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A.O. Adesemoye

J.S. Mayorquin

D.H. Wang

M. Twizeyimana

S.C. Lynch

See next page for additional authors

Authors

A.O. Adesemoye, J.S. Mayorquin, D.H. Wang, M. Twizeyimana, S.C. Lynch, and Akif Eskalen

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A. O. Adesemoye, Department of Microbiology, Adekunle Ajasin University, P.M.B. 001, Akungba-Akoko, Ondo State, Nigeria; and **J. S. Mayorquin, D. H. Wang, M. Twizeyimana, S. C. Lynch,** and **A. Eskalen,** Department of Plant Pathology and Microbiology, University of California, Riverside 92521

Abstract

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Members of the Botryosphaeriaceae family are known to cause Bot gummosis on many woody plants worldwide. To identify pathogens associated with Bot gummosis on citrus in California, scion and rootstock samples were collected in 2010 and 2011 from five citrus-growing counties in California. Symptoms observed on citrus included branch cankers, dieback, and gumming. Various fungal species were recovered from necrotic tissues of branch canker and rootstock samples. Species were identified morphologically and by phylogenetic comparison as 'Eureka' lemon, 'Valencia', 'Washington Navel', 'Fukumoto', grapefruit, 'Satsuma', and 'Meyer' lemon. Species were identified morphologically and by phylogenetic comparison of the complete sequence of the internal transcribed spacer regions, β-tubulin gene, and elongation factor α-1 genes with those of other species in GenBank. A consensus-unrooted most parsimonious tree resulting from multigene phylogenetic analysis showed the existence of three major clades in the Botryosphaeriaceae family. In total, 74 isolates were identified belonging to the Botryosphaeriaceae family, with *Neofusicoccum* spp., *Dothiorella* spp., *Diplodia* spp., (teleomorph *Botryosphaeria*), *Lasiodiplodia* spp., and *Neoscytalidium dimidiatum* (teleomorphs unknown) accounting for 39, 25, 23, 10, and 3% of the total, respectively. On inoculated Eureka lemon shoots, lesion length was significantly different ($P < 0.05$) among 14 isolates recovered from portions of cankered tissues of the original trees. Lesion lengths were significantly longer ($P < 0.05$) for shoots inoculated with isolates of *Neofusicoccum luteum* and shorter for shoots inoculated with isolates of *Dothiorella viticola* ($P < 0.05$) than those of other species. Identifying the distribution and occurrence of these fungal pathogens associated with Bot gummosis is useful for management applications during occasional outbreaks in California.

During the 2012–13 growing season, the U.S. citrus industry produced 11.2 million tons of citrus, worth approximately $3.15 billion. Florida accounted for 63% of U.S. production and California produced 34% of the total amount (43). The California citrus industry comprises numerous varieties, including grapefruit, lemon, lime, orange, tangerine, and many hybrids. Given that the citrus industry is one of California's largest agricultural industries (21), the health of these commodities is critically important.

Growers and county farm advisors have observed branch canker and dieback on various citrus cultivars throughout citrus-growing regions of California for over a decade (G. Bender [San Diego County Farm Advisor] and N. O'Connell [Tulare County Farm Advisor], *personal communication*). Initial symptoms are scattered dieback of twigs and branches, where portions have dead outer bark over a sunken canker. The dead bark may exude gum; the cambial layer of wood underneath the bark may turn brown to yellowish. In an initial survey, *Neofusicoccum australe* and *N. parvum* (Botryosphaeriaceae family) were recovered from these cankered tissues (2). Isolates were tentatively identified to species shortly after recovery by 98 to 100% homology with available sequences in GenBank. However, several previously unrecorded species associated with citrus in the Botryosphaeriaceae family were recovered from symptomatic tissues of cankered branch samples submitted by local growers and farm advisors for disease identification. This prompted the need for a more detailed survey and study.

Species in the Botryosphaeriaceae family were reported on citrus as early as the1900s (16,30). Since that time, identification and taxonomy of this family has long been complex because the teleomorph is rarely seen in nature. Consequently, anamorphic characteristics have been used for identification. The combination of molecular methods and anamorphic characters has simplified species identification in the Botryosphaeriaceae family (12) but has not recently been done for those associated with citrus.

The impact of species in the Botryosphaeriaceae family on citrus has not been extensively explored. Furthermore, many of these previously unrecorded species on citrus that were recovered from submitted extension samples were the same as those found causing dieback on other crops, which include avocado, grapevine, olive, almond, walnut, and pistachio (19,24,25,41,49,51,54). Thus, it is highly likely that the diversity of fungi in the Botryosphaeriaceae family on citrus is not well understood and, moreover, is causing more damage than previously thought.

The objectives of this study were to (i) determine the occurrence of species in the Botryosphaeriaceae family associated with branch canker and dieback on citrus throughout the major citrus-growing regions of California; (ii) identify these species based on morphological characteristics, sequence data, and phylogenetic analysis; and (iii) determine their pathogenicity on citrus.

