Botryosphaeriaceae Species Associated with Avocado Branch Cankers in California

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Abstract

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Members of the family Botryosphaeriaceae cause branch cankers and dieback on California avocado trees. More intensive pruning, a practice associated with high-density planting that is becoming more common in the California avocado industry, may increase the occurrence of branch canker. This study was undertaken to identify and characterize the Botryosphaeriaceae spp. involved in the branch canker disease complex in order to develop future management strategies. From 2008 to 2009, branch cankers were sampled from four or five trees from each of eight avocado groves in five California counties. Six Botryo-

sphaeriaceae spp. were identified based on morphology as well as phylogenetic analysis of the internal transcribed spacer region (ITS1-5.8S-ITS2) and a partial sequence of the β -tubulin gene. These six species included *Neofusicoccum australe*, *N. luteum*, *N. parvum*, an unknown *Neofusicoccum* sp., *Fusicoccum aesculi*, and *Dothiorella iberica*. Members of the Botryosphaeriaceae were isolated from all avocado-growing regions sampled in California; however, incidence and distribution of species varied. This report is the first description of the isolation of *D. iberica* from avocado branch cankers in California.

California accounts for the majority of U.S. avocado production, followed by Florida and Hawaii. In the 2008–09 growing season, the California avocado industry occupied over 64,000 acres and the annual crop was valued at over \$199 million (2). California avocado fruit are grown year round and a single tree can produce up to 91 kg of fresh fruit each year, although most average about 27 kg.

Canker on California avocado trees (*Persea americana* Mill.) can occur on twigs, branches, or trunks and appears to be caused by a complex of fungal species in the family Botryosphaeriaceae (19,20). Pruning wounds can be infection courts of canker pathogens, as well as wounds due to other mechanical injuries and frost damage. Cankers on avocado may exude reddish sap that dries to a whitish-beige powder. Bark may be cracked, darkly discolored, or slightly sunken. On older cankers, bark may be friable and easily removed from the damaged area. Cankered inner bark and wood are red-brown to brown, instead of the normal pale color. In a cross section of a cankered branch, a characteristic wedge-shaped discoloration extending deep into the xylem may be visible. If the xylem is extensively colonized, the affected branch may rapidly wilt, with leaves quickly turning brown but remaining attached (20).

In 1934, Horne described avocado fruit rot as caused by the fungal pathogen in the genus *Dothiorella*, with fruiting bodies found on dead avocado twigs (15). Because inoculation of healthy twigs did not produce disease, this fungus was not believed to be the cause of branch and twig cankers. He concluded that twig dieback on a tree with heavy foliage was due to shading in competition with other twigs (15). By 1953, *Dothiorella gregaria* (teleomorph *Botryosphaeria ribis*) was believed to be the cause of severe trunk cankers at graft unions of avocado on Guatemalan rootstocks (12). Molecular sequencing technology has led to revision of the taxonomy of the family Botryosphaeriaceae (5,25,28,29). 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. (7). In addition, it has been shown that similar branch cankers on other woody hosts (e.g., grape and almond) are due to a complex of fun-

gal species, of which the most common belong to the family Botryosphaeriaceae (16,36).

Members of the Botryosphaeriaceae have a worldwide distribution and cause cankers and fruit rot on a wide variety of woody hosts (6,8,17,22,27,30,36). Species can survive as saprophytes or parasites and some species can survive as endophytes in symptomless tissue (10). Various species within the Botryosphaeriaceae have been isolated from cankers on avocado from many different countries, including Mexico, New Zealand, Peru, South Africa, Chile, Central and South America, Spain, and the United States (19,20,38).

Because the teleomorphs of the Botryosphaeriaceae are rarely seen in nature or in culture, anamorph characteristics have been primarily used for identification (5,7). Using anamorph characteristics combined with DNA sequence data has allowed elucidation of the species involved in branch canker and fruit rot disease complexes. For example, use of morphological and genomic information has led to the recent clarification of the pathogens involved in branch canker in grape (36), mango (30), and almond (16) and fruit rot in olive (17).

For growers to remain competitive in the international market, they must find ways to manage their groves efficiently and significantly increase production. One way to do this is by decreasing spacing between trees from 6 by 6 m to 3 by 3 m, a practice already employed in Chile and becoming more common in California (13,14). More frequent pruning, such as would occur in a high-density grove, could increase the dissemination of canker-causing members of the Botryosphaeriaceae from tree to tree, leading to an increase in canker incidence and a possible decrease in yield as cankered branches are pruned out. Identifying and characterizing the primary causal agents of this disease will assist in developing the appropriate control measures to reduce yield loss.

