil was later shown to be *L. brasiliense* (Netto *et al.* 2014). Thus, this study represents the first report of the species outside Brazil. Presently this species appears to be restricted to South America where it affects several hosts.

Ismail *et al.* (2012) studied a set of isolates associated with dieback of mango in Egypt that was previously identified as *L. theobromae,* and described *Lasiodiplodia egyptiacae* as novel species. According to the authors this species is closely related to *Lasiodiplodia citricola*, *Lasiodiplodia parva*, and *Lasiodiplodia hormozganensis* but is phylogenetically distinct and can also be distinguished on the morphology of conidia and paraphyses. In our phylogenetic analyses, *L. egyptiacae* formed a separate and well-supported (95 % bootstrap value) clade. However, this clade was found to also include the ex-type strain of *Diplodia laeliocattleyae* (CBS 167.28). This species was described in 1927 from living leaves and pseudobulbs of the cultivated orchid *Laeliocattleya* in Italy (Sibilia 1927). Unfortunately the type material of *D. laeliocattleyae* was not available for study, but from the position of the ex-type strain in our phylogenetic analysis, it is obvious that this species does not belong in the genus *Diplodia*. We have consequently

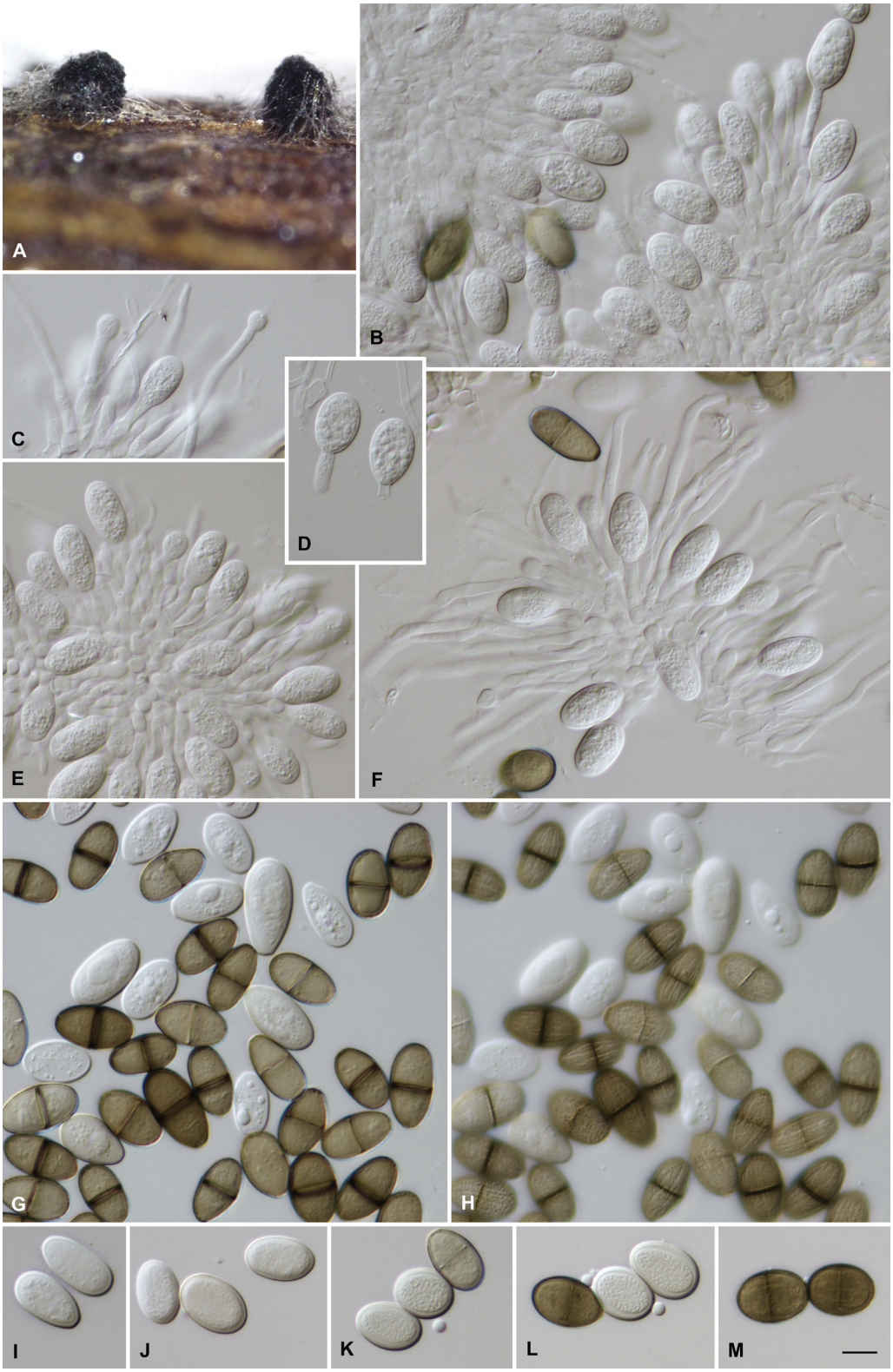

Fig 6 – *Lasiodiplodia* sp. LACAM1. (A). Pycnidia formed on poplar twigs. (B, E). Conidia developing on conidiogenous cells. (C). Detail of long conidiogenous cells. (D). Conidia attached to conidiogenous cell. (F). Conidia developing on conidiogenous cells and paraphyses. (G, H). Hyaline aseptate and brown 1-septate conidia in two focal planes to show the striations in the inner surface of the wall. I–M. Conidia in different stages of maturation. Scale bars: B–M = 10 μm.

transferred it to *Lasiodiplodia* as *L. laeliocattleyae* with *L. egyptiacae* as a later synonym. Apart from a report from mango in Egypt, this species has also been associated with stem-end rot of mango in Brazil (Marques *et al.* 2013), and postharvest stem-end rot of coconut in Brazil (Rosado *et al.* 2016).

