Phylogeny, Distribution, and Pathogenicity of *Lasiodiplodia* Species Associated With Cankers and Dieback Symptoms of Persian Lime in Mexico

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Abstract

Persian lime (*Citrus latifolia* Tan.) is an important and widely cultivated fruit crop in several regions of Mexico. In recent years, severe symptoms of gummosis, stem cankers, and dieback were detected in the Persian lime-producing region in the states of Veracruz and Puebla, Mexico. The aims of this study were to identify the species of *Lasiodiplodia* associated with these symptoms, determine the distribution of these species, and test their pathogenicity and virulence on Persian lime plants. In 2015, symptomatic samples were collected from 12 commercial Persian lime orchards, and 60 *Lasiodiplodia* isolates were obtained. Fungal identification of 32 representative isolates was performed using a phylogenetic analysis based on DNA sequence data of the internal transcribed spacer region and part of the translation elongation factor 1- α and β -tubulin genes. Sequence analyses were carried out using the Maximum Likelihood and Bayesian Inference methods. Six *Lasiodiplodia* species were

identified as *Lasiodiplodia pseudotheobromae*, *Lasiodiplodia theobromae*, *Lasiodiplodia brasiliense*, *Lasiodiplodia subglobosa*, *Lasiodiplodia citricola*, and *Lasiodiplodia iraniensis*. All *Lasiodiplodia* species of this study are reported for the first time in association with Persian lime in Mexico and worldwide. *L. pseudotheobromae* (46.9% of isolates) was the most frequently isolated species followed by *L. theobromae* (28.1%) and *L. brasiliense* (12.5%). Pathogenicity on Persian lime young plants using a mycelial plug inoculation method showed that all identified *Lasiodiplodia* species were able to cause necrotic lesions and gummosis, but *L. subglobosa*, *L. iraniensis*, and *L. pseudotheobromae* were the most virulent.

Keywords: Botryosphaeriaceae, *Citrus latifolia*, fungal diversity, pathogenicity, phylogenetic analysis

Mexico occupies the second place in world production of limes and lemons with a total of 2.4 million tonnes (Food and Agriculture Organization [FAO] 2016). Currently, this country is considered the main producer and exporter of Persian lime (*Citrus latifolia* Tan.) worldwide, with a cultivated area of 82,900 ha and a total production of 1.01 million tonnes (SIAP 2013). The main production area for Persian lime in Mexico is concentrated in the central coastal region of the Gulf of Mexico, and 46% of production is exported to the U.S.A., Europe, Canada, and Japan. Fruits are used fresh, and they are also used for the extraction of pectin and essential oils for foreign markets (Mendoza-Tornez et al. 2016). Dieback of Persian lime trees is a disease that decreases the production and longevity of orchards in the lime-producing region of the states of Puebla and Veracruz, Mexico, but the causative agents are unknown.

The Botryosphaeriaceae family (Dothideomycetes, Botryosphaeriales) includes 23 genera and 187 species, with cosmopolitan distribution on a broad range of woody hosts occurring as endophytes, saprobes, or destructive plant pathogens (Dissanayake et al. 2016; Phillips et al. 2013; Slippers and Wingfield 2007; Slippers et al. 2013). Several Botryosphaeriaceae species have been previously associated with citrus diseases, including *Diplodia* spp., *Dothiorella* spp., *Neofusicoccum* spp. (Adesemoye et al. 2014), *Neoscytalidium* spp. (Adesemoye et al. 2014; Mayorquin et al. 2016), and *Lasiodiplodia* spp. (Abdollahzadeh et al. 2010; Adesemoye et al. 2014;

Al-Sadi et al. 2013; Alves et al. 2008; Coutinho et al. 2017; Davis et al. 1987; Ferrari et al. 1996; Guajardo et al. 2018; Linaldeddu et al. 2015).

The main features that distinguish the genus *Lasiodiplodia* from other closely related genera are the presence of pycnidial paraphyses and longitudinal striations on mature conidia (Abdollahzadeh et al. 2010). Currently, the identification of *Lasiodiplodia* species by morphology is impossible, and it is necessary to use DNA sequence data, preferably combining sequences from multiple loci, such as internal transcribed spacer (ITS), β -tubulin (BT2), and translation elongation factor 1- α (TEF1) genes, for accurate identification (Chen et al. 2014a, 2014b; Coutinho et al. 2017; Machado et al. 2014; Phillips et al. 2013; Rosado et al. 2016; Slippers et al. 2014; Zhou et al. 2015).

Previous studies have associated *Lasiodiplodia* spp. with symptoms of dieback, gummosis, and canker in a wide diversity of *Citrus* species, including sweet orange (*Citrus sinensis* Osbeck) in the U.S.A., Venezuela, Oman, Algeria, and Brazil (Al-Sadi et al. 2013; Coutinho et al. 2017; Davis et al. 1987; Ferrari et al. 1996; Linaldeddu et al. 2015); acid lime (*Citrus aurantifolia* S.), sweet lime (*Citrus limettoides* Tan.), and mandarin (*Citrus reticulata* Blanco) in Oman (Al-Sadi et al. 2013); sour orange (*Citrus aurantium* L.) in the U.S.A. and Suriname (Alves et al. 2008; Davis et al. 1987); grapefruit (*Citrus paradisi* Macfad) in the U.S.A. and Venezuela (Davis et al. 1987; Ferrari et al. 1996); lemon (*Citrus limon* L. Osbeck) in Venezuela and Chile (Ferrari et al. 1996; Guajardo et al. 2018); and *Citrus* species in Iran and the U.S.A. (Abdollahzadeh et al. 2010; Adesemoye et al. 2014). However, there are no studies of *Lasiodiplodia* species affecting Persian lime trees worldwide.

The aims of this study were to identify the species of *Lasiodiplodia* associated with these symptoms by phylogenetic inference, determine the distribution of these species, and test their pathogenicity and virulence on young Persian lime plants.

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Materials and Methods

Field survey. In September 2015, a survey was conducted in 12 commercial Persian lime orchards distributed in the states of Puebla and Veracruz in the central coastal region of the Gulf of Mexico. Symptomatic plant tissues (4 to 14 years old) showing stem cankers, gummosis, and branches dieback were collected (Fig. 1). Disease incidence was calculated using the following formula:

$I(\%) = (ni/N) \times 100$, where I = incidence of disease, ni = total number of symptomatic trees, and N = total number of evaluated trees.