Materials and Methods

Fungal collections and isolations. Five citrus-growing counties in California—including Riverside, San Diego, San Luis Obispo, Tulare, and Ventura—were visited in 2010 and 2011 (Table 1). Approximately 15 to 20 pieces of trunk and branch with canker symptoms were collected from 10 randomly selected trees (approximately 5 to 60 years old) in each of 5 to 10 orchards per county, depending on orchard density. Samples were transported in a cooler to the laboratory at the University of California, Riverside. Trunk and branch pieces were rinsed with deionized water to remove organic debris, dried with paper towels, then briefly flamed after dipping in 95% ethanol for 3 s. Small sections (approximately 2 to 3 mm^2) at the margin of necrotic wood tissue of cankered

Corresponding author: A. Eskalen, E-mail: akif.eskalen@ucr.edu

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branches were cut with a sterile scalpel and five to six pieces were placed onto potato dextrose agar (PDA; Difco Laboratories) amended with 0.01% tetracycline (Sigma-Aldrich) (PDA-tet). Cultures were incubated in the dark for 4 days at 25°C. Pure cultures of fungal isolates were obtained by transferring hyphal tips from colony margins onto fresh PDA-tet plates.

Molecular characterization and phylogenetic analyses. Genomic DNA from fungal isolates was extracted using the following modifications of the methods described by Cenis (10). Approximately 50 µg of mycelium was scraped from the surface of a pure culture grown on PDA-tet and added to a 2.0-ml screw-cap tube with 500 µl of extraction buffer and approximately 300 mg of 1.0-mm-diameter glass beads (BioSpec Products). Tubes were then placed into an MP Bio Fast Prep-24 instrument (MP Biomedicals Inc.) at 4 M/s for 30 s. Polymerase chain reaction (PCR) was conducted in a thermal cycler (Bio-Rad Laboratories, Inc.) using three different markers: Bt2a and Bt2b for the β-tubulin gene; internal transcribed spacer (ITS)4 and ITS5 for the ITS1, ITS2, and 5.8S regions of ribosomal DNA (52); and EF1-728F and EF1-986R for translation elongation factor (EF) α-1 (48,52). Each PCR reaction contained 12.5 µl of GoTaq Green Master Mix (Promega Corp.), 9.3 µl of PCR-grade water, 0.6 µl of 10 µM each primer, and 2 µl of DNA template. The reaction protocol for ITS and β-tubulin included an initial preheat at 94°C for 2 min; followed by 35 cycles of denaturation at 94°C for 15 s, annealing at 58°C for 15 s, and extension at 72°C for 45 s; and final extension at 72°C for 5 min (35). The translation EF α-1 included an initial denaturation at 95°C for 8 min; followed by 35 cycles of 95, 58, and 72°C for 15, 20, and 60 s, respectively; and a final extension at 72°C for 5 min (7).

The PCR products were separated by gel electrophoresis in 1.5% agarose gels in 1× Tris-boric acid-EDTA (TBE) buffer and were photographed with Dark Reader DR88X transilluminator (Clare Chemical Research). The PCR products were purified using Isopure PCR purification kit (Denville Scientific Inc.). The quality of the PCR products was estimated using NanoDrop Spectrophotometer ND-1000 (Thermal Scientific). Sequencing was done at the Institute for Integrative Genome Biology, University of California, Riverside. Sequences were edited using Sequencher software 4.6 (Gene Codes, Corp.) and locally aligned using ClustalX 2.1-Mac OSX (Conway Institute, UCD) (38). Sequences were used to search for similar sequences using the BLAST program (version 2.0; National Center for Biotechnology Information, United States National Institutes of Health).

Sequences of representative isolates of the Botryosphaeriaceae family from citrus cankers in the present study (Table 1) and sequences from other studies in GenBank (Table 2) were used in a phylogenetic analysis performed using MEGA 5.0. The *Guignardia philoprina* (CBS447.68) sequence from GenBank was chosen as an outgroup. Maximum parsimony analysis was performed using the heuristic search with a close-neighbor interchange branch-swapping option. Alignment gaps were treated as missing data. Support for each branch in the inferred trees was evaluated by 1,000 bootstrap replications. Prior to the heuristic search of the most-parsimonious tree, a partition homogeneity test performed in PAUP* (version 4.0b10; Sinauer Associates) using 1,000 random repartitions was conducted to determine whether the ITS, β-tubulin, and EF sequence data could be combined. Tree length, consistency index (CI), retention index (RI), rescaled consistency index (RC), and homoplasy index (HI) were calculated using MEGA5.0.

Morphological identification. Isolates were grown on PDA-tet for the initial identification (11,24). Fungal isolates were transferred to oatmeal agar medium for conidial development and further morphological characterization. Oatmeal agar was prepared by autoclaving 30 g of oat and 15 g of agar in 1 liter of distilled water. Plates were incubated at 25°C under a 12-h daily photoperiod for 2 to 4 weeks to allow pycnidium formation. The length and width of 50 conidia/isolate were measured using a compound microscope (Olympus BX40 with a Leica DFC420 camera) and the SPOT Imaging Software (Diagnostic Instruments. The conidial characteristics observed were compared with those that were reported in previous studies (6,11,24,27,35,36,53).