The objective of this study was to identify the species of Botryosphaeriaceae associated with the avocado canker disease complex in California and evaluate their distribution. Morphological and molecular methods were used to characterize these species. Anamorph names will be used for members of the Botryosphaeriaceae family identified in this study.

Materials and Methods

Field survey and fungal isolation. Between September 2008 and March 2009, four or five symptomatic avocado trees ('Hass') in each of eight avocado groves in five counties (Table 1; Fig. 1) were sampled for *Dothiorella* canker in order to verify the associ-

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ated Botryosphaeriaceae spp. The groves selected were located in the major avocado production areas in California. Mature trees, at least 10 years old, were sampled for visible branch cankers with depressed and darkly discolored bark. Cankers with active oozing of reddish sap as well as those with dried sap were sampled. Usually, a branch section was cut about 6 in. proximal and distal to the canker and packed on ice for transport back to the lab.

In the laboratory, surface dirt and debris were rinsed off branch samples with deionized water and cankers were photographed. Cankers were surface disinfested with 95% ethanol and flame sterilized, and the discolored outer bark was cut away. Small pieces of inner bark and xylem tissue from the lesion margins were placed onto 4% potato dextrose agar (PDA) (Becton, Dickinson and Co., Sparks, MD) amended with tetracycline hydrochloride (0.01%, wt/vol) (PDA-tet) in 85-mm-diameter petri dishes. Cultures were incubated at room temperature for 3 to 5 days. Pure cultures were

obtained by transferring hyphal tips from the colony margin onto fresh PDA-tet plates. Isolates were stored at 5°C as mycelial plugs in 1.5-ml microtubes in 1 ml of sterile deionized water.

Morphological characterization. Twenty-seven Botryosphaeriaceae isolates (Table 2), identified based on sequence data (as described below), were further characterized. Isolates for colony morphological characterization were grown in the dark on PDA-tet agar at 25°C. In order to obtain conidia, isolates were plated onto 2% water agar amended with autoclaved pine needles or autoclaved avocado wood chips. Plates were incubated at 25°C under a 12-h daily photoperiod for up to 4 weeks to encourage pycnidium formation. Conidial color and shape and presence or absence of septations were noted. The length and width of 50 conidia per isolate were measured using a compound microscope (Olympus BX40 with a Leica DFC420 camera) and the SPOT Imaging Software

Table 1. Incidence of Botryosphaeriaceae and *Phomopsis* spp. in the main avocado production areas of California, September 2008 to March 2009

County	Groves ^a	Branch cankers ^b	Botryo. spp. (%) ^c	<i>Phomopsis</i> spp. (%) ^d
San Luis Obispo	2	46	18 (39)	7 (15)
Santa Barbara	1	48	18 (38)	8 (17)
Ventura	2	71	34 (48)	3 (4)
Riverside	2	12	10 (83)	1 (8)
San Diego	1	10	2 (20)	1 (10)
Total:	8	187	82 (44) ^e	20 (11) ^f

^a Number of groves sampled.

^b Number of branch cankers sampled.

^c Number of branch cankers (and percentage of the total number of branch cankers sampled per county) yielding Botryosphaeriaceae spp.

^d Number of branch cankers (and percentage of the total number of branch cankers sampled per county) yielding *Phomopsis* spp.

^e Total number of branch cankers (and percentage of the total number of branch cankers sampled) yielding Botryosphaeriaceae spp.

^f Total number of branch cankers (and percentage of the total number of branch cankers sampled) yielding *Phomopsis* spp.

Fig. 1. California counties in which various species of Botryosphaeriaceae were detected in this study.

Table 2. Botryosphaeriaceae isolates from *Persea americana* in California used in this study

