Botryosphaeriaceae Fungi as Causal Agents of Dieback and Corky Bark in Rambutan and Longan

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Abstract

Fungi in the Botryosphaeriaceae family cause dieback, fruit rots, and stem cankers in many tropical fruit trees. To identify which species of Botryosphaeriaceae were present in tropical fruit in Puerto Rico and the symptoms they cause in rambutan and longan, a disease survey was conducted throughout the island from 2008 to 2016. Diseased organs of rambutan, longan, mango, and tangerine were collected and 39 isolates belonging to the Botryosphaeriaceae family were isolated and identified. Phylogenetic analysis of three nuclear genes identified nine species: six *Lasiodiplodia* spp. and three *Neofusicoccum* spp. All 39 isolates were inoculated on healthy 1-year-old rambutan and longan seedlings to confirm their pathogenicity. Dieback on both rambutan and longan was observed at 14 days after inoculation (DAI). Fourteen isolates from seven Botryosphaeriaceae species (*Lasiodiplodia brasiliensis*, *L. hormozganensis*, *L. iraniensis*, *L. pseudotheobromae*, *L.*

theobromae, *Neofusicoccum batangarum*, and *N. parvum*) caused dieback in rambutan. Five of these pathogenic isolates were collected from rambutan, four from longan, two from mango, and three from tangerine. Ten isolates of four *Lasiodiplodia* spp. caused dieback in longan: *L. hormozganensis*, *L. iraniensis*, *L. pseudotheobromae* and *L. theobromae*. Of these, three were collected from rambutan, three from longan, one from mango, and three from tangerine. Abundant development of pycnidia on branches, called corky bark, were observed on both rambutan and longan 60 DAI. Cross-inoculations showed that pathogenicity is wide in spectrum, indicating that different planting alternatives should be considered for better crop management.

Keywords: fruit, fungi, pathogen detection, pathogen diversity, tree fruit, tropical plants

Rambutan (*Nephelium lappaceum* L.) and longan (*Dimocarpus longan* L.) are exotic tropical trees in the family Sapindaceae. Native to Southeast Asia, they are popular worldwide for their fruit. In 2017, global rambutan and longan production was estimated at 3.4 and 1.4 million tons, respectively (Altendorf 2018). The major producers were Indonesia and Thailand for rambutan, and China and Thailand for longan (Altendorf 2018). In the United States, production is concentrated in Hawaii and Puerto Rico for rambutan and in California, Florida, and Hawaii for longan (Crane et al. 2005; Goenaga and Jenkins 2011).

Diseases of rambutan and longan affect fruit, stems, leaves, and inflorescences (Chamswang et al. 2012; Nishijima et al. 2002; Rossman et al. 2007, 2012; Sangchote et al. 1998; Serrato-Díaz et al. 2011, 2014b, 2015a). Fruit rot is the most important disease in rambutan and is reported to cause up to 30% yield losses in pre and postharvest (Farungsang et al. 1992; Nishijima et al. 2002; Pordesimo and Luna-Ilag 1982; Sangchote et al. 1998; Sivakumar et al. 1997, 2000). It is caused by a complex of pathogenic fungi that include *Calonectria hongkongensis*, *Colletotrichum* spp., *Diaporthe* sp., *Gloeophthalotrichum* spp. *Lasmenia* sp., *Lasiodiplodia theobromae*, and *Pestalotiopsis* sp. (Farungsang et al. 1992; Lombard et al. 2014; Nishijima et al. 2002; Pordesimo and Luna-Ilag 1982; Ploetz 2003; Sangchote et al. 1998; Serrato-Díaz et al. 2011, 2012, 2013a,b, 2015b, 2017; Sivakumar et al. 1997, 2000). Diseases in longan have been less studied. Fruit rot caused by *L. theobromae* is the most important disease, and has been reported in China, Puerto Rico, and Thailand (Sardsud et al. 1998; Serrato-Díaz et al. 2014b; Sun

et al. 2018). Other diseases such as sooty molds on fruit, inflorescence blight, and vascular and flower necrosis have also been reported affecting longan (Serrato-Díaz et al. 2010, 2014b, 2015a).

Another severe disease of the Sapindaceae family is corky bark. It is associated with stem canker and, in severe cases, dieback on branches (Booth and Ting 1964; Rossman et al. 2010). Corky bark of Sapindaceae trees was originally reported from Malaysia (Booth and Ting 1964), and later from Honduras, Hawaii, and Puerto Rico, (Rossman et al. 2007, 2010, 2012). The only pathogen reported for corky bark is *Dolabra nepheliae* C. Booth & W. P. Ting. (class Dothideomycetes). Although the fungus was reported in 1964 in Malaysia and eventually in Honduras, Hawaii, and Puerto Rico, Koch's postulates have not been fulfilled. Therefore, whether or not this fungus causes any of the symptoms attributed and observed in the field remains to be validated (Booth and Ting 1964; Rossman et al. 2007, 2010, 2012).