Isolates. Fragments of branches (4 to 5 mm in diameter) were collected from the margin between necrotic and healthy tissues, surface sterilized with 1% sodium hypochlorite for 1 min, rinsed three times with sterile distilled water, and dried on sterile paper. Fragments were placed in petri plates containing 2% potato dextrose agar (PDA;

Fig. 1. Symptoms of dieback and canker of Persian lime (*Citrus latifolia*) associated with *Lasiodiplodia* spp. in Mexico. **A**, Tree showing dieback of branches. **B**, Tree exhibiting severe defoliation. **C and D**, Sunken cankers on branches. **E**, Branch with longitudinal cracking of the bark. **F**, Branch with canker and internal tissue necrosis. **G**, Diseased trunk with severe wood necrosis.

Difco, Detroit, MI) supplemented with 0.5 g/L streptomycin sulfate (Sigma-Aldrich Co, St. Louis, MO) and incubated at 25°C in the dark. Pure cultures were obtained by transferring hyphal tips from the colony margin onto fresh PDA and incubated at 25°C in the dark. The isolates used in this study were deposited in the Culture Collection of Phytopathogenic Fungi of the Department of Agricultural Parasitology at the Chapingo Autonomous University. Stock cultures were stored in 2.0-ml cryovials in 15% glycerol at -80°C and sterile water at 4°C.

DNA extraction, polymerase chain reaction amplification, and sequencing. Aerial mycelium from 6-day-old culture was scraped directly from the medium using a sterile spatula and placed in 2-ml microtubes. Total genomic DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA) following the manufacturer's recommendation. DNA concentrations were quantified using a NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, Madison, WI), and the samples were diluted to 100 ng/μl for polymerase chain reaction (PCR).

Table 1. GenBank accession numbers of DNA sequences of *Lasiodiplodia* spp. included in the phylogenetic study^a

Species	Isolate code	Host	Location	GenBank accession number		
				ITS	TEF1	BT2
<i>Lasiodiplodia avicenniae</i>	CMW41467	<i>Avicennia marina</i>	South Africa	KP860835	KP860680	KP860758
<i>Lasiodiplodia avicenniae</i>	LAS199 (DNA)	<i>Avicennia marina</i>	South Africa	KU587957	KU587947	KU587868
<i>Lasiodiplodia brasiliense</i>	CMM4015	<i>Mangifera indica</i>	Brazil	JX464063	JX464049	N/A
<i>Lasiodiplodia brasiliense</i>	CMW35884	<i>Adansonia madagascariensis</i>	Madagascar	KU887094	KU886972	KU887466
<i>Lasiodiplodia brasiliense</i>	UACH257	<i>Citrus latifolia</i>	Mexico	MH277910	MH286537	MH279903
<i>Lasiodiplodia brasiliense</i>	UACH266	<i>Citrus latifolia</i>	Mexico	MH277911	MH286535	MH279905
<i>Lasiodiplodia brasiliense</i>	UACH267	<i>Citrus latifolia</i>	Mexico	MH277912	MH286536	MH279906
<i>Lasiodiplodia brasiliense</i>	UACH269	<i>Citrus latifolia</i>	Mexico	MH277913	MH286538	MH279904
<i>Lasiodiplodia brugulerae</i>	CMW41470	<i>Bruguiera gymnorrhiza</i>	South Africa	KP860833	KP860678	KP860756
<i>Lasiodiplodia brugulerae</i>	CMW42480	<i>Bruguiera gymnorrhiza</i>	South Africa	KP860832	KP860677	KP860755
<i>Lasiodiplodia caatinguensis</i>	IBL271	<i>Anacardium occidentale</i>	Brazil	KT154756	KT154750	KT154763
<i>Lasiodiplodia caatinguensis</i>	CMM1325	<i>Citrus sinensis</i>	Brazil	KT154760	KT008006	KT154767
<i>Lasiodiplodia chinensis</i>	CGMCC3.18061	Unknown	China	KX499889	KX499927	KX500002
<i>Lasiodiplodia chinensis</i>	CGMCC3.18044	<i>Vaccinium uliginosum</i>	China	KX499875	KX499913	KX499988
<i>Lasiodiplodia citricola</i>	UACH262	<i>Citrus latifolia</i>	Mexico	MH277948	MH286541	MH279934
<i>Lasiodiplodia citricola</i>	CBS124707	<i>Citrus</i> sp.	Iran	GU945354	GU945340	KU887505
<i>Lasiodiplodia citricola</i>	IRAN1521C	<i>Citrus</i> sp.	Iran	GU945353	GU945339	KU887504
<i>Lasiodiplodia crassisporea</i>	CBS118741	<i>Santalum album</i>	Australia	DQ103550	EU673303	KU887506
<i>Lasiodiplodia crassisporea</i>	CMW13488	<i>Eucalyptus urophylla</i>	Venezuela	DQ103552	DQ103559	KU887507
<i>Lasiodiplodia euphorbicola</i>	CMM3609	<i>Jatropha curcas</i>	Brazil	KF234543	KF226689	KF254926
<i>Lasiodiplodia euphorbicola</i>	CMM3651	<i>Jatropha curcas</i>	Brazil	KF234553	KF226711	KF254937
<i>Lasiodiplodia exigua</i>	BL184	<i>Retama raetam</i>	Tunisia	KJ638318	KJ638337	N/A
<i>Lasiodiplodia exigua</i>	CBS137785	<i>Retama raetam</i>	Tunisia	KJ638317	KJ638336	KU887509
<i>Lasiodiplodia gilanensis</i>	IRAN1523C	Unknown	Iran	GU945351	GU945342	KU887511
<i>Lasiodiplodia gilanensis</i>	IRAN1501C	Unknown	Iran	GU945352	GU945341	KU887511
<i>Lasiodiplodia gonubiensis</i>	CBS115812	<i>Syzgium cordatum</i>	South Africa	AY639595	DQ103566	DQ458860
<i>Lasiodiplodia gonubiensis</i>	CMW14078	<i>Syzgium cordatum</i>	South Africa	AY639594	DQ103567	EU673126
<i>Lasiodiplodia gravistriata</i>	CMM4564	<i>Anacardium humile</i>	Brazil	KT250949	KT250950	N/A
<i>Lasiodiplodia gravistriata</i>	CMM4570	<i>Anacardium humile</i>	Brazil	KT250948	KT266814	N/A
<i>Lasiodiplodia hyalina</i>	CGMCC3.17975	<i>Acacia confusa</i>	China	KX499879	KX499917	KX499992
<i>Lasiodiplodia hyalina</i>	CGMCC3.18383	Unknown	China	KY767661	KY751302	KY751299
<i>Lasiodiplodia hormozganensis</i>	IRAN1500C	<i>Olea</i> sp.	Iran	GU945355	GU945343	KU887515
<i>Lasiodiplodia hormozganensis</i>	IRAN1498C	<i>Mangifera indica</i>	Iran	GU945356	GU945344	KU887514
<i>Lasiodiplodia iraniensis</i>	IRAN1502C	<i>Juglans</i> sp.	Iran	GU945347	GU945335	KU887517
<i>Lasiodiplodia iraniensis</i>	IRAN1520C	<i>Salvadora persica</i>	Iran	GU945348	GU945336	KU887516
<i>Lasiodiplodia iraniensis</i>	UACH275	<i>Citrus latifolia</i>	Mexico	MH271621	MH286542	MH279933
<i>Lasiodiplodia iraniensis</i>	CMM3610	<i>Jatropha curcas</i>	Brazil	KF234544	KF226690	KF254927
<i>Lasiodiplodia iraniensis</i>	CMW36237	<i>Adansonia digitata</i>	Mozambique	KU887121	KU886998	KU887499
<i>Lasiodiplodia laeliocattleyae</i>	BOT 29	<i>Mangifera indica</i>	Egypt	JN814401	JN814428	N/A
<i>Lasiodiplodia laeliocattleyae</i>	CBS130992	<i>Mangifera indica</i>	Egypt	JN814397	JN814424	KU887508
<i>Lasiodiplodia lignicola</i>	CBS134112	Deadwood	Thailand	JX646797	KU887003	JX646845
<i>Lasiodiplodia macrosporea</i>	CMM3833	<i>Jatropha curcas</i>	Brazil	KF234557	KF226718	KF254941
<i>Lasiodiplodia mahajangana</i>	CMW27818	<i>Terminalia catappa</i>	Madagascar	FJ900596	FJ900642	FJ900631
<i>Lasiodiplodia mahajangana</i>	CMW27801	<i>Terminalia catappa</i>	Madagascar	FJ900595	FJ900641	FJ900630
<i>Lasiodiplodia margaritaceae</i>	CBS122519	<i>Adansonia gibbosa</i>	Australia	EU144050	EU144065	KU887520
<i>Lasiodiplodia margaritaceae</i>	CBS122065	<i>Adansonia gibbosa</i>	Australia	EU144051	EU144066	N/A