Species ^a	Origin ^b	Collected	Isolate	GenBank number	
				ITS ^c	β-Tubulin
<i>Neofusicoccum australe</i>	Riverside	September 2008	UCR160	HQ529741	HQ529713
<i>N. australe</i>	Ventura	November 2008	UCR508	HQ529743	HQ529718
<i>N. australe</i>	Ventura	November 2008	UCR509	GQ857659	GQ857665
<i>N. australe</i>	San Luis Obispo	March 2009	UCR739	GQ857662	GQ857668
<i>N. luteum</i>	Riverside	September 2008	UCR211	GQ857658	GQ857664
<i>N. luteum</i>	Ventura	November 2008	UCR455	HQ529756	HQ529726
<i>N. luteum</i>	Ventura	November 2008	UCR516	HQ529757	HQ529727
<i>N. luteum</i>	Santa Barbara	November 2008	UCR524	HQ529762	HQ529732
<i>N. luteum</i>	Santa Barbara	November 2008	UCR526	HQ529763	HQ529733
<i>N. luteum</i>	Ventura	November 2008	UCR539	HQ529758	HQ529728
<i>N. luteum</i>	Santa Barbara	November 2008	UCR594	HQ529759	HQ529729
<i>N. luteum</i>	Riverside	September 2008	UCR654	HQ529760	HQ529730
<i>N. parvum</i>	San Diego	October 2008	UCR295	HQ529765	HQ529734
<i>N. parvum</i>	Ventura	November 2008	UCR531	GQ857661	GQ857667
<i>N. parvum</i>	San Luis Obispo	March 2009	UCR735	HQ529766	HQ529735
<i>N. parvum</i>	San Luis Obispo	March 2009	UCR736	HQ529767	HQ529736
<i>N. parvum</i>	San Luis Obispo	March 2009	UCR737	GQ857660	GQ857666
<i>Neofusicoccum</i> sp.	Ventura	November 2008	UCR468	HQ529771	HQ529739
<i>Neofusicoccum</i> sp.	Ventura	November 2008	UCR532	HQ529769	HQ529740
<i>Neofusicoccum</i> sp.	San Luis Obispo	March 2009	UCR749	HQ529770	HQ529738
<i>Fusicoccum aesculi</i>	Ventura	November 2008	UCR453	HQ529750	HQ529721
<i>F. aesculi</i>	Ventura	November 2008	UCR454	HQ529751	HQ529722
<i>F. aesculi</i>	Ventura	November 2008	UCR652	GQ857657	GQ857663
<i>F. aesculi</i>	San Luis Obispo	March 2009	UCR750	HQ529752	HQ529723
<i>Dothiorella iberica</i>	Ventura	November 2008	UCR452	HQ529753	HQ529724
<i>D. iberica</i>	San Luis Obispo	March 2009	UCR732	HQ529754	HQ529725
<i>D. iberica</i>	San Luis Obispo	March 2009	UCR740	HQ529755	HQ529722

^a Anamorphs of Botryosphaeriaceae spp. were identified based on morphology and phylogenetic analyses.

^b Counties in the state of California in the United States.

^c ITS = internal transcribed spacer.

(v4.7.0.35; Diagnostic Instruments, Inc., MI). Mean and standard deviation of conidial measurements were obtained using summary statistics in SigmaPlot 11.2 (Systat Software Inc., San Jose, CA).

DNA isolation, amplification, and phylogenetic analyses. Total genomic DNA was extracted from each isolate (Table 2) using a modification of the method as described by Ceniz (3). Oligonucleotide primers ITS4 and ITS5 were used to amplify the ITS1, ITS2, and 5.8S regions of rDNA (37). A partial sequence of the β -tubulin nuclear gene was amplified using the Bt2a and Bt2b primers (11). Each 30- μ l polymerase chain reaction (PCR) mixture included 20.25 μ l of PCR-grade water, 3 μ l of ThermoPol Reaction buffer, 0.6 μ l of dNTP mix, 2.25 μ l of each primer, 0.15 μ l of NEB Taq polymerase, and 1.5 μ l of DNA template. PCR reactions were carried out in a thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA) using the following program for both ITS and Bt2 primers: an initial preheat at 94°C for 2 min, followed by 25 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 15 s, and extension at 72°C for 45 s. A final extension at 72°C was held for 5 min. The PCR products were separated by gel electrophoresis in 1.5% agarose gels in 1 $\times$ Tris-boric acid-EDTA (TBE) buffer and were photographed under UV light after staining with SYBR Green (Invitrogen, Eugene, OR). PCR products were purified using the QIAquick PCR purification kit (Qiagen Inc., Valencia, CA). ITS and β -tubulin regions were sequenced in both directions at the Institute for Integrative Genome Biology facility of the University of California, Riverside.

Sequences were edited using the program Sequencher 4.6 (Gene Codes Corp., Ann Arbor, MI), locally aligned using ClustalX 2.1-Mac OSX (Conway Institute, UCD Dublin) (33), and manually aligned using MacClade 4.08 OSX (Sinauer Associates, Inc., Sunderland, MA) (18). Phylogenetic analysis was completed using PAUP (version 4.0b10; Sinauer Associates; 32). Alignment gaps were treated as missing data. Sequences from isolates obtained in the survey (Table 2) were compared with sequences in GenBank (Table 3) using *Guignardia* sp. CBS447.68 as the outgroup. Separate and combined analyses were run for the ITS and β -tubulin data sets. A partition homogeneity test was used to determine whether the ITS and β -tubulin regions could be combined. Maximum parsimony analysis was performed using the heuristic search option (NNI branch swapping). Bootstrap values were evaluated using

1,000 replicates and 100 random sequence additions to test branch strength. Tree length, consistency index (CI), retention index (RI), rescaled consistency index (RC), and homoplasy index (HI) were recorded for all analyses.