Worldwide, species in the Botryosphaeriaceae family have been reported to cause stem cankers and dieback in tropical and subtropical fruit such as almond, avocado, citrus, grapevine, mamey sapote, mango, mangosteen, and pistachio, as well as rambutan and longan (Adesemoye et al. 2014; Burruano et al. 2008; Inderbitzin et al. 2010; Ismail et al. 2012; Javier-Alva et al. 2009; MAG 2007; McDonald and Eskalen 2011; Michailides et al. 2002; Muhammad et al. 2009; Sakalidis et al. 2011; Sardsud et al. 1998; Úrbez-Torres et al. 2009; Vazquez-Lopez et al. 2009; Zea-Bonilla et al. 2007). Mango and citrus are the tropical crops most affected by the Botryosphaeriaceae family: around 14 species of Botryosphaeriaceae have been reported in mango causing dieback, stem end rot, and gummosis and 10 species have been identified from dieback, bot gummosis, and canker in citrus (Abdollahzadeh et al. 2013; Adesemoye et al. 2014; Marques et al. 2013a,b; Mayorquin et al. 2016; Polizzi et al. 2009; Sakalidis et al. 2011).

In surveys conducted in Puerto Rico from 2008 to 2013, samples of diseased tissue of mango, rambutan, and longan showing symptoms of fruit rot, inflorescence blight, leaf blight, dieback, and rachis necrosis were collected. From this survey, 260 fungal isolates in the Botryosphaeriaceae family were identified, suggesting that they play an important role in tropical plant diseases and

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potentially could be the causative agents of dieback in rambutan and longan, as well as in other fruit crops (Serrato-Diaz et al. 2013c).

The purpose of this study was to identify species of Botryosphaeriaceae from rambutan and longan and test whether they can cause dieback and corky bark. Also, we hypothesized that these fungi can cause cross-infection, with fungal isolates from one tropical fruit crop able to cause disease in others crops.

Materials and Methods

Collection of plant material and isolation of Botryosphaeriaceae spp. Isolates of Botryosphaeriaceae from rambutan (60 randomly selected trees from six plots), longan (60 randomly selected trees from six plots), mango (1,500 randomly selected trees from a germplasm collection), and tangerine (2 randomly selected trees from an experiment) showing dieback, fruit rot, inflorescence blight, and leaf blight were collected throughout Puerto Rico from 2008 to 2016, as reported by Serrato-Diaz et al. (2013c). Sampling effort was inconsistent; members of the Botryosphaeriaceae family and other fungi were also isolated. Isolations were done according to Serrato-Diaz et al. (2013c).

Diseased plant tissues of rambutan, longan, mango, and tangerine were disinfested with 70% ethanol, rinsed with sterile deionized-distilled water for 1 min, and plated on potato dextrose agar (PDA) amended with streptomycin at 0.125 g/liter and ampicillin at 0.25

g/liter. Purified isolates were grown in potato dextrose broth and mycelium was collected after 8 days for DNA extraction.

DNA extraction, PCR, and sequencing. Genomic DNA was extracted from 39 fungal isolates using the Dneasy Plant Mini Kit (Qiagen). DNA was amplified from regions of three nuclear genes: the internal transcribed spacer (ITS)1-5.8S-ITS2 (ITS) region of the nuclear ribosomal DNA, the β -tubulin gene (β tub), and the trans-elongation factor 1- α gene (EF1- α). The ITS region was amplified using primer pair ITS1/ITS4 (White et al. 1990). The partial sequences of the β tub and EF1- α genes were amplified using primer pairs Bt2a/Bt2b for β tub and EF1-728F/EF1-986R and EF1-688F/EF1-1251R for EF1- α (Alves et al. 2008; Carbone and Kohn 1999; Glass and Donaldson 1995). PCRs using Amplitaq Gold (Applied Biosystems) were conducted according to Alves et al. (2004). PCR products were purified using QIA Quick PCR Kit (Qiagen) and sequenced at the Sequencing and Genotyping Facility at the University of Puerto Rico. Sequences were edited using Sequencher (version 5.0; Gene Codes Corporation) and deposited in NCBI GenBank (Table 1).

Phylogenetic inference. Phylogenetic trees were constructed using sequences from 39 isolates of Botryosphaeriaceae and ex-type specimens for *Neofusicoccum* spp. and *Lasiodiplodia* spp. (Table 2) (Yang et al. 2017). *Diplodia mutila* ex-type CBS 112553 and *D. seriata* ex-type CBS 112555 were added as outgroups

Table 1. Source of isolates and accession numbers for sequences of internal transcribed spacer (ITS)1-5.8S-ITS2, β -tubulin, and transelongation factor EF1- α genes of Botryosphaeriaceae used in this study