(Continued on next page)

^a Newly deposited sequences are shown in bold. BL, personal number of B. T. Linaldeddu; BOT, A. M. Ismail, Plant Pathology Research Institute, Giza, Egypt; BT2, β-tubulin; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CGMCC, China General Microbiological Culture Collection, Beijing, China; CMM, Culture Collection of Phytopathogenic Fungi "Professora Maria Menezes," Universidade Federal Rural de Pernambuco, Recife, Brazil; CMW, Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa; CPC, working collection of P. W. Crous housed at the CBS; N/A, sequence not available in GenBank; IBL, personal culture collection of I. B. L. Coutinho; IRAN, Culture Collection of the Iranian Research Institute of Plant Protection, Tehran, Iran; ITS, internal transcribed spacer; STE-U, Culture Collection of the Department of Plant Pathology, University of Stellenbosch, Stellenbosch, South Africa; TEF1, translation elongation factor 1-α; UACH, Culture Collection of Phytopathogenic Fungi of Department of Agricultural Parasitology, Chapingo Autonomous University, Texcoco, Mexico; UCD, Phaff Yeast Culture Collection, Department of Food Science and Technology, University of California, Davis, U.S.A.; WAC, Department of Agriculture Western Australia Plant Pathogen Collection, Perth, Australia.

The ITS region of ribosomal DNA was amplified using the primers ITS5/ITS4 (White et al. 1990). Parts of TEF1 and BT2 genes were amplified using the primers EF1-688F/EF1-1251R (Alves et al. 2008) and Bt2A/Bt2B (Glass and Donaldson 1995), respectively. The amplification conditions were as follows: an initial denaturation step at 94°C for 2 min followed by 35 cycles of denaturation at 94°C for 30 s; annealing at 55°C for the ITS region, 54°C for the TEF1 gene, and 58°C for BT2 gene for 30 s; extension at 72°C for 1 min; and a final extension step at 72°C for 10 min. The PCR assays were

conducted in a Bio-Rad C1000 thermocycler (Bio-Rad Laboratories, Hercules, CA). The PCR products were separated by electrophoresis in 1% agarose gel stained with ethidium bromide and viewed under ultraviolet light. The amplified PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and sequenced by Macrogen (Macrogen Inc., Seoul, Korea) in both directions with the same primers that were used for the PCR reactions.