Results

Field survey and morphological characterization. Cankers on trees in avocado groves were mostly on branches, characterized by darkened and depressed areas of bark, and sometimes oozed reddish sap. On older cankers, the bark was easily removed, and a dried, white powder exudate was frequently present. In the xylem tissue, cankers were reddish-brown in color and variable in shape. Branch cross sections revealed that some discoloration extended into the center with a characteristic wedge shape. Dieback of the affected branch also was usually present.

Botryosphaeriaceae spp. were present in all eight groves sampled and were isolated from 82 of 187 cankers (Table 1). Members of the Botryosphaeriaceae were the most abundant fungi isolated from avocado branch cankers in California, followed by *Phomopsis* spp. (Table 1). Other sporadically isolated fungi from avocado branch cankers were identified as belonging to the genera *Bionectria*, *Colletotrichum*, *Schizophyllum*, *Fusarium*, *Eutypella*, and *Phaeoacremonium*, based on morphological and DNA sequence evidence.

There were five distinct groups of Botryosphaeriaceae isolates based on colony color and growth characteristics. The first group had buff-colored, aerial hyphae in patchy tufts that covered the surface of the medium in an 85-mm petri plate in 2 to 3 days, with some isolates growing up the sides of the plate. Isolates in this group had either very pale yellow to bright yellow pigment in the center on the reverse of the plate, which is characteristic of *Neofusicoccum australe* and *Neofusicoccum luteum* (22,29). With age, the reverse of the colony became a progressively darker greenish-gray color, with the top of the culture showing dense hyphae that were appressed in the middle but fluffier at the margin. By day 9 to 10, the reverse of the colony was completely black, with the top of the culture showing moderate to dense, appressed hyphae with color varying from light to very dark gray-black. Conidial morphology and DNA analyses (below) were used to identify the two species in this group as *N. australe* (Slippers, Crous & M.J.

Table 3. Sequences from GenBank used in the phylogenetic study

Isolate ^a	Species	Host	Origin	GenBank number	
				ITS ^b	β -Tubulin
UCD1314So	<i>Neofusicoccum australe</i>	<i>Vitis vinifera</i>	California	DQ008323	DQ008346
UCD1467So	<i>N. australe</i>	<i>V. vinifera</i>	California	DQ233610	DQ233631
CMW6837	<i>N. australe</i>	<i>Acacia</i> sp.	Australia	AY339262	AY339254
UCD2057Te	<i>N. luteum</i>	<i>V. vinifera</i>	California	DQ233604	DQ233625
UCD2089Te	<i>N. luteum</i>	<i>V. vinifera</i>	California	DQ233605	DQ233626
UCD2090Te	<i>N. luteum</i>	<i>V. vinifera</i>	California	DQ233606	DQ233627
CMW9076	<i>N. luteum</i>	<i>Malus $\times$ domestica</i>	New Zealand	AY236946	AY236922
CMW10310	<i>N. luteum</i>	<i>V. vinifera</i>	Portugal	AY339259	AY339251
UCD642So	<i>N. parvum</i>	<i>V. vinifera</i>	California	DQ008328	DQ008351
UCD646So	<i>N. parvum</i>	<i>V. vinifera</i>	California	DQ008329	DQ008352
UCD759St	<i>N. parvum</i>	<i>V. vinifera</i>	California	DQ233611	DQ233632
CMW7798	<i>N. parvum</i>	<i>Magnifera indica</i>	Australia	AY615183	AY615170
CMW7799	<i>N. parvum</i>	<i>Persea americana</i>	Australia	AY615184	AY615171
PD283	<i>N. arbuti</i>	<i>Arbutus menziesii</i>	United States	GU251154	GU251814
PD284	<i>N. arbuti</i>	<i>A. menziesii</i>	United States	GU251153	GU251813
PD301	<i>N. nonquaesitum</i>	<i>Vaccinium</i> sp.	Chile	GU251164	GU251824
PD302	<i>N. nonquaesitum</i>	<i>Vaccinium</i> sp.	Chile	GU251165	GU251825
CMW8000	<i>Fusicoccum aesculi</i>	<i>Prunus</i> sp.	Switzerland	AY236949	AY236927
SDAU07164	<i>F. aesculi</i>	<i>Punica granata</i>	China	FJ171720	FJ238529
SDAU08.56	<i>F. aesculi</i>	<i>Populus</i> sp.	China	FJ214104	FJ238527
UCD1439SLO	<i>Dothiorella iberica</i>	<i>V. vinifera</i>	California	EF202008	EF202015
UCD1448SLO	<i>D. iberica</i>	<i>V. vinifera</i>	California	EF202009	EF202016
CBS 113188	<i>D. iberica</i>	<i>Quercus suber</i>	Spain	AY573198	EU673097
CBS447.68	<i>Guignardia philoprina</i>	<i>Taxus baccata</i>	The Netherlands	FJ824768	FJ824779

^a Acronyms of culture collections: UCD = University of California, Davis; CMW = Culture Collection Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; SDAU = Shandong Agricultural University; CBS = Centraalbureau Schimmelcultures, Utrecht, The Netherlands.