Table 1. Representative isolates of species in the Botryosphaeriaceae recovered from citrus samples with branch canker in this study

Species	Isolate[b]	County[c]	Host	Variety	GenBank number[a]		
					ITS	β-tubulin	EF
Diplodia mutila	UCP130*	Tulare	*Citrus sinensis*	Fukumoto	JF271751	JF271769	JF271787
D. mutila	UCR1665	Ventura	*C. limon*	Lisbon lemon	JQ659283	JQ659295	JQ659271
D. mutila	UCR1670	San Diego	*C. sinensis*	Valencia	JQ659284	JQ659296	JQ659272
Diplodia seriata	UCR 1653	San Diego	*C. sinensis*	Valencia	JQ659282	JQ659294	JQ659270
D. seriata	UCR 1687	Ventura	*C. limon*	Lisbon lemon	JQ659285	JQ659297	JQ659273
D. seriata	UCR 1795	San Diego	*C. latifolia*	Bearss Lime	JQ659286	JQ659298	JQ659274
Dothiorella iberica	UCP124*	Tulare	*C. sinensis*	Fukumoto	JF271750	JF271768	JF271786
Dothiorella viticola	UCP61*	Tulare	*C. sinensis*	Valencia	JF271747	JF271765	JF271783
D. viticola	UCP105*	Tulare	*C. sinensis*	Parent Washington	JF271748	JF271766	JF271784
D. viticola	UCP134*	Tulare	*C. sinensis*	Parent Washington	JF271752	JF271770	JF271788
D. viticola	UCR1104	San Diego	*C. sinensis*	Valencia	JF271757	JF271775	JF271792
D. viticola	UCR1796	San Diego	*C. latifolia*	Bearss Lime	JQ659287	JQ659299	JQ659275
Lasiodiplodia parva	UCR 1056	Tulare	*Citrus* sp.	Unknown	JQ659278	JQ659290	JQ659265
L. parva	UCR 1057	Tulare	*Citrus* sp.	Unknown	JQ659279	JQ659291	JQ659266
Neofusicoccum australe	UCR1099*	San Diego	*C. sinensis*	Valencia	JF271756	JF271774	JF271791
N. australe	UCR1110*	Riverside	*C. unshiu*	Satsuma	JF271758	JF271776	JF271793
N. australe	UCR1111*	Riverside	*C. unshiu*	Satsuma	JF271759	JF271777	JF271794
N. luteum	UCR1097*	San Diego	*C. sinensis*	Valencia	JF271755	JF271773	JF271790
N. luteum	UCR1177*	SLO	*C. limon*	Lemon	JF271763	JF271781	JF271797
N. luteum	UCR1180*	SLO	*C. limon*	Lemon	JF271764	JF271782	JF271798
N. mediterraneum	UCP114*	Tulare	*C. sinensis*	Fukumoto	JF271749	JF271767	JF271785
N. parvum	UCR1166*	Ventura	*C. limon*	Meyer Lemon	JF271762	JF271780	JF271796
N. parvum	UCR1170	Ventura	*C. limon*	Meyer Lemon	JQ659280	JQ659292	JQ659268
N. parvum	UCR1171	Ventura	*C. limon*	Meyer Lemon	JQ659281	JQ659293	JQ659269
N. parvum	UCR1798	Tulare	*C. sinensis*	Cara Cara	JQ659288	JQ659300	JQ659276
Neoscytalidium dimidiatum	UCR1087*	San Diego	*C. paradisi*	Red Blush Grapefruit	JF271754	JF271772	JF271789

[a] Three gene loci (internal transcribed spacer [ITS]1-5.8S-ITS2 regions, β-tubulin gene, and elongation factor [ET] α-1) were sequenced and sequences were deposited in GenBank.

[b] UCP = University of California, Riverside Citrus Project and UCR = University of California, Riverside. Asterisks show isolates used in the pathogenicity test.

[c] SLO = San Luis Obispo.

Pathogenicity test. Three isolates each of eight species in the Botryosphaeriaceae family that were recovered from citrus cankers were used in the pathogenicity test (Table 1). These randomly selected isolates were used to inoculate 30-cm-long detached healthy green shoots collected from 1- to 2-year-old 'Eureka' lemon trees. One wound per shoot was made using a 3-mm cork borer. A 3-mm-diameter mycelial plug from 3- to 5-day-old culture growing on PDA-tet was placed on the freshly wounded surface of each shoot, and the inoculated area was covered with Parafilm (2). Inoculated shoots and controls (mock-inoculated with agar plugs) were covered with petroleum jelly at their ends to prevent desiccation and incubated at 25°C in moist chambers for 4 weeks. After incubation, lesion length was recorded on each shoot. The experiment was arranged in a completely randomized design with five replications and was conducted twice. Small pieces of necrotic tissue from the edges of each lesion were placed on PDA-tet in plates and incubated at room temperature. Fungal colonies were confirmed morphologically and using molecular identification techniques.

Data analysis. Lesion length data from both runs were tested for homogeneity of error variance by analysis of variance (ANOVA) in SAS (version 9.2; SAS Institute Inc.). Because no heterogeneity was detected, data were pooled for the final ANOVA analysis. Means lesion lengths among isolates were compared using a Tukey's honestly significant difference mean separation test at $\alpha = 0.05$.

Results

Fungal collections and isolations. Cankers were found on the main trunk, branches, twigs, and exposed roots (roots above ground; Fig. 1), with sampled trees ranging in age from approximately 5 to 60 years old. On heavily infected trees, especially young trees, death of branches or death of the entire plant was observed. Botryosphaeriaceae isolates were recovered from rootstock samples, including 'Carrizo' citrange (*Citrus sinensis* (L.) Osb. × *Poncirus trifoliata* L.), 'Macrophylla' (*C. macrophylla* Wester), 'Sour orange' (*C. aurantium* L.), and 'Volkameriana' (*C. volkameriana* L.); and scions 'Bearss' lime (*C. latifolia* Tan.), 'Cara Cara Pink' orange (*C. sinensis* (L.) Osb.), Eureka lemon (*C. limon* Burm.f.), 'Fukumoto' orange (*C. sinensis*), grapefruit (*C. paradisi* Macf.), lemon (*C. limon*), 'Lisbon' lemon (*C. limon*), 'Meyer' lemon (*C. meyeri*), 'Parent Washington' orange (*C. sinensis*), 'Red Blush' grapefruit (*C. paradisi* Macf.), 'Satsuma' orange (*C. unshiu* Marc.), and 'Valencia' orange (*C. sinensis*).