Phylogenetic analyses. Forward and reverse sequences were assembled using the Staden Package (Staden et al. 1998). Sequences

Table 1. (Continued from previous page)

Species	Isolate code	Host	Location	GenBank accession number		
				ITS	TEF1	BT2
<i>Lasiodiplodia mediterranea</i>	CBS137784	<i>Vitis vinifera</i>	Italy	KJ638311	KJ638330	KU887522
<i>Lasiodiplodia mediterranea</i>	CBS137783	<i>Quercus ilex</i>	Italy	KJ638312	KJ638331	KU887521
<i>Lasiodiplodia missouriana</i>	UCD2193MO	<i>Vitis</i> sp.	U.S.A.	HQ288225	HQ288267	HQ288304
<i>Lasiodiplodia missouriana</i>	UCD2199MO	<i>Vitis</i> sp.	U.S.A.	HQ288226	HQ288268	HQ288305
<i>Lasiodiplodia parva</i>	CBS494.78	Cassava field soil	Colombia	EF622084	EF622064	EU673114
<i>Lasiodiplodia parva</i>	CBS456.78	Cassava field soil	Colombia	EF622083	EF622063	KU887523
<i>Lasiodiplodia plurivora</i>	STE-U5803	<i>Prunus salicina</i>	South Africa	EF445362	EF445395	KU887524
<i>Lasiodiplodia plurivora</i>	STE-U4583	<i>Vitis vinifera</i>	South Africa	AY343482	EF445396	KU887525
<i>Lasiodiplodia pontae</i>	IBL12	<i>Spondias purpurea</i>	Brazil	KT151794	KT151791	KT151797
<i>Lasiodiplodia pseudotheobromae</i>	CMM3887	<i>Jatropha curcas</i>	Brazil	KF234559	KF226722	KF254943
<i>Lasiodiplodia pseudotheobromae</i>	CBS116459	<i>Gmelina arborea</i>	Costa Rica	EF622077	EF622057	EU673111
<i>Lasiodiplodia pseudotheobromae</i>	UACH258	<i>Citrus latifolia</i>	Mexico	MH277917	MH286514	MH279929
<i>Lasiodiplodia pseudotheobromae</i>	UACH259	<i>Citrus latifolia</i>	Mexico	MH277926	MH286522	MH279919
<i>Lasiodiplodia pseudotheobromae</i>	UACH260	<i>Citrus latifolia</i>	Mexico	MH277921	MH286518	MH279924
<i>Lasiodiplodia pseudotheobromae</i>	UACH261	<i>Citrus latifolia</i>	Mexico	MH277919	MH286516	MH279926
<i>Lasiodiplodia pseudotheobromae</i>	UACH265	<i>Citrus latifolia</i>	Mexico	MH277916	MH286513	MH279930
<i>Lasiodiplodia pseudotheobromae</i>	UACH271	<i>Citrus latifolia</i>	Mexico	MH277920	MH286517	MH279925
<i>Lasiodiplodia pseudotheobromae</i>	UACH274	<i>Citrus latifolia</i>	Mexico	MH277923	MH286520	MH279922
<i>Lasiodiplodia pseudotheobromae</i>	UACH276	<i>Citrus latifolia</i>	Mexico	MH277915	MH286512	MH279931
<i>Lasiodiplodia pseudotheobromae</i>	UACH277	<i>Citrus latifolia</i>	Mexico	MH277918	MH286515	MH279927
<i>Lasiodiplodia pseudotheobromae</i>	UACH278	<i>Citrus latifolia</i>	Mexico	MH277927	MH286524	MH279918
<i>Lasiodiplodia pseudotheobromae</i>	UACH279	<i>Citrus latifolia</i>	Mexico	MH277922	MH286519	MH279923
<i>Lasiodiplodia pseudotheobromae</i>	UACH281	<i>Citrus latifolia</i>	Mexico	MH277924	MH286521	MH279921
<i>Lasiodiplodia pseudotheobromae</i>	UACH283	<i>Citrus latifolia</i>	Mexico	MH277914	MH286511	MH279932
<i>Lasiodiplodia pseudotheobromae</i>	UACH286	<i>Citrus latifolia</i>	Mexico	MH277925	MH286523	MH279920
<i>Lasiodiplodia pseudotheobromae</i>	UACH268	<i>Citrus latifolia</i>	Mexico	MH277928	MH286525	MH279928
<i>Lasiodiplodia pyriformis</i>	CBS121771	<i>Acacia mellifera</i>	Namibia	EU101308	EU101353	KU887528
<i>Lasiodiplodia pyriformis</i>	CBS121770	<i>Acacia mellifera</i>	Namibia	EU101307	EU101352	KU887527
<i>Lasiodiplodia rubropurpurea</i>	CMW15207	<i>Eucalyptus grandis</i>	Australia	DQ103554	DQ103572	KU887530
<i>Lasiodiplodia rubropurpurea</i>	CBS118740	<i>Eucalyptus grandis</i>	Australia	DQ103553	DQ103571	EU673136
<i>Lasiodiplodia thailandica</i>	CPC22795	<i>Mangifera indica</i>	Thailand	KJ193637	KJ193681	N/A
<i>Lasiodiplodia thailandica</i>	CPC22755	<i>Phyllanthus acidus</i>	Thailand	KJ193637	KJ193681	N/A
<i>Lasiodiplodia theobromae</i>	CBS111530	Unknown	Unknown	EF622074	EF622054	KU887531
<i>Lasiodiplodia theobromae</i>	CBS164.96	Fruit along coral reef coast	New Guinea	AY640255	AY640258	KU887532
<i>Lasiodiplodia theobromae</i>	UACH263	<i>Citrus latifolia</i>	Mexico	MH277691	MH286528	MH279908
<i>Lasiodiplodia theobromae</i>	UACH264	<i>Citrus latifolia</i>	Mexico	MH277692	MH286534	MH279909
<i>Lasiodiplodia theobromae</i>	UACH272	<i>Citrus latifolia</i>	Mexico	MH277693	MH286533	MH279915
<i>Lasiodiplodia theobromae</i>	UACH273	<i>Citrus latifolia</i>	Mexico	MH277694	MH286526	MH279907
<i>Lasiodiplodia theobromae</i>	UACH280	<i>Citrus latifolia</i>	Mexico	MH277695	MH286527	MH279910
<i>Lasiodiplodia theobromae</i>	UACH284	<i>Citrus latifolia</i>	Mexico	MH277696	MH286532	MH279914
<i>Lasiodiplodia theobromae</i>	UACH285	<i>Citrus latifolia</i>	Mexico	MH277697	MH286531	MH279913
<i>Lasiodiplodia theobromae</i>	UACH287	<i>Citrus latifolia</i>	Mexico	MH277698	MH286530	MH279912
<i>Lasiodiplodia theobromae</i>	UACH288	<i>Citrus latifolia</i>	Mexico	MH277699	MH286529	MH279911
<i>Lasiodiplodia sterculiae</i>	CBS342.78	<i>Sterculia oblonga</i>	Germany	KX464140	KX464634	KX464908
<i>Lasiodiplodia subglobosa</i>	CMM4046	<i>Jatropha curcas</i>	Brazil	KF234560	KF226723	KF254944
<i>Lasiodiplodia subglobosa</i>	CMM3872	<i>Jatropha curcas</i>	Brazil	KF234558	KF226721	KF254942
<i>Lasiodiplodia subglobosa</i>	UACH270	<i>Citrus latifolia</i>	Mexico	MH271619	MH286539	MH279916
<i>Lasiodiplodia subglobosa</i>	UACH282	<i>Citrus latifolia</i>	Mexico	MH271620	MH286540	MH279917
<i>Lasiodiplodia venezuelensis</i>	CBS118739	<i>Acacia mangium</i>	Venezuela	DQ103547	DQ103568	KU887533
<i>Lasiodiplodia venezuelensis</i>	WAC12540	<i>Acacia mangium</i>	Venezuela	DQ103548	DQ103569	KU887534
<i>Lasiodiplodia viticola</i>	UCD2604MO	<i>Vitis</i> sp.	U.S.A.	HQ288228	HQ288270	HQ288307
<i>Lasiodiplodia viticola</i>	UCD2553AR	<i>Vitis</i> sp.	U.S.A.	HQ288227	HQ288269	HQ288306
<i>Lasiodiplodia vitis</i>	CBS124060	<i>Vitis vinifera</i>	Italy	KX464148	KX464642	KX464917
<i>Neoscytalidium dimidiatum</i>	CBS145.78	<i>Homo sapiens</i>	United Kingdom	KF531816	KF531795	KF531796