^b ITS = internal transcribed spacer.

Wingf.) Crous, Slippers & A.J.L. Phillips and *N. luteum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips (9).

Colonies of the second group had gray-white, dense, fluffy, aerial mycelium, with the reverse of the colony showing a dark greenish center becoming light green-gray and then white at the edges. The colony covered the entire medium surface by about day 3. By day 10, the reverse of the culture was dark green-gray to black and the upper surface of the culture showed dense appressed dark gray hyphae with scattered aerial light gray to white tufts. Conidial morphology and DNA analyses (below) were used to identify these isolates as *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips (9).

Isolates of the third group had white to light gray, thin, fluffy hyphae and covered the surface of the medium by day 4. By day 10, the upper surface of the colonies were light to medium-dark gray to black with dense, appressed hyphae and with scattered whitish tufts, and were less dense near the center. The reverse of the plate was dark green-black. Conidial morphology and DNA analyses (below) were used to identify these isolates as *Fusicoccum aesculi* Corda (9).

The fourth group had white-gray to light yellow, dense, cottony mycelium that covered the surface of the medium by day 4 to 5. The reverse varied from white-beige to light yellow with a dark greenish-gray center. By day 10, the surface of the colony showed scattered patches of dark green-gray hyphae, usually whiter at the edges. The reverse showed variable patches of color from whitish beige to yellow to green-gray. By day 15, the dark green-gray areas were converging, with patchy whitish edges. Conidial morphology and DNA analyses (below) closely group these isolates with a recently described species, *Neofusicoccum nonquaesitum*, but which will be referred to as *Neofusicoccum* sp. in this article.

The fifth group had thin white or gray or brown hyphae, slightly fluffy, almost to the edge of the plate by day 7. By day 10, the upper surface showed thin to moderately dense, appressed hyphae, with color ranging from light to dark green-gray to brown, with buff edges. The reverse of the plate showed dark green-black hyphae with buff outer edges. Conidial morphology and DNA analyses (below) were used to identify these isolates as *Dothiorella iberica* A.J.L. Phillips, J. Luque & A. Alves (9).

Conidial characteristics separated the Botryosphaeriaceae family into two groups. The first group consisted of five of the six species

noted above (*N. australe*, *N. luteum*, *N. parvum*, *Neofusicoccum* sp., and *F. aesculi*), with conidia that were hyaline and thin walled. The other group consisted of one species (*D. iberica*), with conidia that were pigmented and thick walled. The conidial measurements obtained for these Botryosphaeriaceae spp. in California are shown in Table 4, along with previously reported measurements (16,22–24,29).

Phylogenetic analyses. Of the 613 ITS sequence nucleotides analyzed, 72 were parsimony informative. Maximum parsimony analysis of the ITS region resulted in one most-parsimonious tree (length = 183, CI = 0.809, RI = 0.946, RC = 0.765, and HI = 0.191; Fig. 2). The ITS tree contained two major clades, the first of which (100% bootstrap support) was resolved into one group corresponding to *F. aesculi*. The second major clade (77% bootstrap support) was resolved into two subclades. The first subclade (74% bootstrap support) corresponded to species with *Neofusicoccum* anamorphs, and this was resolved further into *N. luteum*, *N. australe*, *N. parvum*, and one *Neofusicoccum* sp. closely related to *N. nonquaesitum*. The second subclade (100% bootstrap support) corresponded to *D. iberica*.

Of the 484 β -tubulin sequence nucleotides analyzed, 100 were parsimony informative. Maximum parsimony analysis of the β -tubulin region resulted in one most-parsimonious tree (length = 233, CI = 0.841, RI = 0.955, RC = 0.804, and HI = 0.159; Fig. 3). This tree contained two major clades, the first of which (100% bootstrap support) corresponded to *D. iberica*. The second major clade (84% bootstrap support) resolved into two subclades. The first subclade (72%) corresponded to *N. luteum*, *N. australe*, *N. parvum*, and one *Neofusicoccum* sp. closely related to *N. nonquaesitum*. The second subclade (100% bootstrap support) corresponded to *F. aesculi*. Also noted is the reversal of the placement of the *F. aesculi* clade with the *D. iberica* clade in the β -tubulin tree.