Based on phylogenetic analyses, colony morphology, and conidial characteristics (see below), 74 isolates belonging to the Botryosphaeriaceae family were identified (Fig. 2). Of the total Botryosphaeriaceae isolates recovered from samples, 37% were recovered from Tulare County and 28% were recovered from San Diego County. Ventura, Riverside, and San Luis Obispo Counties had 18, 12, and 5% of the total, respectively. *Neofusicoccum* spp. accounted for 39% of all isolates, and the remaining isolates were identified as *Dothiorella* spp. (25%), *Diplodia* spp. (23%), *Lasiodiplodia* spp. (10%), and *Neoscytalidium dimidiatum* (3%). Among *Neofusicoccum* spp., *Neofusicoccum parvum* was the most frequently recovered (36%), followed by *N. australe and N. luteum* that each accounted for 27% of the *Neofusicoccum* spp. recovered. *N. mediterraneum* had the lowest number (10%). Other fungal genera sporadically isolated from citrus cankers were *Alternaria*, *Phomopsis,* and *Cladosporium*.

Phylogenetic analysis. The nonsignificant partition-homogeneity test ($P = 0.180$) indicated congruence between ITS, β-tubulin, and EF α-1 sequence sets and, thus, the three sets were combined for further phylogenetic analysis. Of the 1,219 nucleotides analyzed, 394 were parsimony informative. Maximum parsimony analysis yielded 300 trees (length = 1045, CI = 0.74, RI = 0.93, RC = 0.69, HI = 0.26).

A consensus-unrooted most-parsimonious tree showed the existence of three major clades in the Botryosphaeriaceae isolates. The

Table 2. Sequences and their description of the Botryosphaeriaceae species obtained from GenBank used in the phylogenetic study

Isolate[b]	Species	Host	Origin	GenBank number[a]		
				ITS	β-Tubulin	EF
PD75	*Diplodia mutila*	*Ilex* sp.	United States	GU251119	GU251779	GU251251
CBS112553	*D. mutila*	*Vitis vinifera*	Portugal	AY259093	DQ458850	AY573219
UCD288Ma	*D. mutila*	*V. vinifera*	California	DQ008313	DQ008336	EU012411
SDZ-01	*Diplodia seriata*	*Vitis* sp.	China	HQ629954	HQ629956	HQ629958
SDZ-02	*D. seriata*	*Vitis* sp.	China	HQ629955	HQ629957	HQ629959
GA-422	*D. seriata*	*Prunus persica*	China	HQ660463	HQ660477	HQ660489
CBS119049	*D. seriata*	*Vitis* sp.	Italy	DQ458889	DQ458857	DQ458874
CBS113188	*Dothiorella iberica*	*Quercus suber*	Spain	AY573198	EU673097	EU673278
CBS115041	*D. iberica*	*Q. ilex*	Spain	AY573202	EU673096	AY573222
CBS117006	*Dothiorella viticola*	*V. vinifera*	Spain	AY905555	EU673103	AY905562
UCD1435SLO	*D. viticola*	*V. vinifera*	California	EF202007	EF202014	EF202021
CBS117009	*D. viticola*	*V. vinifera*	Spain	AY905554	EU673104	AY905559
CMW28309	*Lasiodiplodia parva*	*Terminalia ivorensis*	Cameroon	GQ469962	GQ469894	GQ469904
CMW28333	*L. parva*	*T. superba*	Cameroon	GQ469961	GQ469892	GQ469903
PD298	*Neofusicoccum australe*	*Vaccinium* sp.	Chile	GU251220	GU251880	GU251352
UCD1314So	*N. australe*	*Vitis vinifera*	California	DQ008323	DQ008346	GU294732
CMW6837	*N. australe*	*Acacia* sp.	Australia	AY339262	AY339254	AY339270
CBS110497	*N. luteum*	*V. vinifera*	Portugal	EU673311	EU673092	EU673277
CMW10310	*N. luteum*	*Vitis* sp.	Portugal	AY339259	AY339251	AY339267
UCD2057Te	*N. luteum*	*V. vinifera*	California	DQ233604	DQ233625	EU012414
CBS121558	*N. mediterraneum*	*V. vinifera*	California	GU799463	GU799461	GU799462
UCCE825A	*N. mediterraneum*	*Juglans regia*	California	HM443604	HM443606	HM443608
UCD720SJ	*N. mediterraneum*	*V. vinifera*	California	GU799452	GU799475	GU799483
UCD642So	*N. parvum*	*V. vinifera*	California	DQ008328	DQ008351	GU294741
PD17	*N. parvum*	*Prunus dulcis*	United States	GU251143	GU251803	GU251275
PD59	*N. parvum*	*Prunus dulcis*	United States	GU251146	GU251806	GU251278
PD105	*Neoscytalidium dimidiatum*	*Ficus* sp.	United States	GU251108	GU251768	GU251240
PD103	*N. dimidiatum*	*Ficus* sp.	United States	GU251106	GU251766	GU251238
PD104	*N. dimidiatum*	*Ficus* sp.	United States	GU251107	GU251767	GU251239
CBS447.68	*Guignardia philoprina*	*Taxus baccata*	The Netherlands	FJ824768	FJ824779	FJ824773

[a] Sequences of three gene loci (internal transcribed spacer [ITS]1-5.8S-ITS2 regions, β-tubulin gene, and elongation factor [EF] α-1) were used.