of the type and reference strains of *Lasiodiplodia* species of each locus analyzed were downloaded from GenBank and combined with the newly generated sequences (Table 1). Multiple sequence alignments for each locus independently were performed using the MEGA 7.0.14 (Kumar et al. 2016), and manual adjustments were done where necessary. Alignment of each locus was loaded in SequenceMatrix v.1.8 (Vaidya et al. 2011) to build the concatenated matrix.

The phylogeny for each locus (ITS, TEF1, and BT2) and the concatenated matrix were inferred under the Maximum Likelihood (ML) and Bayesian Inference (BI) criterion. The ML analyses were done in RAxML-HPC2 (Stamatakis 2014), and Bayesian phylogenetic estimates were done in MrBayes 3.2.6 (Ronquist et al. 2012) implemented on the CIPRES Science Gateway portal (<https://www.phylo.org/portal2/home.action>). The ML tree searches were performed under the GTRGAMMA model with 1,000 pseudo-replicates. The BI trees were constructed using the GTR + I + G (ITS), HKY + G (TEF1), and GTR + G (BT2) nucleotide substitution models, with four runs and four MCMC chains per run; Markov chains were run over 100,000,000 generations, and trees and parameter values were sampled every 10,000 generations. Sequences generated in this study were deposited in GenBank (Table 1), and the alignments and tree are in TreeBASE (<http://purl.org/phylo/treebase/phyloids/study/TB2:S23111>).

Pathogenicity and virulence tests. Pathogenicity of 32 representative isolates was tested on healthy 18-month-old Persian lime plants obtained from a commercial nursery. For inoculation, branches of Persian lime plants were wounded using a sterile scalpel, and a colonized PDA plug (5-mm diameter) from a 6-day-old culture was placed on a wounded area. The inoculation site was sealed with parafilm to prevent desiccation. Four branches (two per plant) were inoculated with each isolate. Ten control branches were inoculated with noncolonized PDA plugs. Immediately after inoculation, each plant was covered with a plastic bag for 72 h to maintain humidity. All plants were maintained in a glasshouse under natural light conditions, and the temperature was regulated at $30\text{ }^{\circ}\text{C} \pm 5\text{ }^{\circ}\text{C}$ by air conditioning. Virulence of isolates was assessed 30 days after inoculation by removing the bark and measuring the lesion length in the wood with a digital caliper. The experiment was conducted twice. Differences in virulence caused by *Lasiodiplodia* species were determined by one-way analysis of variance, and means were compared by LSD test at $\alpha = 0.05$ using SAS (version 9.1; SAS Institute, Cary, NC).

Results

Field survey. Symptoms of gummosis, stem cankers, and dieback were detected in all surveyed orchards of Persian lime distributed in the states of Puebla and Veracruz. The highest incidence of disease was observed in the municipality of Hueytamalco with 97% followed by Martinez de la Torre with 96.5% and Acateno with 94.3%. The lowest incidence rates were detected in Tlapacoyan and Papantla with 89 and 86%, respectively.

Collection of samples and isolation. In this study, a total of 60 *Lasiodiplodia* isolates were obtained from Persian lime diseased tissues collected in 12 commercial orchards (5 isolates per orchard) distributed in the municipalities of Martinez de la Torre, Tlapacoyan, and Papantla in the state of Veracruz as well as in Acateno and Hueytamalco in the state of Puebla, Mexico. Based on colony morphology, a subsample of 32 representative isolates (at least two per orchard) was used for the phylogenetic analyses and pathogenicity tests.

Phylogenetic analyses. The isolates of *Lasiodiplodia* species were identified based on ML and BI phylogenetic analyses of the ITS region and parts of TEF1 and BT2 genes. The combined ITS, TEF1, and BT2 datasets consisted of 100 taxa, including 1 outgroup taxon. The alignment contained 1,309 characters, of which 348 corresponded with the TEF1 gene, 430 corresponded with the BT2 gene, and 531 corresponded with the ITS region. The combined datasets resulted in six well-supported clades corresponding to previously described *Lasiodiplodia* species (Fig.