Isolates	Fungal species	Symptom ^a	Source	GenBank accession numbers		
				ITS	β -Tubulin	EF1- α
2. BO2-PRRF35	<i>Neofusicoccum parvum</i>	Fruit rot	Rambutan	MK282737	MK294088	MK294155
6. BO6-PRRF64	<i>N. parvum</i>	Fruit rot	Rambutan	MK282739	MK294090	MK294157
49. Bt8-RH23B	<i>Lasiodiplodia iraniensis</i>	Leaf blight	Rambutan	MK282716	MK294105	MK294134
50. Bt9-RH25	<i>L. iraniensis</i>	Leaf blight	Rambutan	MK282717	MK294106	MK294135
52. Bt21-RH8	<i>L. iraniensis</i>	Leaf blight	Rambutan	MK282718	MK294107	MK294136
78. Bt23-RC8C	<i>L. pseudotheobromae</i>	Dieback	Rambutan	MK282723	MK294114	MK294141
80. BO8-PRRF58	<i>L. pseudotheobromae</i>	Fruit rot	Rambutan	MK282725	MK294112	MK294143
97. BO11-PRRF54	<i>L. parva</i>	Fruit rot	Rambutan	MK282730	MK294119	MK294148
253. RFISAB4	<i>N. batangarum</i>	Fruit rot	Rambutan	MK282731	MK294082	MK294149
254. RFISAB2	<i>N. batangarum</i>	Fruit rot	Rambutan	MK282732	MK294083	MK294150
255. RFISAJJ3	<i>N. mangiferae</i>	Fruit rot	Rambutan	MK282738	MK294089	MK294156
66. Bt3-RC6A	<i>L. iraniensis</i>	Dieback	Rambutan	MK282709	MK294102	MK294127
68. Bt5-RC4B	<i>L. iraniensis</i>	Dieback	Rambutan	MK282710	MK294103	MK294128
69. Bt6-RC2B	<i>L. iraniensis</i>	Dieback	Rambutan	MK282706	MK294099	MK294124
89. PHLO6	<i>L. pseudotheobromae</i>	Inflores	Longan	MK282724	MK294113	MK294142
105. PHLO9	<i>L. theobromae</i>	Inflores	Longan	MK282714	MK294094	MK294132
115. FLLOPR1	<i>L. iraniensis</i>	Inflores	Longan	MK282705	MK294098	MK294123
122. FLLOPRC1	<i>L. brasiliensis</i>	Inflores	Longan	MK282704	MK294096	MK294122
123. FLLOPRC2	<i>L. brasiliensis</i>	Inflores	Longan	MK282703	MK294095	MK294121
124. LFPR2B	<i>L. hormozganensis</i>	Fruit rot	Longan	MK282729	MK294118	MK294147
129. LFPR6B	<i>L. hormozganensis</i>	Fruit rot	Longan	MK282728	MK294117	MK294146
85. PHLO2	<i>L. iraniensis</i>	Inflores	Longan	MK282719	MK294108	MK294137
140. LFPR11A	<i>L. iraniensis</i>	Fruit rot	Longan	MK282707	MK294100	MK294125
113. FLLOPR3	<i>L. pseudotheobromae</i>	Inflores	Longan	MK282722	MK294111	MK294140
151. LFPR1B	<i>L. hormozganensis</i>	Fruit rot	Longan	MK282726	MK294115	MK294144
152. Bt33-LFPR3	<i>L. iraniensis</i>	Fruit rot	Longan	MK282708	MK294101	MK294126
134. LFPR12A	<i>L. hormozganensis</i>	Fruit rot	Longan	MK282727	MK294116	MK294145
120. FLLOPR5	<i>L. theobromae</i>	Inflores	Longan	MK282711	MK294091	MK294129
43. K15C	<i>N. parvum</i>	Inflores	Mango	KC631662	KC631653	KC631658
212. FLMAISA8	<i>L. brasiliensis</i>	Inflores	Mango	MK282702	MK294097	MK294120
284. 25P	<i>N. batangarum</i>	Inflores	Mango	MK282733	MK294084	MK294151
286. 129T	<i>L. iraniensis</i>	Inflores	Mango	MK282715	MK294104	MK294133
294. 405T	<i>N. parvum</i>	Inflores	Mango	MK282734	MK294085	MK294152
295. 317T	<i>N. batangarum</i>	Inflores	Mango	MK282735	MK294086	MK294153
302. 230T	<i>N. batangarum</i>	Inflores	Mango	MK282736	MK294087	MK294154
310. MDBH3	<i>L. pseudotheobromae</i>	Dieback	Tangerine	MK282721	MK294110	MK294139
311. MDB51	<i>L. pseudotheobromae</i>	Dieback	Tangerine	MK282720	MK294109	MK294138
312. MDBH2	<i>L. theobromae</i>	Dieback	Tangerine	MK282712	MK294092	MK294130
313. MDBH4	<i>L. theobromae</i>	Dieback	Tangerine	MK282713	MK294093	MK294131

^a Inflores = inflorescence blight.