An isolate of a *Lasiodiplodia* sp. (LACAM1) obtained in this study could not be unequivocally attributed to any of the currently known species in the genus. Morphologically, it closely resembles *L. parva* and has identical conidial dimensions. In the combined ITS and *tef1-α* phylogenetic analysis it was placed between *L. citricola* and *L. parva* (Fig 3), while in individual gene genealogies it clustered with either one or the other (Fig S1). Gene genealogy concordance can be useful to identify hybrid individuals because hybrids should group with different species in different single-gene genealogies (O'Donnell *et al.* 2000; Taylor *et al.* 2000). Thus, we hypothesize that this isolate may be a hybrid between *L. citricola* and *L. parva*. However, since only two loci were used, future studies including several single-copy nuclear genes (e.g. O'Donnell *et al.* 2000) and additional isolates should be carried out in order to confirm this hypothesis. Another possibility is that *L. citricola* and *L. parva* represent a single species and the small differences seen between them represent intraspecific genetic variability. Our results indicate that more studies using additional loci apart from ITS and *tef1-α* are needed to clarify issues related to species recognition and hybridisation in the genus *Lasiodiplodia*.

Machado *et al.* (2014) described four new species of *Lasiodiplodia*, including *Lasiodiplodia jatrophicola*, associated with collar and root rot of the biofuel plant *Jatropha curcas* in Brazil. The authors suggested that *L. jatrophicola* is closely related to *L. iraniensis* (Abdollahzadeh *et al.* 2010), but can be distinguished phylogenetically, and also morphologically due to its larger conidia and smaller paraphyses. In our phylogenetic analyses, *L. jatrophicola* formed a sub-clade with only moderate bootstrap support (78 %) within the *L. iraniensis* clade. At the molecular level the two species are separated based on very minor differences, a single nucleotide difference in the ITS sequence and no fixed polymorphisms in the *tef1-α* sequences. Despite the reported morphological differences, it is well known that morphological characters are quite variable in these fungi and of little value for species discrimination (Phillips *et al.* 2013; Slippers *et al.* 2014). Thus, *L. jatrophicola* is considered as a synonym of *L. iraniensis*. This species is known to occur on mango in Iran (Abdollahzadeh *et al.* 2010) and Western Australia (Sakalidis *et al.* 2011) and is reported here for the first time on this host in Peru.