2). The first clade with nine isolates (Culture Collection of Phytopathogenic Fungi of Department of Agricultural Parasitology, Chapingo Autonomous University, Texcoco, Mexico [UACH] 263, UACH264, UACH272, UACH273, UACH280, UACH284, UACH285, UACH287, and UACH288) clustered with *Lasiodiplodia theobromae* (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands [CBS] 111530 and CBS164.96), with 83% bootstrap support. The second clade with four isolates (UACH257, UACH266, UACH267, and UACH269) clustered with *Lasiodiplodia brasiliense* (Culture Collection of Phytopathogenic Fungi “Professora Maria Menezes,” Universidade Federal Rural de Pernambuco, Recife, Brazil [CMM] 4015 and Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa [CMW] 35884), with 86% bootstrap support. In the third clade, only one isolate (UACH275) clustered with *Lasiodiplodia iraniensis* (CMM3610 and CMW36237), with 100% bootstrap support. The fourth clade with two isolates (UACH270 and UACH282) clustered with *Lasiodiplodia subglobosa* (CMM3872 and CMM4046), with 97% bootstrap support. The fifth clade had the highest number of isolates (UACH258, UACH259, UACH260, UACH261, UACH265, UACH268, UACH271, UACH274, UACH276, UACH277, UACH278, UACH279, UACH281, UACH283, and UACH286), which clustered with *Lasiodiplodia pseudotheobromae* (CBS116459 and CMM3887), with 85% bootstrap support. The sixth clade has only one isolate (UACH262) clustered with *Lasiodiplodia citricola* (CBS124707 and Culture Collection of the Iranian Research Institute of Plant Protection, Tehran, Iran 1521C), with 100% bootstrap support (Fig. 2).

Distribution of *Lasiodiplodia* species. *L. pseudotheobromae* was the most frequently isolated species (46.9%) from Persian lime trees followed by *L. theobromae* (28.1%), *L. brasiliense* (12.5%), *L. subglobosa* (6.3%), *L. iraniensis* (3.1%), and *L. citricola* (3.1%). The distribution of *Lasiodiplodia* species varied among the five populations distributed in the states of Puebla and Veracruz, Mexico (Fig. 3). *L. pseudotheobromae* was found in all populations. *L. theobromae* and *L. subglobosa* were found in at least one population of each state. *L. brasiliense* was recorded only in the two populations from the state of Puebla. *L. iraniensis* was identified only in the Papantla population. *L. citricola* was only isolated from an orchard located in Acateno, Puebla.

Pathogenicity and virulence on branches. Thirty days after inoculation, all isolates belonging to the six species of *Lasiodiplodia* identified in this study were pathogenic on Persian lime young plants using a mycelial plug inoculation method. The symptoms produced on the wood were necrotic lesions that extended upward and downward from the inoculation site (Fig. 4). All isolates of *Lasiodiplodia* induced the formation of gum exudations on the branches (Fig. 4), whereas no disease symptoms were observed on the control plants. *Lasiodiplodia* isolates were consistently recovered from the diseased branches, whereas no *Lasiodiplodia* spp. were isolated from healthy control plants, thus fulfilling Koch’s postulates. The two experiments did not differ significantly ($P = 0.05$); thus, the data from both experiments were combined for statistical analyses. The mean lesion lengths caused by species of *Lasiodiplodia* for the combination of two experiments are shown in Fig. 5.

There were significant differences ($P = 0.001$) in internal necrosis length produced by the different *Lasiodiplodia* species. The longest mean lesions (>19 mm) were produced by *L. subglobosa*, *L. iraniensis*, and *L. pseudotheobromae*, which were the most virulent species. In addition, these species caused longitudinal cracks on the bark of the branches. However, the shorter mean lesions (<14 mm) were induced by *L. theobromae*, *L. brasiliense*, and *L. citricola*, which were considered the least virulent species.

Discussion

This study showed the first findings about *Lasiodiplodia* species infecting Persian lime trees in Mexico. Six *Lasiodiplodia* species were isolated from stem cankers and dieback of branches, and they

were identified based on phylogenetic analyses of combined ITS, BT2, and TEF1 sequence datasets. These species included *L. pseudotheobromae*, *L. theobromae*, *L. brasiliense*, *L. subglobosa*, *L. citricola*, and *L. iraniensis*. All *Lasioidiplodia* species of this study are reported for the first time in association with Persian lime in Mexico and worldwide. A detailed morphological characterization of

Lasioidiplodia isolates was not performed, because the identification of Botryosphaeriaceae species using morphology is virtually impossible, and it should be only used to distinguish between genera and complement the DNA phylogeny for the description of new species (Ismail et al. 2012; Osorio et al. 2017; Phillips et al. 2013; Slippers et al. 2014). The most appropriate way to differentiate species within

Fig. 2. Phylogenetic tree of *Lasioidiplodia* species inferred from a concatenated alignment of an internal transcribed spacer, β -tubulin, and translation elongation factor 1- α sequence alignments. Bootstrap values by the Maximum Likelihood method and probabilities by the Bayesian Inference analyses are shown on the respective branches. Bootstrap values below 80% and posterior probabilities below 0.90 are not shown. The tree is rooted to *Neoscytalidium dimidiatum* CBS 145.78. Epi- and ex-type strains are indicated in bold. The scale bar indicates the average number of substitutions per site.

Fig. 3. Collection sites of six *Lasiodiplodia* spp. associated with Persian lime (*Citrus latifolia*) dieback in five populations distributed in the states of Puebla (PUE) and Veracruz (VER), Mexico. Circles represent association frequency of each species with Persian lime trees showing symptoms of dieback in each population sampled, v is the number of commercial orchards sampled in each population, and n is the number of isolates analyzed in each population. *L. brasiliense*, *Lasiodiplodia brasiliense*; *L. citricola*, *Lasiodiplodia citricola*; *L. iraniensis*, *Lasiodiplodia iraniensis*; *L. pseudotheobromae*, *Lasiodiplodia pseudotheobromae*; *L. subglobosa*, *Lasiodiplodia subglobosa*; *L. theobromae*, *Lasiodiplodia theobromae*.

Fig. 4. Symptoms developed in the branches of young Persian lime (*Citrus latifolia*) plants artificially inoculated with *Lasiodiplodia* spp. 30 days after inoculation. **A and B**, Branches producing gum exudations. **C**, Symptomatic branch with necrosis and gummosis. **D**, Branch with necrosis around inoculation site.

Botryosphaeriaceae is through of DNA sequence data, preferably combining sequences from multiple loci (Dissanayake et al. 2016; Phillips et al. 2013; Rodríguez-Gálvez et al. 2017; Slippers et al. 2014).

