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Identification and pathogenicity of Botryosphaeriaceae species associated with coast live oak (*Quercus agrifolia*) decline in southern California

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Abstract: Symptoms of decline have been observed on dying coast live oak (*Quercus agrifolia*) trees in areas throughout southern California that are both infested and uninfested by the gold-spotted oak borer (GSOB). The purpose of this study was to identify and assess the pathogenicity of several anamorph species of the Botryosphaeriaceae, including *Diplodia corticola*, *Dothiorella iberica* and *Diplodia agrifolia* sp. nov., that were recovered consistently from symptomatic tissues. Species were identified morphologically and by phylogenetic analyses of the complete sequence of the internal transcribed spacer (ITS) of the rDNA and partial sequences of β-tubulin and elongation factor (*EF1-α*) genes. Results from morphological assessments and phylogenetic analyses support the erection of a new species closely related to *D. mutila*, described herein as *Diplodia agrifolia* sp. nov. Pathogenicity of all species was verified by wound inoculation of 1 y old coast live oak seedlings under controlled conditions. Isolates of *D. corticola* were the most aggressive tested, and isolates of *D. agrifolia* were the second most aggressive. Both species caused bleeding symptoms on inoculated seedlings. Seedlings inoculated with *D. corticola* died within 4 wk, with the pathogen progressing up and down through the xylem in advance of living phloem and moving throughout the taproot in 70% of inoculated seedlings. Colonization and re-isolation was successful for all species. All three fungal species represent newly recorded fungal pathogens of coast live oak in California. Results from the pathogenicity test suggest that these fungi play a role in the decline of coast live oaks throughout southern California.

Key words: Bot canker, *Botryosphaeria*, coast live oak, *Diplodia*, *Dothiorella*, gold-spotted oak borer

Introduction

Coast live oak (*Quercus agrifolia*) is a common evergreen tree that dominates oak woodlands throughout the central coast of California and provides critical habitat to wildlife and value to Native American communities throughout its range (Callaway and Davis 1998, Garcia et al. 1991, Rogers-Martinez 1992). It is a key species used for rehabilitation projects for watersheds and wildlife habitat (Goldner 1984, Howald and D'Antonio 1990, Perala et al. 1991) and a highly valued ornamental tree for urban landscapes in California (Burger et al. 1997).

Since 2002, mortality of coast live oak in San Diego County, California, had been estimated by an aerial survey at 17 000 trees (Coleman and Seybold 2008a, b, 2010, 2011; Coleman et al. 2011) and even larger estimates of mortality are being provided by sequential aerial image analysis (Tom Scott unpubl). In 2008, a cause was identified: infestations of a formerly unrecognized insect pest, the gold-spotted oak borer (GSOB) *Agrilus auroguttatus* Schaeffer (Coleoptera: Buprestidae)—a flat-headed borer presumed to have been introduced from either Arizona or Mexico on infested firewood (Coleman and Seybold 2008a, b, 2010, 2011; Hespenheide and Bellamy 2009; Coleman et al. 2011). With 67% of coast live oak trees in GSOB-infested areas showing evidence of attack (Coleman and Seybold 2008a), the role this pest plays in the oak mortality is unequivocal. In addition, oak mortality was observed at the same time as the GSOB discovery in areas as far as 20 miles north of the insect infestation. These separate areas of mortality distant from GSOB activity and the appearance of symptoms of crown thinning with branch cankering, bark cracking and/or peeling, staining or bleeding on the bole often associated with bark cankers on dying trees in both GSOB-infested and uninfested areas prompted a survey by the authors to assess the occurrence of potential pathogens throughout these representative regions.

Isolates from this 2009–2011 survey were tentatively identified to species shortly after recovery by 98–100%

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TABLE I. Distribution and frequency of fungal species recovered from field survey

GSOB presence[b]	Site/fungal species[a]	*D. corticola*	*D. agrifolia*	*Do. iberica*
+	Marian Bear Regional Park	4	0	0
+	Pine Creek, Cleveland National Forest	4	2	4
+	William Heisi County Park	0	1	5
+	Samataguma private property	0	0	5
−	Wilderness Gardens Preserve	5	0	4
−	Santa Rosa Plateau	5	1	5
−	San Timoteo Canyon	4	1	5
Margin	Santa Ysabel and Volcan Mountain Preserves	2	0	5
Margin	Mataguay Scout Camp	0	1	4

[a] Number of plots with recovered fungal species out of five plots per site.

[b] GSOB presence: site infested by GSOB (+); site non-infested by GSOB (−); site infested by GSOB but on the advancing margin of the infestation (Margin).

homology with available sequences in GenBank. Several anamorphs of Botryosphaeriaceae species commonly referred to as "bot canker" fungi were consistently recovered from bleeding trunk and branch cankers more frequently than other fungal species. Species of Botryosphaeriaceae commonly cause cankers, dieback, fruit rots and other symptoms and are important plant pathogens of many hosts (Sinclair et al. 1993). The most frequently recovered species from coast live oak, referred to herein by their common anamorphs, were *Diplodia corticola* (teleomorph *Botryosphaeria corticola*) Phillips, Alves et Luque, *Dothiorella iberica* (teleomorph *B. iberica*) Phillips, Luque et Alves, and to a lesser degree *Diplodia mutila* (teleomorph *B. stevensii*) Fries in Montagne (TABLE I, Lynch et al. 2010, Shannon Lynch, unpubl).