Table 2. Ex-type specimens of *Neofusicoccum*, *Lasiodiplodia*, and *Diplodia* spp. used in this study and their GenBank accession numbers

Species	Culture collection	GenBank accession numbers ^a		
		ITS	EF1	β-Tubulin
<i>Diplodia mutila</i>	CBS 112553 (ex-type)	AY259093	AY573219	DQ458850
	CBS 230.30	DQ458886	DQ458869	DQ458849
<i>D. seriata</i>	CBS 112555 (ex-type)	AY259094	AY573220	DQ458856
	CBS 119049	DQ458889	DQ458874	DQ458857
<i>Lasiodiplodia brasiliensis</i>	CMM 4015 (ex-type)	JX464063	JX464049	–
	CMM 2185	KC484800	KC481530	–
	CMM 2186	KC484812	KC481542	–
<i>L. citricola</i>	IRAN1522C (ex-type)	GU945354	GU945340	–
	IRAN1521C	GU945353	GU945339	–
<i>L. crassisporea</i>	CBS 118741 (ex-type)	DQ103550	EU673303	–
	CMW13488	DQ103552	DQ103558	–
<i>L. egyptacea</i>	CBS13092 (ex-type)	JN814397	JN814424	–
	BOT-29	JN814401	JN814428	–
<i>L. gilanensis</i>	IRAN1523C (ex-type)	GU945351	GU945342	–
	IRAN1501C	GU945352	GU945341	–
<i>L. gonubiensis</i>	CBS 115812 (ex-type)	DQ458892	DQ458877	DQ458860
	CMW14078	AY639594	DQ103567	–
<i>L. jatrophiicola</i>	CBS 176.26	KX464136	KX464629	KX464900
	CBS 111.13	KX464135	KX464628	KX464899
<i>L. hormozganensis</i>	IRAN1500C (ex-type)	GU945355	GU945343	–
	IRAN1498C	GU945356	GU945344	–
<i>L. iraniensis</i>	IRAN921C (ex-type)	GU945346	GU945334	–
	IRAN1502C	GU945347	GU945335	–
<i>L. margaritacea</i>	CBS 122519 (ex-type)	KT852959	KU887520	–
	CBS 122065	EU144051	EU144066	–
<i>L. parva</i>	CBS 456.78 (ex-type)	EF622083	EF622063	–
	CBS 494.78	EF622084	EF622064	EU673114
<i>L. pseudotheobromae</i>	CBS 116459 (ex-type)	EF622077	EF622057	EU673111
	CBS 447.62	EF622081	EF622060	EU673112
<i>L. rubropurpurea</i>	WAC12535 (ex-type)	DQ103553	EU673304	EU673136
	WAC 12536	DQ103554	DQ103572	–
<i>L. theobromae</i>	CBS 164.96 (ex-type)	AY640255	AY640258	EU673110
	CMW9074	AY236952	AY236901	AY236930
<i>L. venezuelensis</i>	WAC12539 (ex-type)	DQ103547	EU673305	EU673129
	WAC 12540	DQ103548	DQ103569	–
<i>Neofusicoccum batangarum</i>	CMW28363 (ex-type)	FJ900607	FJ900653	FJ900634
	CMW28320	FJ900608	FJ900654	FJ900635
<i>N. cordaticola</i>	CMW13992 (ex-type)	EU821898	EU821868	EU821838
	CMW14056	EU821903	EU821873	EU821843
<i>N. kwambonambiense</i>	CMW14023 (ex-type)	EU821900	EU821870	EU821840
	CMW14140	EU821919	EU821889	EU821859
<i>N. mangiferae</i>	CMW7024 (ex-type)	AY615185	DQ093221	AY615172
	CMW7797	AY615186	DQ093220	AY615173
<i>N. parvum</i>	CMW9081 (ex-type)	AY236943	AY236888	AY236917
	CBS 110301	AY259098	AY573221	EU673095
<i>N. ribis</i>	CMW7772 (ex-type)	AY236935	AY236877	AY236906
	CMW7773	AY236936	AY236878	AY236907
<i>N. umdonicola</i>	CMW14058 (ex-type)	EU821904	EU821874	EU821844
	CMW14060	EU821905	EU821875	EU821845
<i>N. brasiliense</i>	CMM1285 (ex-type)	JX513628	JX513608	KC794030
	CMM1269	JX513629	JX513609	KC794032
<i>N. algeriense</i>	CBS 719.85 (ex-type)	KX464151	KX464646	KX464921
	CBS 113072	KX464150	KX464645	KX464920
<i>N. andinum</i>	CBS 117453 (ex-type)	AY693976	AY693977	KX464923
	CBS 117452	DQ306263	DQ306264	KX464922
<i>N. occulatum</i>	CBS 128008 (ex-type)	MH864743	EU339509	EU339472
	CBS 256.80	KX464175	KX464696	KX464973
<i>N. pistaciarum</i>	CBS 113083	NR147367	KX464712	KX464998
	CBS 113084	KX464187	KX464713	KX464999
<i>N. arbuti</i>	CBS 116131	–	KF531792	KF531793
	CBS 116573	KX464153	KX464648	KX464925
<i>N. macroclavatum</i>	CBS 114497	–	KX464695	KX464972
<i>N. terminaliae</i>	CBS 125263	MH863478	–	KX465052
	CBS 125264	GQ471805	GQ471783	KX465053
<i>N. stellenboschiana</i>	CBS 282.70	KX464225	KX464758	KX465051
<i>N. corticosae</i>	CBS 118099	KX464168	KX464681	KX464957
	CBS 120081	–	KX464682	KX464958

^a ITS = internal transcribed spacer, EF1 = transelongation factor EF1-α, and – indicates no sequences available in GenBank.