L. pseudotheobromae was the most frequently isolated species from Persian lime plants with dieback symptoms, and it occurred in the five populations analyzed in this study. This fungal species has been previously associated with diseases on some citrus species, including sour orange in Suriname (Alves et al. 2008), *Citrus* species in Iran (Abdollahzadeh et al. 2010), and lemon in Turkey (Awan et al. 2016). Similarly, this fungus has been commonly isolated from mango (*Mangifera indica* L.) tissues with symptoms of dieback and/or stem end rot worldwide (Ismail et al. 2012; Marques et al. 2013; Munirah et al. 2017; Rodríguez-Gálvez et al. 2017; Sakalidis et al. 2011; Trakunyingcharoen et al. 2014; 2015). In addition, it has been recorded in Brazil on fruit trees, including cashew (*Anacardium occidentale* L.) (Coutinho et al. 2017; Netto et al. 2017), hog plum (*Spondias purpurea* L.), and *Tamarindus indica* L. (Coutinho et al. 2017). Results of pathogenicity tests revealed that isolates of *L. pseudotheobromae* were more virulent in Persian lime branches than *L. theobromae* isolates. These results agree with those reported by Begoude et al. (2010, 2011) and Chen et al. (2011), who found that *L. pseudotheobromae* was more virulent than *L. theobromae* when inoculated on stems of tropical almond (*Terminalia catappa*), *Eucalyptus* spp., and *Terminalia* spp. In contrast to our results, Correia et al. (2016), Marques et al. (2013), and Netto et al. (2017) determined that *L. theobromae* isolates were more virulent than *L. pseudotheobromae* isolates when inoculated on mango fruits, detached green shoots of grapes, and detached green shoots of cashew.

L. theobromae was the second most abundant species isolated from Persian lime orchards in Mexico. This species has a cosmopolitan distribution, particularly in tropical and subtropical regions, where it causes a variety of diseases on a wide range of host plants (Punithalingam 1980). Studies based on the combination of morphological characters and DNA sequence data confirmed that this fungus is a common pathogen in fruit crops of economic importance, such as mango (Abdollahzadeh et al. 2010; Al-Jabri et al. 2017; Al-Sadi et al. 2013; de Oliveira Costa et al. 2010; Ismail et al. 2012; Marques et al. 2013; Munirah et al. 2017; Ni et al. 2012; Rodríguez-Gálvez et al. 2017; Sakalidis et al. 2011; Trakunyingcharoen et al. 2014), papaya (*Carica papaya* L.) (Netto et al. 2014), coconut palm (*Cocos nucifera* L.) (Rosado et al. 2016), kiwifruit (*Actinidia chinensis* Planch.) (Zhou et al. 2015), *Vitis* spp. (Correia et al. 2016; Rodríguez-Gálvez et al. 2015), cashew (Coutinho et al. 2017; Netto et al. 2017), hog plum, and *Talisia esculenta* (Coutinho et al. 2017). Similarly, *L. theobromae* has been recorded as the second most frequently isolated species after *L. pseudotheobromae* in symptomatic tissues of the ornamental tree known as tropical almond in Madagascar and South Africa (Begoude et al. 2010). Regarding citrus species, Davis et al. (1987) recorded a trunk disease of young citrus trees caused by two isolates of *L. theobromae* in Texas, U.S.A. and also showed that some isolates were apparently avirulent. This pathogen also has been associated with dieback and gummosis symptoms on acid lime, sweet orange, and sweet lime in Oman (Al-Sadi et al. 2013) and lemon in Venezuela (Ferrari et al. 1996) and Chile (Guajardo et al. 2018). In Mexico, *L. theobromae* was previously associated as the causal agent of bot canker disease of grapevines based on morphological and cultural characters as well as analyses of ITS, BT2, and TEF1 sequences (Úrbez-Torres et al. 2008). Subsequently, other studies have mentioned the occurrence of *L. theobromae* as a common pathogen on fruit crops in Mexico, including *Pouteria sapota* (Vásquez-López et al. 2009), mango (Sandoval-Sánchez et al. 2013), and physic nut (Uc-Vázquez et al. 2017). However, all of these isolates were identified based on morphological characteristics and analysis of the ITS region, and therefore, it is necessary to verify and update the inventory of Botryosphaeriaceae species associated with the main fruit crops in Mexico by analysis of sequences of at least three genes. In this study, *L. theobromae* was less virulent than *L. pseudotheobromae* in inoculated young plants of Persian lime.

This finding contrasts with that found by Marques et al. (2013), who determined that *L. theobromae* was more virulent than *L. pseudotheobromae* in inoculated mango fruits. In the case of citrus, Al-Sadi et al. (2014) found that *L. theobromae* was more virulent than *Lasiodiplodia hormozganensis* on inoculated twigs of acid lime and sweet lime, because *L. hormozganensis* induced symptoms of gummosis and dieback, similar to those induced by *L. theobromae* observed in our study.

L. brasiliense was the third most common species of all of the isolates examined in our study. This species was previously reported to be a pathogen of mango (Coutinho et al. 2017; Netto et al. 2014; Rodríguez-Gálvez et al. 2017), papaya (Netto et al. 2014), coconut palm (Rosado et al. 2016), table grapes (Correia et al. 2016), sapodilla (*Manilkara zapota* L.), hog plum (Coutinho et al. 2017), cashew (Netto et al. 2017), custard apple (*Annona squamosa* L.) (Cardoso et al. 2017), and apple (Martins et al. 2018) in Brazil. In addition, this fungus was recorded on *Tectona grandis* in Thailand (Doilom et al. 2015) and *Eucalyptus* hybrid (Li et al. 2018) and mango (Zhang et al. 2018) in China. Thus, this study represents the first report of this fungus on a citrus worldwide. In 2017, Cruywagen et al. (2017) considered *L. brasiliense* as a hybrid that could have arisen from hybridization between *L. theobromae* and another currently unknown species; however, additional studies are needed to confirm this hybrid status. In our study, *L. brasiliense* and *L. theobromae* exhibited low levels of virulence, whereas *L. pseudotheobromae* was highly virulent in Persian lime young plants. A recent study by Netto et al. (2014) showed that *L. brasiliense*, *L. theobromae*, and *L. pseudotheobromae* did not differ with respect to their virulence in papaya fruits. In contrast to our results, Correia et al. (2016) found that *L. brasiliense* and *L. theobromae* were highly virulent species, and *L. pseudotheobromae* exhibited an intermediate virulence in detached green shoots of grapes. Rosado et al. (2016) observed that *L. theobromae* was highly virulent in coconut fruits compared with *L. brasiliense* and *L. pseudotheobromae*. Subsequently, Netto et al. (2017) noted that *L. brasiliense* and *L. theobromae* exhibited intermediate virulence, and *L. pseudotheobromae* presented a low level of virulence in detached green shoots of cashew. All of the studies mentioned above show a wide range of virulence of *L. brasiliense* isolates in different tissues of fruit crop hosts.