[b] CBS = Centraalbureau Schimmelcultures, Utrecht, The Netherlands; CMW = Culture Collection Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; UCD = University of California, Davis. CBS447.68 (*Guignardia philoprina*) was added as an outgroup.

first major clade (93% bootstrap support) consisted of *Neofusicoccum* spp., and could be further resolved into four terminal subclades corresponding to isolates of *N. luteum*, *N. australe*, *N. mediterraneum*, and *N. parvum* isolates. The second major clade was divided into two subclades, the first (100% bootstrap support) consisting of *Neoscytalidium dimidiatum* isolates, with the second subclade (100% bootstrap support) consisting of isolates of *Diplodia* spp. and *Lasiodiplodia pava*. The *Diplodia* subclade had two subdivisions: *Diplodia mutila* and *D. seriata*. The third major clade had two subclades corresponding to *Dothiorella iberica* (100% bootstrap support) and *D. viticola* (100% bootstrap support) (Fig. 3).

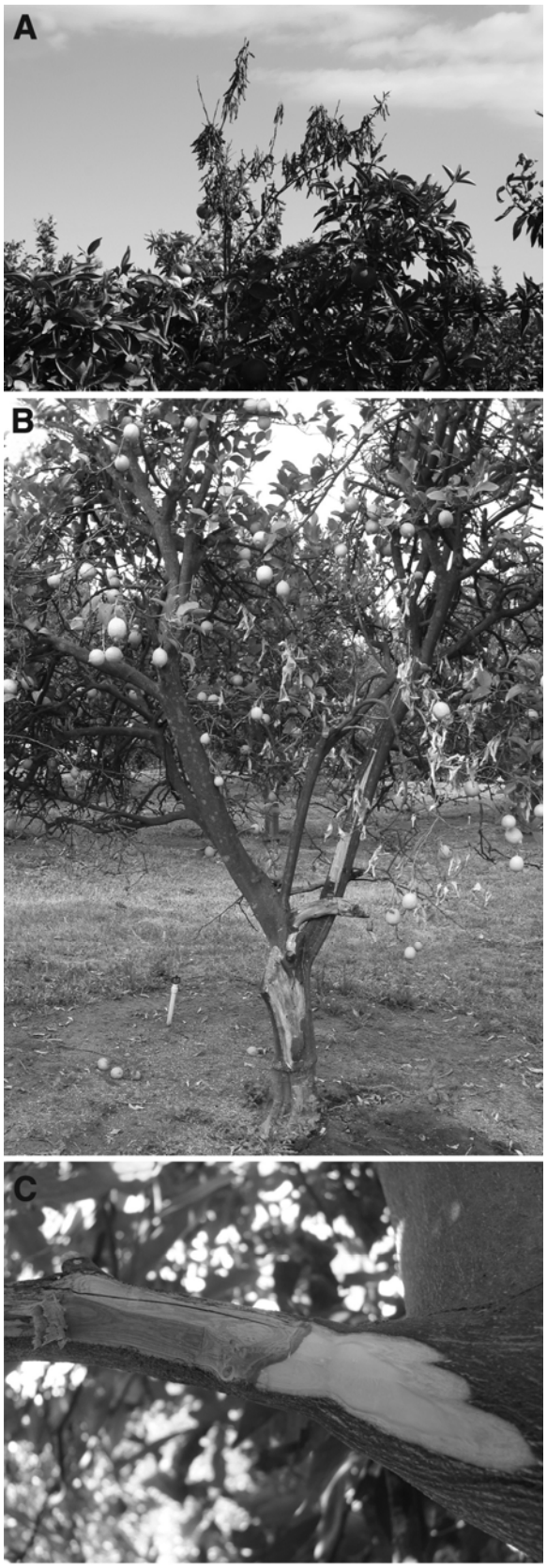

Fig. 1. Symptoms of Bot gummosis of citrus, including **A,** branch dieback on 'Satsuma' mandarin; **B,** trunk and branch cankers on 'Eureka' lemon; and **C,** a cankered branch on 'Valencia' orange.

Morphological identification. Two distinct groups were morphologically distinguished among *Neofusicoccum* spp. isolates. The first group consisted of isolates with conidia that were hyaline, aseptate, and fusiform. Cultural growth was characterized by either very pale yellow to bright yellow pigment in the center on the reverse side of the plate. These characteristics and conidial measurements (Table 3) matched those earlier described for *Neofusicoccum australe* and *N. luteum* (24,35). Isolates in the second group had conidia that were hyaline, broadly ellipsoid to fusoid, and aseptate. This matched descriptions for *N. mediterraneum* and *N. parvum* (Table 3) (24,36). Conidia of *N. mediterraneum* were larger than those of *N. parvum* (Table 3) (11).

Species of *Dothiorella* were characterized by conidia that were dark colored and septate early in their development, while still attached to conidiogenous cells (28). There were two species: *D. iberica*, which had conidia that had an internally verruculose wall and one septum; and *D. viticola*, which had conidia that were brown, oblong to subcylindrical, one-septum and occasionally slightly constricted at the septum, moderately thick-walled, externally smooth, and had rounded ends with a truncate base (48).