(Phillips et al. 2013; Yang et al. 2017). Sequences were aligned using SATé (Kato and Standley 2013) and MAFFT with RAXML for the tree estimator with 10 iterations. The three genes were concatenated using Sequence Matrix to give 1,412 and 1,453 characters for *Neofusicoccum* and *Lasiodiplodia* spp., respectively, including alignment gaps. Maximum-likelihood trees were constructed to infer the phylogeny of the Botryosphaeriaceae family using RAXML with GTRCAT as the default model, 25 γ categories, and the automatic Bootstrap MRE implemented in CIPRES Science Gateway portal (Miller et al. 2010; Stamatakis 2006a,b).

Pathogenicity tests. Cross inoculations using 39 isolates of Botryosphaeriaceae (collected from rambutan, longan, mango, and tangerine showing dieback symptoms) were performed on 246 healthy 1-year-old rambutan and longan seedlings (Table 1). A first group of rambutan and longan seedlings (42 seedlings each) was inoculated only with isolates collected from their respective original host (14 isolates for rambutan and 14 for longan). A second group of 75 rambutan seedlings was inoculated with 14 isolates collected from longan, 7 isolates from mango, and 4 isolates from tangerine. A third group of 75 longan seedlings was inoculated with 14 isolates from rambutan, 7 from mango, and 4 from tangerine. Three seedlings

per isolate were inoculated with 5-mm mycelial disks from pure 8-day-old cultures on PDA (Serrato-Diaz et al. 2014a,b). Twelve control seedlings were inoculated with PDA disks only. Each seedling was wounded with a scalpel on two lateral branches and the main trunk. Mycelial disks containing the fungus were placed directly on the wounds. Seedlings were kept in a humid chamber using plastic bags for 8 days under greenhouse conditions and observed for dieback at 8, 14, 30, and 60 days after inoculation (DAI).

Differences between longan and rambutan in number of isolates causing dieback were tested for significance using Fisher's exact test. Differences among isolation sources (both host species and host organ from which isolates were obtained) and number of isolates causing dieback were tested for significance using exact contingency tests. For these tests, data from the three inoculation groups were combined.

Results

Phylogenetic inference. The best trees obtained with maximum-likelihood showed nine clades corresponding to three *Neofusicoccum* spp. and six *Lasiodiplodia* spp. In the *Neofusicoccum* phylogenetic tree (Fig. 1), five isolates clustered with the *Neofusicoccum*

Fig. 1. Best maximum-likelihood tree of genus *Neofusicoccum* based on the concatenated sequences of β -tubulin, internal transcribed spacer, and translation elongation factor EF1- α . Bootstrap support values are shown at the nodes and isolates and hosts used in this study are marked. The tree was rooted to the outgroups *Diplodia seriata* (CBS 119049 and CBS 112553) and *D. mutila* (CBS 230.30 and CBS 112553).

batangarum clade (bootstrap support [BS] = 88), four with the *N. parvum* clade (BS = 98), and one in the *N. mangiferae* clade (BS = 99). In the *Lasiodiplodia* tree (Fig. 2), 4 isolates clustered with the *L. hormozganensis* clade (BS = 80), 4 with the *L. theobromae* clade (BS = 81), 3 with the *L. brasiliensis* clade (BS = 99), 11 with the *L. iraniensis* clade (BS = 95), 6 with the *L. pseudotheobromae* clade (BS = 100), and 1 with the *L. parva* clade (BS = 99).

Dieback and corky bark symptoms on rambutan. Vascular necrosis (Fig. 3B and C) and necrosis of branches (Fig. 3A) were observed 8 DAI. At 14 DAI, dieback was observed: diseased branches turned from light brown to dark brown, starting from the inoculated area and spreading to the leaves (Fig. 3D). The main stems turned light brown to dark brown, with vascular necrosis that eventually caused the death of the plant (Figs. 3C and 4C). At 30 DAI,

Fig. 2. Best maximum-likelihood tree of genus *Lasiodiplodia* based on the concatenated sequences of β -tubulin, internal transcribed spacer, and translation elongation factor EF1- α . Bootstrap support values are shown at the nodes and isolates and hosts used in this study are marked. The tree was rooted to the outgroups *Diplodia seriata* (CBS 119049 and CBS 112555) and *D. mutila* (CBS 230.30 and CBS 112553).

light brown pycnidia were observed on branches and main stems, turning dark brown with age. Corky bark symptoms were observed 60 DAI (Fig. 5). Control seedlings inoculated with PDA disks showed no symptoms of dieback.