A partition homogeneity test indicated that the ITS and β -tubulin datasets could be combined ($P = 0.33$). Of the 1,095 nucleotides analyzed, 172 were parsimony informative. Maximum parsimony analysis yielded one most-parsimonious tree (length = 416, CI = 0.824, RI = 0.950, RC = 0.783, and HI = 0.175). The topology of this tree (Fig. 4) was consistent with the topology of the ITS tree (Fig. 2). Two major clades were resolved, the first of which (100% bootstrap support) corresponded to *F. aesculi*. The second major clade (77% bootstrap support) was resolved into two subclades. The first subclade (72% bootstrap support) corresponded to *N.*

Table 4. Conidial measurements of Botryosphaeriaceae fungi from California used in this study and comparison with previous studies

Species, isolate	Botryosphaeriaceae spp. in this study		Botryosphaeriaceae spp. from previous studies	
	Conidial size (μm) ^a	Mean $\pm$ SD (μm) ^b	Conidial size (μm) ^c	Source
<i>Neofusicoccum australe</i>				
UCR508	(15–)19–21 $\times$ (6–)7–8	18.9 $\pm$ 1.2 $\times$ 6.9 $\pm$ 0.4	(18–)23–26(–30) $\times$ 5–6(–7.5)	29
UCR509	(13–)22–27 $\times$ (5–)7–8	21.8 $\pm$ 3.4 $\times$ 7.0 $\pm$ 1.0	...	...
UCR745	(12–)19–23 $\times$ (5–)7–8	19.3 $\pm$ 1.8 $\times$ 7.2 $\pm$ 0.9	...	...
<i>N. luteum</i>				
UCR444	(20–)27–34 $\times$ (6–)7–8	27.4 $\pm$ 2.9 $\times$ 6.9 $\pm$ 0.6	(14–)20–24(–32) $\times$ (5–)6–7(–9)	22
UCR516	(17–)21–24 $\times$ (7–)7–8	20.7 $\pm$ 1.7 $\times$ 7.3 $\pm$ 0.5	(15–)18–22.5(–24) $\times$ 4.5–6(–7.5)	23
<i>N. parvum</i>				
UCR295	(9–)16–22 $\times$ (5–)7–8	15.4 $\pm$ 2.6 $\times$ 6.5 $\pm$ 0.6	(11–)14–18(–23) $\times$ 5–7(–10)	22
UCR531	(17–)20–22 $\times$ (6–)6–7	19.9 $\pm$ 1.1 $\times$ 6.4 $\pm$ 0.5	(12–)15–20(–24) $\times$ (4–)4.5–6(–7.5)	23
UCR736	(12–)17–20 $\times$ (6–)7–8	16.9 $\pm$ 1.5 $\times$ 7.1 $\pm$ 0.6	...	...
<i>Neofusicoccum</i> sp.			<i>N. nonquaesitum</i>	16
UCR532	(13–)20–26 $\times$ (6–)7–8	19.8 $\pm$ 2.8 $\times$ 6.7 $\pm$ 0.5	(17.2–)23.2 $\pm$ 2.7 (–29.1) $\times$ (5.6–)7.6 $\pm$ 1.0 (–10.6)	...
UCR749	(20–)24–31 $\times$ (6–)7.5–9	24.2 $\pm$ 2.2 $\times$ 7.4 $\pm$ 0.8	...	...
<i>Fusicoccum aesculi</i>				
UCR454	(21–)23–28 $\times$ (6–)7–8	23.4 $\pm$ 1.5 $\times$ 6.9 $\pm$ 0.4	(15–)20–26(–32) $\times$ (4–)5–6(–9)	22
UCR652	(21–)26–32 $\times$ (6–)7–9	25.8 $\pm$ 2.2 $\times$ 7.2 $\pm$ 0.7	(18–)21–28.5(–30) $\times$ (3.5–)4–4.5(–6)	23
UCR750	(11–)21–26 $\times$ (4–)6–8	20.6 $\pm$ 2.5 $\times$ 6.2 $\pm$ 0.7	...	...
<i>Dothiorella iberica</i>				
UCR452	(17–)24–30 $\times$ (11–)14–19	23.8 $\pm$ 2.2 $\times$ 13.9 $\pm$ 1.5	(17.2–)23–23.4(–28.6) $\times$ (8.1–)10.8–11(–16)	24
UCR681	(19–)24–26 $\times$ (9–)10–11	23.5 $\pm$ 1.3 $\times$ 9.9 $\pm$ 0.6	...	...
UCR732	(22–)24–27 $\times$ (8–)10–12	24.2 $\pm$ 1.2 $\times$ 9.8 $\pm$ 0.7	...	...

^a Minimum size, mode, and maximum size in length and width of 50 conidia from each isolate.

^b SD = standard deviation.

^c Minimum size, most common values, and maximum size for length and width of conidia.

luteum, *N. australe*, *N. parvum*, and one *Neofusicoccum* sp. closely related to *N. nonquaesitum*. The second subclade (100% bootstrap support) corresponded to *D. iberica*.