Diplodia spp. had thick-walled spores which, after release from pycnidia, became dark colored and septate. Two species could be distinguished among this group of isolates: *Diplodia mutila* which had conidia that were dark brown to black, thick-walled, oblong or cylindrical with rounded ends, and had conidiogenous cells that were holoblastic and cylindrical; and *D. seriata* (teleomorph: *Botryosphaeria obtusa*) (29,48), which had conidia that were aseptate, thick-walled, and hyaline with a smooth outer surface and rough inner surface but became brown before they were released from the pycnidia and conidiogenous cells.

Lasiodiplodia spp. had conidia that were similar to those of *Diplodia* spp., except there were no striations in the conidial wall of *Diplodia* spp. One species, *L. parva*, was identified. It had conidia that were initially hyaline, aseptate, and ellipsoid to ovoid, with granular content and broadly rounded ends, and became pigmented, verruculose, and ovoid (1).

Neoscytalidium dimidiatum isolates had conidia that were ellipsoid to ovoid and hyaline, with an acutely rounded apex and truncate base, and were initially aseptate. Conidiogenous cells were hyaline, intermingled with paraphyses. The mycelium was composed of branched, septate, brown hyphae, which disarticulated into 0- to 1-septate phragmospores. The conidial measurements obtained for these Botryosphaeriaceae spp. in California on citrus are shown in Table 3, along with previously reported measurements (6,11,24,27,35,36).

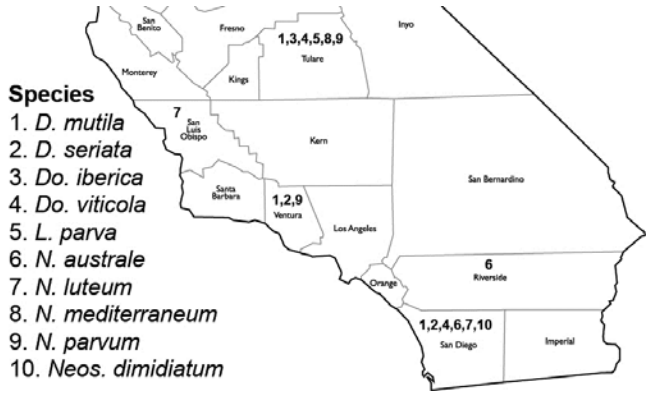

Fig. 2. California counties in which various species of Botryosphaeriaceae were detected in this study.

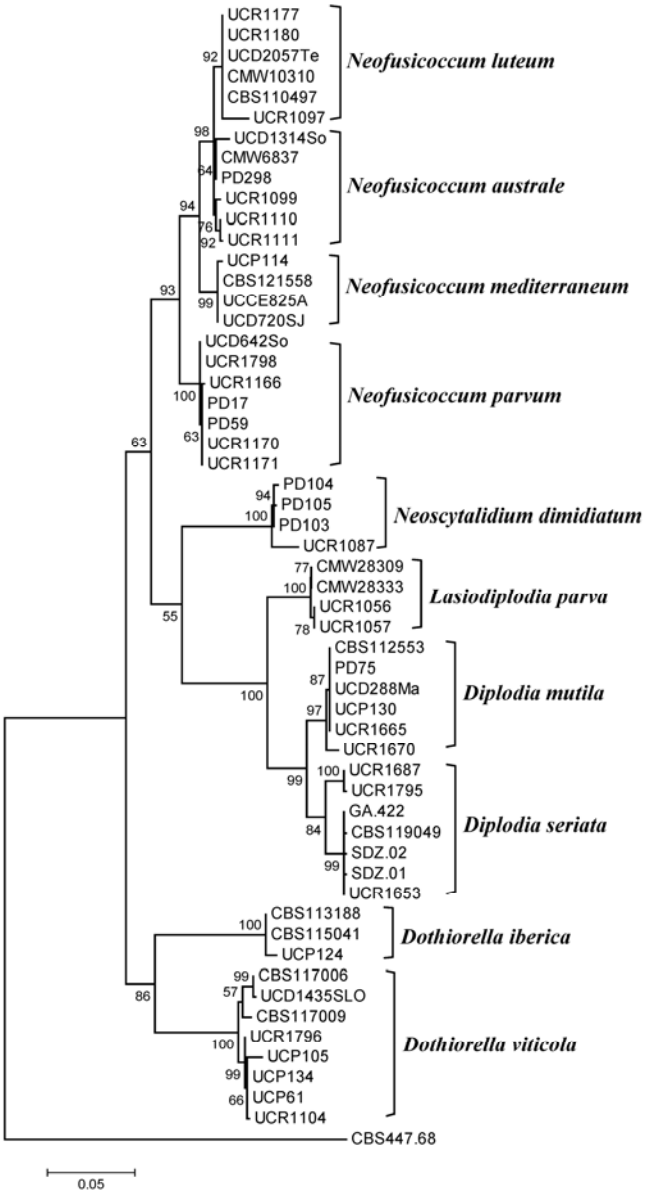

Fig. 3. Most parsimonious unrooted tree based on internal transcribed spacer (ITS)1, 5.8S ribosomal DNA, ITS2, partial β-tubulin gene, and elongation factor α-1 sequences of isolates of species in the Botryosphaeriaceae family inferred from maximum parsimony analysis using MEGA 5. Numbers on branches are bootstrap values >50% in 1,000 replicates. Isolate CBS447.68 (*Guignardia philoprina*) was added as an outgroup. CBS = Centraalbureau Schimmelcultures, Utrecht, The Netherlands; CMW = Culture Collection Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; UCD = University of California, Davis; UCP = University of California, Riverside Citrus Project; UCR = University of California, Riverside.