Of the 39 isolates of Botryosphaeriaceae evaluated, 13 caused dieback on rambutan (Table 3). Of these, three isolates were collected from rambutan showing fruit rot and one from rambutan with dieback, two from longan with fruit rot, one from longan and three from mango with inflorescence blight, and three from tangerine with dieback (Fig. 4). Seven species (*L. brasiliensis*, *L. hormozganensis*, *L. iraniensis*, *L. pseudotheobromae*, *L. theobromae*, *N. parvum*, and *N. batangarum*) caused dieback on rambutan in Puerto Rico. All *Lasiodiplodia* spp. and *Neofusicoccum* spp. were reisolated from pathogenicity tests, fulfilling Koch's postulates.

Dieback and corky bark symptoms on longan. Of the 39 isolates of Botryosphaeriaceae evaluated, cross inoculations showed that 10 isolates caused dieback on longan (Table 3). Of these, three isolates were from rambutan causing leaf blight and dieback symptoms, three from longan with fruit rot and inflorescence blight, one from mango with inflorescence blight, and three from tangerine with dieback on branches (Fig. 6). Isolates 124, 129, 66, and 313 caused dieback at 8 DAI and the remaining isolates caused dieback at 14 DAI. At 30 DAI, light-brown pycnidia that turned dark brown with age developed on the branches and trunk. Corky bark symptoms were observed on longan branches at 60 DAI (Fig. 5). No symptoms were observed in control seedlings. Four species belonging to the Botryosphaeriaceae family

(*L. hormozganensis*, *L. iraniensis*, *L. pseudotheobromae*, and *L. theobromae*) caused dieback of longan. All four *Lasiodiplodia* spp. were reisolated from pathogenicity tests, fulfilling Koch's postulates.

The difference in number of isolates causing dieback on rambutan versus longan was not significant (Fisher's exact test, $P = 0.62$). The proportion of pathogenic isolates did not differ significantly among original hosts (rambutan, longan, mango, and tangerine, $P = 0.18$). Similarly, the number of pathogenic isolates obtained from different organs (leaf, fruit, branch, and inflorescence) did not differ significantly ($P = 0.28$).

Discussion

Maximum-likelihood analysis supported identification of nine species in the Botryosphaeriaceae family among the fungi isolated from tropical fruit trees with a variety of symptoms. Five of these belong to the genus *Lasiodiplodia* (*L. brasiliensis*, *L. hormozganensis*, *L. iraniensis*, *L. pseudotheobromae*, and *L. theobromae*) and three to genus *Neofusicoccum* (*N. batangarum*, *N. mangiferae*, and *N. parvum*). Only *L. theobromae* has been previously reported causing dieback in rambutan orchards (in Costa Rica), and one *Lasiodiplodia* sp. was previously reported to cause dieback of longan shoots (in Thailand) (MAG 2007; Sardud et al. 1998). To our knowledge, this is the first report of *L. hormozganensis*, *L. iraniensis*, and *L. pseudotheobromae* causing dieback and corky bark on rambutan and longan trees, and the first report of *N. parvum*, *N. batangarum*, and *L. brasiliensis* causing dieback and corky bark on rambutan.

Fig. 3. Branch necrosis, vascular necrosis, and dieback on rambutan. A, Branch necrosis at 8 days after inoculation (DAI); B and C, vascular necrosis at 8 DAI; and D, dieback at 14 DAI.

Fig. 4. Dieback on 1-year-old rambutan seedlings caused by Botryosphaeriaceae isolates obtained from dieback, fruit rot, and inflorescence blight of A, rambutan; B, longan; C, mango; D, tangerine; and E, control. Arrows show dieback at 14 days after inoculation.

Of the nine species tested, seven (*L. brasiliensis*, *L. hormozganensis*, *L. iraniensis*, *L. pseudotheobromae*, *L. theobromae*, *N. batangarum*, and *N. parvum*) caused dieback on rambutan in Puerto Rico and only four (*L. hormozganensis*, *L. iraniensis*, *L. pseudotheobromae*, and *L. theobromae*) caused dieback in longan. Although longan and rambutan are closely related members of the same family, our findings suggest greater resistance of longan trees to species of Botryosphaeriaceae compared with rambutan.