Little information has been available on the pathogenicity of the aforementioned species on coast live oak. *D. corticola* was recovered from coast live oaks that were inoculated with *Phytophthora ramorum* and colonized by ambrosia beetles in Marin County; *Do. sarmentorum*, which is closely related to *Do. iberica*, was recovered from *P. ramorum*-inoculated trees not attacked by beetles (Erbilgin et al. 2008). Apart from recent confirmation of *D. corticola* as a pathogen on mature coast live oak trees in the field (Lynch et al. 2010), these species have been described as decay fungi and endophytes, without testing to confirm pathogenicity (Alves et al. 2004, Phillips et al. 2005, Erbilgin et al. 2008). All three Botryosphaeriaceae species are pathogens to varying degrees and *D. corticola* and *D. mutila* are known factors contributing to the decline of oak species in Europe (Luque et al. 1989, 2008; Sánchez et al. 2003; Alves et al. 2004). However, the status of these and other remaining fungi as pathogens on coast live oak in North America remains unknown.

The objectives of this study were to (i) use morphological and molecular data to identify species of Botryosphaeriaceae associated with canker symptoms on coast live oak and (ii) determine pathogenicity of Botryosphaeriaceae recovered from coast live oak at both GSOB-infested and uninfested sites in southern California. Through the course of this study, it became apparent that a third objective was necessary: to resolve the taxonomic status of Botryosphaeriaceae being recovered. Phylogenetic study of the species and isolates obtained in this study has enabled us to erect a new *Diplodia* species described herein.

MATERIALS AND METHODS

Field survey and fungal isolation.—Isolates were collected from diseased symptomatic coast live oak wood in GSOB-infested and uninfested locations of San Diego and Riverside counties, California (TABLES I, II). Between Dec 2009 and May 2010, 45 permanent fixed-radius plots were established at nine sites (TABLE I, five plots per location). Plots were distributed at least 200 m apart over a random compass bearing from the initial location and recorded with global positioning system (GPS) equipment (Garmin® 60CSx, 5–10 m accuracy).

Phloem with bleeding or staining symptoms or insect exit holes was extracted from the trunk with a sterile plug cutter (2.54 cm diam) with a portable electric drill. Holes were filled with wooden dowel pieces (2.54 cm diam) and covered with wound sealant. Branch and twig samples from cankered, symptomatic crowns were collected with sterile pruning shears or saws. Samples were returned to the lab for fungal isolations and identification. In 2011, all plots were revisited and sampled.

The outer surface of crown samples was washed with sterile de-ionized water, briefly flamed then removed with a paring knife. All samples were cut in half and pieces excised from the leading margin of clean necrotic tissue were plated onto potato dextrose Agar (Difco™) amended with 100 µg