Pathogenicity test. The 14 isolates significantly differed ($P < 0.05$) in the size of lesions produced on Eureka lemon shoots (Fig. 4). Isolates of *Neofusicoccum luteum* had mean lesion lengths (10 cm each) that were significantly ($P < 0.05$) longer than those of other species. They were followed by mean lesion lengths of *N. australe* and *N. parvum* isolates (lesion lengths of 8.2 to 8.8 cm). *Dothiorella viticola* had significantly ($P < 0.05$) shorter mean lesion lengths compared with those of other species (Fig. 3). In reisolations, all fungal pathogens used in inoculation were successfully recovered (80 to 100%) when small pieces of inoculated shoots were placed on agar medium.

Discussion

In California, the importance and diversity of species in the Botryosphaeriaceae family in association with branch cankers on multiple hosts, including grapevine (49,44,50), olive (51), avocado (24,25,41,54), pistachio, (26) almond (19), and walnut (39), is increasingly becoming better understood (42). This study shows that citrus is now among the crops that are affected by species in the Botryosphaeriaceae family in California.

The taxonomy of the Botryosphaeriaceae family has significantly changed since species in this family were first discovered on citrus. Initially, *Diplodia natalensis* Pole-Evans was reported to be the cause of citrus dieback, gummosis, and fruit rot (5,9,17). It was considered widespread in the Mediterranean basin (20), and reported in all citrus-growing areas of North and South America, all Mediterranean citrus-growing countries, South Africa (23), Japan, China, India, Australia, Indonesia, the Philippines (15,31), and the islands of the West Indies (16). However, further taxonomic revisions reported *D. natalensis* to be a synonym of *Botryosphaeria rhodina* (Berk. & M.A. Curtis) Arx (anamorph *L. theobromae* (Pat.) Griffon & Maubl.) (3). Later, the disease was referred to as Dothiorella gummosis, because it was believed to be caused by *Dothiorella gregaria* Sacc. (teleomorph *B. ribis*) (37). New information has revealed that the genus name *Dothiorella* has been misapplied to anamorphs with hyaline, aseptate conidia of the type normally associated with *Fusicoccum* and *Neofusicoccum* spp. (12). Moreover, further phylogenetic studies have shown that *B. ribis* is synonymous with *B. parva* (anamorph *N. parvum*) (35). Given this new information, the disease can no longer be referred to as 'Dothiorella gummosis'. Thus, we refer to this disease caused by species in the Botryosphaeriaceae family as 'Bot gummosis' of citrus.

In other citrus-growing countries, the fungus '*B. ribis*' was reported as the most common cause of gummosis on lemon in the Mediterranean basin (31), and caused disease in artificial inoculations of lemon, sour orange, and tangerine and sweet orange in Sicily (33). Along with *Diplodia auranti*, it was recovered among other fungi in association with cankers on lemon, orange, and sour orange on citrus in Sicily (18). Former authors believe them to be wound parasites (18), occurring on plants weakened by various diseases, and causing limited damage (14). Given our current understanding of the diversity of species in the Botryosphaeriaceae family in this study, and that they were aggressive on excised Eu-

Table 3. Conidial size of Botryosphaeriaceae isolates from citrus branch canker used in this study and comparison with previous studies

Species	Conidial size (µm) of Botryosphaeriaceae isolates[a]		Reference
	In this study	In previous studies	
Diplodia mutila	28.9 ± 2.1 × 16.6 ± 1.7	23.1 ± 2.7 × 11.9 ± 1.5	29
D. seriata	29.1 ± 1.6 × 11.8 ± 0.6	…	…
Dothiorella viticola	28.1 ± 1.3 × 13.8 ± 0.9	…	…
D. iberica	25.0 ± 2.2 × 8.5 ± 1.3	23.8 ± 2.2 × 13.9 ± 1.5	24
Lasiodiplodia parva	26.0 ± 3.4 × 13.4 ± 1.9	22.6 × 12.2	6
Neofusicoccum australe	17.3 ± 3.6 × 6.6 ± 0.5	18.9 ± 1.2 × 6.9 ± 0.4	24
N. luteum	19.0 ± 2.0 × 6.3 ± 0.8	19.7 × 5.6	34,35
N. mediterraneum	27.1 ± 2.0 × 6.8 ± 0.9	24 × 6.0	11
N. parvum	16.4 ± 2.6 × 7.2 ± 1.3	17.2 ± 1.6 × 5.6 ± 0.6	29
Neoscytalidium dimidiatum	7.6 ± 1.2 × 4.8 ± 1.1	12.8 × 5.0	36

[a] Numbers are mean and standard deviation (in some cases) of length × width of 50 conidia from each species.

reka Lemon shoots, it is likely that the diversity and impact of this family on citrus worldwide is greater than previously understood.