Lasiodiplodia marypalme and *Lasiodiplodia euphorbiicola* were described in the same year from Brazil on respectively *Carica papaya* and *J. curcas* (Machado *et al.* 2014; Netto *et al.* 2014). However, the ITS and *tef1-α* sequences from both species are 100 % identical and the phylogenetic analysis presented here showed them to be indistinguishable. This is also consistent with a recent study by Correia *et al.* (2016) where *L. marypalme* was reduced to synonymy with *L. euphorbiicola*.

Chen *et al.* (2015) described *Lasiodiplodia americana* as a distinct species based on morphological characters and DNA sequence data from the ITS, *tef1-α* and beta-tubulin gene regions. The authors suggested that *L. americana* is phylogenetically closely related to *Lasiodiplodia mahajangana*, *L. theobromae*, and *L. iraniensis*, but that it can be distinguished from these species by the size and shape of their conidia and also due to its different optimum growth temperature. However, we clearly show that it is phylogenetically indistinguishable from *Lasiodiplodia exigua*, a species recently described by Linaldeddu *et al.* (2015) and that was not included in the phylogenetic analyses performed by Chen *et al.* (2015). Pairwise comparisons showed that ITS sequences of both species are 100 % identical. Also, *tef1-α* sequences have two nucleotide differences but these are located in the binding sites of primers EF1-728F and EF1-986R (Carbone & Kohn 1999) frequently used to amplify and sequence this gene region in many *Lasiodiplodia* species and used also by Chen *et al.* (2015). Thus, to avoid introducing errors in alignments and consequently in phylogenies, these regions must not be included in phylogenetic analyses. Therefore, *L. exigua* and *L. americana* are regarded as synonyms, and because *L. exigua* was published first, this species name is retained.

Lasiodiplodia species are frequently reported as the most aggressive pathogens of mango among the wide diversity of *Botryosphaeriaceae* found on this host. However, several studies show that there is some variability in aggressiveness both at the species and isolate level (e.g. Costa *et al.* 2010; Marques *et al.* 2013).

Pathogenicity trials showed that all species/isolates were pathogenic to mango plants of the cultivar Kent. Under the conditions used for the assays, symptom developed rapidly and lesions were readily visible after 14 d. *Lasiodiplodia theobromae* and *L. jatrophicola* were the most aggressive of the species tested. In the case of *L. jatrophicola* this is noteworthy since this species is here reported for the first time on mango. A large number of isolates of *L. theobromae* were included in the artificial inoculation trials. A high level of variability was found in the aggressiveness of these isolates. This is well illustrated by the fact that the most and least aggressive isolates belong both to *L. theobromae*. The species *L. pseudotheobromae* and *L. laeliocattleyae* were also highly aggressive towards mango, while *Lasiodiplodia* sp. LACAM1 (putative hybrid) was amongst the least aggressive isolates.

Apart from *Lasiodiplodia* spp. other species of *Botryosphaeriaceae* are known to be pathogens of mango (Slippers *et al.* 2005; Costa *et al.* 2010; Sakalidis *et al.* 2011). An interesting aspect of this study is that although an extensive survey was undertaken, no other *Botryosphaeriaceae* species were found apart from *Lasiodiplodia* spp. in the region of Piura, Peru. Rodríguez-Gálvez *et al.* (2015) performed an extensive survey in vineyards in the same region and reported a similar situation identifying only *L. theobromae* from table grapes with Botryosphaeria dieback symptoms. It is possible, that other species of *Botryosphaeriaceae* occur in this region but *Lasiodiplodia* spp. are clearly dominant. Given that these species are typically tropical and subtropical pathogens, it is conceivable that they are better adapted to the environmental conditions of the region.

This study shows that *L. theobromae* is the main agent of dieback of mango in the region of Piura, Peru. Nevertheless, other *Lasiodiplodia* species are also associated with this disease

and proved to be pathogenic to mango and thus their relevance and impact should not be overlooked. The substantial increase on the disease incidence reported here is reason for concern. It is of the utmost importance to establish the pathway of infection in order to develop suitable management strategies in order to prevent or reduce disease impact. The results from our survey suggest that *Lasiodiplodia* spp. infect mango through pruning wounds. Pruning wounds provide not only a point of entry to pathogens but are also a source of stress for the host plant. This in combination with environmental stress factors (e.g. drought, elevated temperatures) may be the leading cause of the reported increase in disease incidence.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funbio.2016.06.004.

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