Analysis of the longan genome has revealed more pathogen resistance genes in the nucleotide-binding site leucine-rich repeat (NBS-LRR) and LRR receptor-like kinase (RLK) families than in other fruit such as grape, kiwifruit, mei (*Myrica rubra*), orange, papaya, and peach (Morales-Cruz et al. 2015; Yuling et al. 2017). Higher expression or greater diversity of resistance proteins in longan could explain resistance of longan against Botryosphaeriaceae isolates. Several studies showed that NBS-LRR and LRR-RLK genes conferred resistance in a variety of plants, including *Arabidopsis*, rice, peanut, wheat, and barley, against pathogens such as *Erysiphe cruciferarum*, *E. cichoracearum*, *Magnaporthe oryzae*, *Aspergillus flavus*, and *Fusarium graminearum*, respectively (Chen et al. 2015; Fukuoka et al. 2014; Ma et al. 2015; Song et al. 2017; Thapa et al. 2018; Xiao et al. 2001; Yang et al. 2013; Zhang et al. 2015). However, more studies need to be conducted to elucidate which genes in longan are responsible for resistance to *Neofusicoccum* spp.

Cross inoculations showed that species in the Botryosphaeriaceae family have a broad host range in tropical fruit crops. Isolates of Botryosphaeriaceae collected from mango, longan, and tangerine were pathogenic on rambutan, and isolates collected from mango, rambutan, and tangerine were pathogenic on longan. *L. theobromae* and *N. parvum* are known to have broad host ranges, including tropical and subtropical fruit crops (Khanzada et al. 2004; Punithalingam 1976; Sakalidis et al. 2013; Slippers and Wingfield 2007). Worldwide, over 500 hosts for *L. theobromae* (Punithalingam 1976, 1980) and 90 hosts

for *N. parvum* have been reported (Sakalidis et al. 2013; Slippers and Wingfield 2007).

In previous reports, these fungi were identified using morphology alone; a phylogenetic species concept based on DNA sequence data was not applied until recently. With the addition of DNA sequence data, several new species have been identified in the *L. theobromae* and *N. parvum*/*N. ribis* complexes (Abdollahzadeh et al. 2010; Alves et al. 2008; Burgess et al. 2006; Ismail et al. 2012; Pavlic et al. 2009a,b; Sakalidis et al. 2013). It is probable that some of the studies mentioned above might today be referred to the *L. theobromae* and *N. parvum* species complexes rather than the species sensu stricto. It is possible that these species complexes include several species with narrow host ranges rather than one species with a broad host range. This is the case for *L. iraniensis*, *L. pseudotheobromae*, *L. hormozganensis*, and *L. brasiliensis*, which recently were identified within the *L. theobromae* complex using DNA sequences. They cause diseases including cankers, dieback, gummosis, necrosis, fruit rot, and stem end rot in economically important plants such as *Acacia mangium*, *Bougainvillea spectabilis*, cashew, citrus, coconut, eucalyptus, grape, mango, palm, *Ricinus communis*, and *Salvadora persica* (Abdollahzadeh et al. 2010; Al-Sadi et al. 2013; Castro-Medina et al. 2014; Custódio et al. 2018; Dissanayake et al. 2015; Ismail et al. 2012; Kwon et al. 2017; Li et al. 2015; Marques et al. 2013a; Munirah et al. 2017; Rodríguez-Gálvez et al. 2017; Rosado and Machado 2016; Sakalidis et al. 2011).

Many fungi of the Botryosphaeriaceae family are endophytes, becoming pathogens when the host is stressed by environmental factors (Chethana et al. 2016; Félix et al. 2016; Paolinelli-Alfonso et al. 2016; Slippers and Wingfield 2007; Yan et al. 2018). For example, *L. theobromae* is an opportunistic pathogen of woody plants that increases in virulence at high temperatures, producing cellulases and lignocellulases, 18 chitin-binding gene families, and heat-shock proteins. These enzymes and proteins allow the fungus to colonize plant

Fig. 5. Corky bark symptoms on rambutan and longan branches at 60 days after inoculation. **A**, Corky bark symptoms on rambutan; **B**, corky bark symptoms on longan; and **C**, profuse pycnidia production on branches.

tissue through degradation of the cell wall and protection from host defense (Félix et al. 2016; Paolinelli-Alfonso et al. 2016; Yan et al. 2018). Recently, analysis of Botryosphaeriaceae genomes (*Botryosphaeria dothidea*, *L. theobromae*, and *N. parvum*) revealed

cytochrome P450 mono-oxygenase genes, involved in fungal adaptation to new ecological niches. These genes can provide protection during the infection through the detoxification of phytotoxins (Chen et al. 2014; Yan et al. 2018).