In this study, the presence and diversity of species in the Botryosphaeriaceae family associated with Bot gummosis of citrus in California is presented for the first time using phylogenetic analyses for identification. These species belong to five genera—including *Diplodia*, *Dothiorella*, *Neofusicoccum*, *Neoscytalidium*, and *Lasiodiplodia*—and are pathogenic when inoculated into excised Eureka lemon shoots. To our knowledge, this is the first report of *L. parva* causing branch canker disease on citrus and any crop in California. Of all species tested, *N. luteum* was the most aggressive to excised lemon shoots. Although disease progress on trunks and large branches of adult trees in the field could not be determined using excised lemon shoots in the present study, host–pathogen compatibility was demonstrated. Further studies will be required to determine disease impact on mature trees and conditions that may contribute to disease development. Pioneering works on this disease in the United States suggest that extremes of cold or heat, severe frost (15), and drought (31) are among various conditions (e.g. variety susceptibility, trees injured or weakened by insects, wind, and mineral nutrient deficiencies) that may contribute to the occurrence and development of the disease. Nevertheless, given that host compatibility was demonstrated for a diverse range of previously unrecorded species in the Botryosphaeriaceae family on citrus, that they were recovered from cankered tissues of citrus trees with symptoms of branch and tree death, and that these species have adversely impacted other hosts, these fungal species may be considered primary pathogens on citrus.

Because the taxonomic understanding, occurrence, and impact of Botryosphaeriacious species among multiple hosts is increasingly becoming better recognized, transmission of these pathogens among hosts that overlap in distribution needs to be explored. Úrbez-Torres et al. (51) showed that all but two fungal species that were identified on olive trees are important pathogens on grapevine and occur in olive orchards either near or adjacent to vineyards throughout the state (51). This appears to be the case in the current study for *N. luteum* on citrus, avocado, olive, and grapevine, which occur in geographically similar areas. Studies also suggest that species may vary by geographical region due to climatic conditions such as temperature (22,44).

Although climate may play a role in fungal distribution, and proximity to suitable hosts may play a role in disease transmission and spread, several of the findings on citrus indicate that fungal distribution may be more complex. For example, optimal growth of *L. theobromae* occurs at 35 to 40°C, and is more prevalent in grapevine vineyards and olive orchards throughout the warmer counties in California (Madera, Merced, Riverside, and Tulare; 22,49–51). However, this species was not recovered from citrus in the present study, where these crops grow. Another example is *D. mutila*, which was detected on citrus in the present study and grapevine in other studies (46,49) in geographically separate regions throughout counties where they both occur. *Dothiorella iberica* occurs on both olive and citrus in Tulare County but has not been detected on citrus in Ventura County, where it infects avocado and olive (24,51). *N. mediterraneum* has a broader distribution on olive (51), almond, pistachio, and walnut (19) but, thus far, has only been detected on citrus in Tulare County. *N. australe* occurs on avocado and citrus in geographically overlapping areas but has not been detected on grapevine, where it has only been reported in northern California. In southern California, *N. parvum* has been found on citrus and avocado (24) but, thus far, has only been reported on grapevine in northern California (44). *Neoscytalidium dimidiatum* has been recovered from English walnut in Tulare County (8) and only citrus in San Diego County. Finally, *Diplodia seriata*, which has not yet been reported to occur south of the northern San Joaquin Valley, has only been recovered from citrus in southern California. Results from the survey in the present study shed light on where these various fungi are found on citrus in relation to other hosts and geography but more survey data will be needed to further substantiate these findings. Nonetheless, the complex distribution of these fungal species on a variety of hosts indicates the need to investigate mechanisms of transmission and infestation between hosts over a geographical range to determine effective strategies for management.

Currently, the management of Bot gummosis on citrus may be achieved by pruning dead limbs and twigs and removing pruning waste from fields prior periods of rain. This strategy is based on results from studies on avocado and grapevine (4,11,13,32,45,47); however, the efficacy of those cultural practices has not been evaluated. Pruning wounds, mechanical injury, frost, and sunburn damage have been reported to serve as points of entry for spores in the Botryosphaeriaceae family on other hosts (11,13). In avocado and grapevine, spores are released from fruiting bodies present on branches or twigs and spread by rain splash and wind, typically between November and April (13,50). Currently, there are no effective fungicides that can be used by growers to prevent cankers on citrus in California. However, the ability for fungicides to control occasional outbreaks of citrus cankers caused by species in the Botryosphaeriaceae family is currently being evaluated, based on studies on avocado (40).

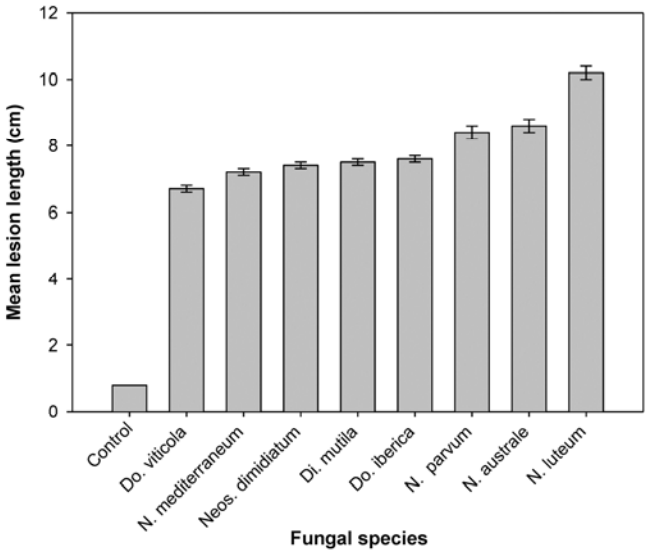

Fig. 4. Mean lesion lengths on excised 'Eureka' lemon shoots inoculated with isolates belonging to eight species of Botryosphaeriaceae. Vertical lines represent standard error of the mean according to Tukey's honestly significant difference mean separation test at $\alpha = 0.05$.

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