Distribution of Botryosphaeriaceae spp. in California. At least two Botryosphaeriaceae spp. were found in all counties sampled in this study (Table 5; Fig. 1). *N. luteum* was the species most frequently isolated from cankers, followed by *N. australe*, *F. aesculi*, *N. parvum*, *D. iberica*, and *Neofusicoccum* sp. Not all species were found in all counties. *N. australe* was found in all five counties. *N. luteum* was found in all counties, except for Riverside County. *N. parvum* was found in San Luis Obispo and Ventura

Counties, in addition to the southern county of San Diego. *F. aesculi*, *D. iberica*, and the unknown *Neofusicoccum* sp. were found only in the two northern counties of San Luis Obispo and Ventura (Table 5; Fig.1).

Discussion

This study represents the first attempt to determine the presence and diversity of Botryosphaeriaceae fungi associated with avocado branch canker in the major avocado production areas of California. Six different Botryosphaeriaceae spp. were isolated from avocado branch cankers. The diversity of species present is similar to that

Fig. 2. Most equally parsimonious tree with bootstrap values obtained from the internal transcribed spacer (ITS)1, 5.8S rDNA region, and ITS2 sequence data using 1,000 replicates generated in PAUP. Asterisks show GenBank reference sequences. The anamorph of each Botryosphaeriaceae member is shown, except for the outgroup, which is named by its teleomorph.

reported on other woody hosts in California (1,16,36) as well as other parts of the world (8,26,27,31). A previous study confirmed that the most abundant Botryosphaeriaceae spp. isolated in this study (*N. luteum*, *N. parvum*, *N. australe*, and *F. aesculi*) caused disease of avocado in California (19). A preliminary study has also shown *D. iberica* and the *Neofusicoccum* sp. to cause disease on avocado (data not shown).

The resulting ITS and combined ITS and β -tubulin phylogenetic trees from our study are consistent with results of other recent research that differentiated the anamorphs of the *Fusicoccum* and *Neofusicoccum* clades based on the 28S rRNA gene and hyaline, thin-walled conidial morphology (5) and the *Dothiorella* clade

based on the ITS and EF1- α genes and pigmented, thick-walled conidial morphology (24). However, our phylogenetic analysis based on the β -tubulin gene sequences shows the placement of the *F. aesculi* clade reversed with the *D. iberica* clade, for reasons which are unclear at this time. Recent work to resolve the placement of the dark-spored genera in the Botryosphaeriaceae family clearly shows *D. iberica* as the sister clade to the *Neofusicoccum* clade (25), as shown in our ITS and combined trees, and as noted in more detail below.

To our knowledge, this article is the first to report on the isolation of *D. iberica* from avocado branch cankers in California. *D. iberica* has been isolated from cankers on grapevine in

Fig. 3. Most equally parsimonious tree with bootstrap values obtained from the partial β -tubulin gene sequence data using 1,000 replicates generated in PAUP. Asterisks show GenBank reference sequences. The anamorph of each Botryosphaeriaceae member is shown, except for the outgroup, which is named by its teleomorph.

California (35). The genus name *Dothiorella* has been the source of much confusion in the past because it has been used in more than one sense. Although avocado branch canker has been known (34) as *Dothiorella* branch or stem canker and *Dothiorella* fruit rot, this nomenclature is outdated for two reasons. First, as has been noted here and elsewhere (30,36), cankers of woody hosts are caused by a number of species in the Botryosphaeriaceae and are not limited to the genus *Dothiorella*. Second, the genus name of *Dothiorella* for branch canker was misapplied by Petrak in 1922, according to Denman (7). Petrak was the first to make the

connection between the teleomorph *Botryosphaeria* and the anamorph *Fusicoccum*, but Petrak referred to *Fusicoccum*, which has hyaline, thin-walled spores, as *Dothiorella*, which has pigmented, thick-walled spores, leading to a long period of misapplication of the name *Dothiorella* (7). In 1999, Crous and Palm synonymized *Dothiorella* with *Diplodia* (4). However, Phillips subsequently separated *Dothiorella* from *Diplodia* based on two fungal isolates which, on phylogenetic analysis (ITS + EF1- α), fell within the *Fusicoccum* clade, but were morphologically distinct from *Fusicoccum* spp. and could not be

Fig. 4. Most equally parsimonious tree with bootstrap values obtained from the internal transcribed spacer (ITS)1, 5.8S rDNA region, ITS2, and partial β -tubulin gene sequence data using 1,000 replicates generated in PAUP. Asterisks show GenBank reference sequences. The anamorph of each Botryosphaeriaceae member is shown, except for the outgroup, which is named by its teleomorph.