Table 3. Botryosphaeriaceae isolates that caused dieback on rambutan and longan

Isolates	Fungi	Source	Dieback in rambutan	Dieback in longan
49.Bt8-RH23B	<i>Lasiodiplodia iraniensis</i>	Rambutan leaves	No	No
50.Bt9-RH25	<i>L. iraniensis</i>	Rambutan leaves	No	Yes
52.Bt21-RH8	<i>L. iraniensis</i>	Rambutan leaves	No	No
80.BO8-PRRF58	<i>L. pseudotheobromae</i>	Rambutan fruit	Yes	No
97.BO11-PRRF54	<i>L. parva</i>	Rambutan fruit	No	No
2. BO2-PRRF35	<i>Neofusicoccum parvum</i>	Rambutan fruit	Yes	No
6.BO6-PRRF64	<i>N. parvum</i>	Rambutan fruit	Yes	No
253.RFISAB4	<i>N. batangarum</i>	Rambutan fruit	No	No
254.RFISAB2	<i>N. batangarum</i>	Rambutan fruit	No	No
255.RFISAJJ3	<i>N. mangiferae</i>	Rambutan fruit	No	No
78.Bt23-RC8C	<i>L. pseudotheobromae</i>	Rambutan branches	No	No
66.Bt3-RC6A	<i>L. iraniensis</i>	Rambutan branches	No	Yes
68.Bt5-RC4B	<i>L. iraniensis</i>	Rambutan branches	No	No
69.Bt6-RC2B	<i>L. iraniensis</i>	Rambutan branches	Yes	Yes
89. PHLO6	<i>L. pseudotheobromae</i>	Longan inflorescences	No	No
113.FLLOPR3	<i>L. pseudotheobromae</i>	Longan inflorescences	No	No
105.PHLO9	<i>L. theobromae</i>	Longan inflorescences	No	No
120.FLLOPR5	<i>L. theobromae</i>	Longan inflorescences	No	No
85.PHLO2	<i>L. iraniensis</i>	Longan inflorescences	No	No
115.FLLOPR1	<i>L. iraniensis</i>	Longan inflorescences	No	Yes
122.FLLOPRC1	<i>L. brasiliensis</i>	Longan inflorescences	Yes	No
123.FLLOPRC2	<i>L. brasiliensis</i>	Longan inflorescences	No	No
124.LFPR2B	<i>L. hormozganensis</i>	Longan fruit	No	Yes
129.LFPR6B	<i>L. hormozganensis</i>	Longan fruit	Yes	Yes
151.LFPR1B	<i>L. hormozganensis</i>	Longan fruit	Yes	No
134.LFPR12A	<i>L. hormozganensis</i>	Longan fruit	No	No
140.LFPR11A	<i>L. iraniensis</i>	Longan fruit	No	No
152.Bt33-LFPR3	<i>L. iraniensis</i>	Longan fruit	No	No
212.FLMAISA8	<i>L. brasiliensis</i>	Mango inflorescences	No	No
286.129T	<i>L. iraniensis</i>	Mango inflorescences	Yes	Yes
43.K15C	<i>N. parvum</i>	Mango inflorescences	No	No
294.405T	<i>N. parvum</i>	Mango inflorescences	Yes	No
284.25P	<i>N. batangarum</i>	Mango inflorescences	No	No
295.317T	<i>N. batangarum</i>	Mango inflorescences	Yes	No
302.230T	<i>N. batangarum</i>	Mango inflorescences	No	No
310.MDBH3	<i>L. pseudotheobromae</i>	Tangerine branches	Yes	Yes
311.MDB51	<i>L. pseudotheobromae</i>	Tangerine branches	Yes	Yes
312.MDBH2	<i>L. theobromae</i>	Tangerine branches	Yes	No
313.MDBH4	<i>L. theobromae</i>	Tangerine branches	No	Yes

Fig. 6. Dieback on 1-year-old longan seedlings caused by Botryosphaeriaceae isolates obtained from dieback, fruit rot and inflorescence blight of **A**, longan; **B**, rambutan; **C**, mango; **D**, tangerine; and **E**, control. Arrows show dieback at 14 days after inoculation.

This study confirms our hypothesis that a variety of species in the Botryosphaeriaceae family can cause vascular necrosis, dieback, and corky bark in rambutan and longan in Puerto Rico. Pathogenicity of *Dolabra nepheliae* (previously identified as the pathogen responsible for corky bark of rambutan) needs to be proven to determine whether this species can also cause corky bark symptoms on rambutan and longan, or if it is a saprotroph feeding on dead organic material left by infections caused by species of Botryosphaeriaceae.

Cross-inoculation with *Lasiodiplodia* and *Neofusicoccum* isolates from different hosts showed that pathogenicity is wide spectrum. In Puerto Rico, the combination of high humidity, high temperatures, wounds caused by wind damage, and proximity among fruit tree crops could facilitate cell penetration and degradation by *Lasiodiplodia* and *Neofusicoccum* spp. and movement between crops. These adaptations to different hosts by isolates of Botryosphaeriaceae indicate that different planting alternatives (for example, annual crops intercalated with woody plants) should be considered for better crop management. The corky bark symptoms observed in the field are the reproductive structures of the Botryosphaeriaceae spp.; therefore, drip irrigation is the best way to avoid dispersion of the pathogen from one tree to another. Because members of the Botryosphaeriaceae family are vascular pathogens, wounds left in the pruning process can facilitate their entrance. These Botryosphaeriaceae fungi are very virulent pathogens with wide host ranges; thus, better management practices for planting, pruning, and irrigation of rambutan, longan, mango, and tangerine are needed in Puerto Rico and worldwide.

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