L. subglobosa was described from physic nut collected in Brazil (Machado et al. 2014), and later, it was reported by Poletto et al. (2016) to be causing stem canker of pecan (*Carya illinoensis* [Wangenh.] Koch). Thus, this study represents the first report of

Fig. 5. Virulence of six *Lasiodiplodia* species associated with dieback of Persian lime (*Citrus latifolia*) as measured by mean internal lesion lengths (millimeters). Data are lesion sizes measured 30 days after inoculation with mycelium-colonized agar plugs inserted into wounded branches of young lime trees. Bars above columns are the standard errors of the means. Columns with the same letter do not differ significantly according to Fisher's LSD test ($P \leq 0.05$). *L. brasiliense*, *Lasiodiplodia brasiliense*; *L. citricola*, *Lasiodiplodia citricola*; *L. iraniensis*, *Lasiodiplodia iraniensis*; *L. pseudotheobromae*, *Lasiodiplodia pseudotheobromae*; *L. subglobosa*, *Lasiodiplodia subglobosa*; *L. theobromae*, *Lasiodiplodia theobromae*.

this species in Mexico and from outside Brazil. Results of pathogenicity tests showed that *L. subglobosa* was the most virulent species in young Persian lime plants, although it was not the most common species.

L. citricola and *L. iraniensis* were less common species and occurred at the same frequency among the isolates obtained from symptomatic Persian lime tissues in Mexico. *L. citricola* was first introduced by Abdollahzadeh et al. (2010) for isolates from *Citrus* species in Iran and later associated with other species causing canker in peach trees (*Prunus persica* L.) (Chen et al. 2013) as well as being fungal species responsible for inducing various symptoms in tissues of English walnut (*Juglans regia* L.) (Chen et al. 2014a) and pistachio (*Pistacia vera* L.) (Chen et al. 2014b) in California, U.S.A.. In Italy, *L. citricola* was associated as causal agent of trunk disease on grapevines (Carlucci et al. 2015). However, Rodríguez-Gálvez et al. (2017) suggested two hypotheses regarding this species. The first hypothesis suggests that there are hybrids isolates between *L. citricola* and *Lasiodiplodia parva*, and the second hypothesis suggest that *L. citricola* and *L. parva* represent a single species, with the small differences between them being a representation of the intraspecific genetic variability. Either hypothesis can be confirmed in additional studies.

L. iraniensis was first described from diseased plants of *Salvadora persica*, *Juglans* sp., mango, *Eucalyptus* sp., *Citrus* sp., and tropical almond collected in Iran (Abdollahzadeh et al. 2010); later, this pathogen was associated with mandarin (Al-Sadi et al. 2013), mango (Al-Sadi et al. 2013; Marques et al. 2013; Rodríguez-Gálvez et al. 2017; Sakalidis et al. 2011), *Bougainvillea spectabilis* (Li et al. 2015), and *Anacardium occidentale* (Netto et al. 2017). According to Netto et al. (2017), *Lasiodiplodia jatrophiicola* is phylogenetically closely related yet clearly distinct from *L. iraniensis*, with larger conidia and shorter paraphyses typical of this species. However, Cruywagen et al. (2017) and Rodríguez-Gálvez et al. (2017) indicated that *L. jatrophiicola* and *L. iraniensis* should be considered as synonyms, because the two species cannot be separated based solely on DNA sequence phylogeny using combined data of ITS and TEF1 sequences. Additional studies are required with a large number of isolates of both species as well as analysis of additional gene sequence data to resolve these uncertainties as indicated by Yang et al. (2017).

Overall, the results from this research showed great similarity to the results from studies performed by Correia et al. (2016), Machado et al. (2014), and Netto et al. (2017) in Brazil, because they identified at least three of the same *Lasiodiplodia* species reported on Persian lime trees in Mexico. These phylogenetic studies have shown the great diversity of *Lasiodiplodia* species associated with diseases in crops of economic importance in both countries. Therefore, it is necessary to explore this diversity in detail through subsequent studies.

Although we identified six *Lasiodiplodia* species associated with Persian lime in Mexico, we did not find some species, such as *Lasiodiplodia hormozganensis* (Al-Sadi et al. 2013), *L. parva* (Adesemoye et al. 2014), *Lasiodiplodia mediterranea* (Linaldeddu et al. 2015), and *Lasiodiplodia caatinguensis* (Coutinho et al. 2017), which have been previously reported on other citrus species worldwide. Our results add to a growing body of data and confirm that there are at least 10 *Lasiodiplodia* species that are able to cause diseases in *Citrus* spp. worldwide.

In conclusion, the findings of this study represent the first detailed research about the phylogeny, distribution, and pathogenicity of six *Lasiodiplodia* species associated with gummosis and dieback symptoms of Persian lime trees in the central coastal region of the Gulf of Mexico and worldwide. Although *L. pseudotheobromae* and *L. theobromae* were the most frequently isolated species, all *Lasiodiplodia* species proved to be pathogenic to young Persian lime plants, with *L. subglobosa*, *L. iraniensis*, and *L. pseudotheobromae* being the most virulent. This complex of *Lasiodiplodia* species is a threat to the Persian lime industry, and additional studies on the impact, biology, fungicide sensitivity, and epidemiology of these fungi are needed for the development of suitable management strategies to reduce the disease impact on the productivity and longevity of Persian lime orchards in Mexico.

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