Table 5. Number of each Botryosphaeriaceae spp. and average air temperature, by county, in the main avocado production areas of California, September 2008 to March 2009

County	<i>Neofusicoccum australe</i>	<i>N. luteum</i>	<i>N. parvum</i>	Unknown <i>Neofusicoccum</i> sp.	<i>Fusicoccum aesculi</i>	<i>Dothiorella iberica</i>	Temperature (°C) ^a
San Luis Obispo	8	0	4	1	3	2	13.1
Santa Barbara	1	17	0	0	0	0	15.0
Ventura	13	9	1	2	8	1	16.4
Riverside	3	7	0	0	0	0	18.2
San Diego	1	0	1	0	0	0	15.8
Total	26	33	6	3	11	3	...

^a Average air temperature, March 2008 to March 2009.

accommodated in this clade (24). Therefore, he placed these two species in the resurrected genus *Dothiorella* as *D. sarmentorum* and *D. iberica* (24). In 2008, a multigene approach (SSU, LSU, ITS, β -tubulin, and EF1- α) was used to further resolve the taxonomy and phylogeny of the dark-spored *Botryosphaeria*-like teleomorphs and their associated anamorphs (25). The result of this study, in regards to *Dothiorella*, shows the *Dothiorella* clade to be more closely related to the *Neofusicoccum* clade than to the *Fusicoccum* clade. Phillips et al. (25) also proposed that the anamorph genus name *Dothiorella* be used for both the anamorph and teleomorph of this clade.

The *Neofusicoccum* sp. closely related to *N. nonquaesitum* cannot be definitively identified as such at this time based on the following considerations. This group was based on two DNA loci of only three total isolates obtained from San Luis Obispo and Ventura Counties from a single host species. In contrast, Inderbitzin et al. (16), in identifying the *N. nonquaesitum* species, used six DNA loci on 132 isolates from 36 plant hosts from five continents. They found that 12 species of *Fusicoccum* and *Neofusicoccum* have conidial dimensions similar to *N. nonquaesitum*. In addition, although our three isolates were within the same terminal clade as the *N. nonquaesitum* sequences from GenBank, our isolates grouped separately from them. Further analysis of these isolates using additional loci would clarify the identity of these isolates.

There was intraspecific genetic variation noted in every terminal clade, except for *D. iberica*. The intraspecific variation noted in the *N. parvum* clade is interesting in that three of our isolates formed a highly supported (99%) subclade within this clade. Similarly, in the *N. australe* clade, two relatively well-supported (71 and 82%) subclades were formed within this clade. Pavlic et al. (21), using genealogical concordance of phylogenetic species recognition with DNA sequence data from five nuclear loci to analyze the *N. parvum*/*N. ribis* complex on a single host, discovered that it consisted of three cryptic species occurring sympatrically, in addition to *N. parvum*. In the current study, the possibility of cryptic species within the *N. parvum* and *N. australe* clades cannot be ruled out. Further genetic analysis with additional DNA loci on a more extensive sampling of the population may help to resolve this intraspecific variation.

Climatic factors do not provide an explanation for differences in the frequencies or location of isolation of the six Botryosphaeriaceae spp. found during this study. *N. australe*, *N. luteum*, and *N. parvum* were found in both the northern and southern counties, with average air temperatures ranging from a low of 13.1°C in San Luis Obispo County to a high of 18.2°C in Riverside County. *F. aesculi*, *D. iberica*, and the unknown *Neofusicoccum* sp. were all isolated only from the northern counties of San Luis Obispo and Ventura. In a study of grapevine canker in California, which encompassed a large number of northern California counties as well as some southern California counties, *N. australe* (*B. australis*) and *N. parvum* (*B. parva*) were detected as far north as Sonoma County, *F. aesculi* (*B. dothidea*) as far north as Mendocino County, and *N. luteum* (*B. lutea*) only in Riverside County (36). Based on the field information from the grapevine canker study and the current study, as well as in vitro studies which showed that Botryosphaeriaceae spp. can tolerate a range of temperatures (36), it seems unlikely that climatic conditions are an important factor in the

distribution of species in California. However, no definite conclusions can be made regarding species distribution on California avocado based on environmental factors without more extensive sampling accompanied by detailed environmental data.

As high-density planting becomes more frequent in California avocado groves, management strategies must be developed to protect pruning wounds from invasion by canker-causing pathogens. Currently, there are no effective fungicides used by growers to prevent infection of pruning wounds on avocado trees. Preliminary in vitro studies (V. McDonald, unpublished) have shown there are chemicals on the market that are effective against Botryosphaeriaceae and *Phomopsis* spp. Studies designed to evaluate the most effective fungicides to protect pruning wounds in the field are now underway.

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