TABLE II. Fungal isolates used in the phylogenetic study

Isolate	Identity	Host	Origin	Collector	GenBank accession no.		
					ITS[a]	β-tubulin	EF1-α[b]
UCROK36	*Dothiorella iberica*	*Quercus agrifolia*	San Diego Co. CA USA	S.C.L. & A.E.	JQ411410	JQ411441	JQ512119
UCROK123	*Do. iberica*	*Q. agrifolia*	San Diego Co. CA USA	S.C.L. & A.E.	JQ411415	JQ411446	JQ512124
UCROK741	*Do. iberica*	*Q. agrifolia*	San Diego Co. CA USA	S.C.L. & A.E.	JQ411417	JQ411448	JQ512126
UCROK874	*Do. iberica*	*Q. agrifolia*	San Diego Co. CA USA	S.C.L. & A.E.	JQ411418	JQ411449	JQ512127
UCROK926[c]	*Do. iberica*	*Q. agrifolia*	San Diego Co. CA USA	S.C.L. & A.E.	JN693503	JQ411457	JQ512134
UCROK958	*Do. iberica*	*Q. agrifolia*	San Diego Co. CA USA	S.C.L. & A.E.	JQ411419	JQ411450	JQ512128
UCROK971[c]	*Do. iberica*	*Q. agrifolia*	San Diego Co. CA USA	S.C.L. & A.E.	JN693504	JQ411458	JQ512136
UCROK1079	*Do. iberica*	*Q. agrifolia*	San Diego Co. CA USA	S.C.L. & A.E.	JQ411414	JQ411445	JQ512123
UCROK1222	*Do. iberica*	*Q. agrifolia*	Riverside Co. CA USA	S.C.L. & A.E.	JQ411404	JQ411435	JQ512113
UCROK1227	*Do. iberica*	*Q. agrifolia*	Riverside Co. CA USA	S.C.L. & A.E.	JQ411405	JQ411436	JQ512114
UCROK1396	*Do. iberica*	*Q. agrifolia*	San Diego Co. CA USA	S.C.L. & A.E.	JQ411406	JQ411437	JQ512115
UCROK1403	*Do. iberica*	*Q. agrifolia*	San Diego Co. CA USA	S.C.L. & A.E.	JQ411407	JQ411438	JQ512116
UCROK1406	*Do. iberica*	*Q. agrifolia*	San Diego Co. CA USA	S.C.L. & A.E.	JQ411408	JQ411439	JQ512117
UCROK1433	*Do. iberica*	*Q. agrifolia*	San Diego Co. CA USA	S.C.L. & A.E.	JN693506	JQ411455	JQ512131
UCROK1472	*Do. iberica*	*Q. agrifolia*	San Diego Co. CA USA	S.C.L. & A.E.	JQ411409	JQ411440	JQ512118
UCROK364	*Diplodia agrifolia*	*Q. agrifolia*	San Diego Co. CA USA	S.C.L. & A.E.	JQ411413	JQ411444	JQ512122
UCROK1314	*D. agrifolia*	*Q. agrifolia*	Riverside Co. CA USA	S.C.L. & A.E.	JQ411411	JQ411442	JQ512120
UCROK1429	*D. agrifolia*	*Q. agrifolia*	San Diego Co. CA USA	S.C.L. & A.E.	JQ411412	JQ411443	JQ512121
UCROK732[c]*	*D. agrifolia*	*Q. agrifolia*	San Diego Co. CA USA	S.C.L. & A.E.	JN693507	JQ411459	JQ517317
UCROK1425[c]	*D. agrifolia*	*Q. agrifolia*	San Diego Co. CA USA	S.C.L. & A.E.	JN693508	JQ411460	JQ517318
UCROK488[c]	*Diplodia corticola*	*Q. agrifolia*	San Diego Co. CA USA	S.C.L. & A.E.	JN693501	JQ411453	JQ512132
UCROK946	*D. corticola*	*Q. agrifolia*	San Diego Co. CA USA	S.C.L. & A.E.	JQ418341	JQ411454	JQ512135
UCROK1192	*D. corticola*	*Q. agrifolia*	San Diego Co. CA USA	S.C.L. & A.E.	JQ411398	JQ411428	JQ512106
UCROK1233	*D. corticola*	*Q. agrifolia*	Riverside Co. CA USA	S.C.L. & A.E.	JQ411399	JQ411429	JQ512107
UCROK1246[c]	*D. corticola*	*Q. agrifolia*	Riverside Co. CA USA	S.C.L. & A.E.	JN693502	JQ411430	JQ512108
UCROK1254	*D. corticola*	*Q. agrifolia*	Riverside Co. CA USA	S.C.L. & A.E.	JQ411400	JQ411431	JQ512109
UCROK1339	*D. corticola*	*Q. agrifolia*	San Diego Co. CA USA	S.C.L. & A.E.	JQ411401	JQ411432	JQ512110
UCROK1407	*D. corticola*	*Q. agrifolia*	San Diego Co. CA USA	S.C.L. & A.E.	JQ411402	JQ411433	JQ512111
UCROK1482	*D. corticola*	*Q. agrifolia*	San Diego Co. CA USA	S.C.L. & A.E.	JQ411403	JQ411434	JQ512112
CBS 115041*	*Botryosphaeria iberica*	*Quercus ilex*	Spain	J. Luque	EU673155	EU673096	AY573222
UCD1439SLO	*B. iberica*	*Vitis vinifera*	CA, USA	J.R. Úrbez-Torres	EF202008	EF202015	EF202022
UCD1448SLO	*B. iberica*	*Vitis vinifera*	CA, USA	J.R. Úrbez-Torres	EF202009	EF202016	EF202023
PD257	*B. iberica*	*Quercus ilex*	Spain		GU251168	GU251828	GU251300
CBS 112553**	*Botryosphaeria stevensii*	*Vitis vinifera*	Portugal	A.J.L. Phillips	AY259093		AY573219
UCD1953SB	*B. stevensii*	*Vitis vinifera*	CA, USA	J.R. Úrbez-Torres	DQ233598	DQ233619	EU012412
Table II. Cont.							
UCD288Ma	*B. stevensii*	*Vitis vinifera*	Madera Co. CA USA	J.R. Úrbez-Torres	DQ008313	DQ008336	EU012411
CBS 112546	*D. corticola*	*Quercus ilex*	Spain		AY259090	EU673310	EU673310
CBS 112549*	*D. corticola*	*Quercus suber*	Portugal		AY259100	AY573227	AY573227
CBS112072	*D. corticola*	*Quercus ilex*	Spain	J. Luque	AY259108		
CBS112073	*D. corticola*	*Quercus suber*	Spain	J. Luque	AY268420		
CBS112074	*D. corticola*	*Quercus suber*	Italy	J. Luque	AY268421		
CBS112076	*D. corticola*	*Quercus suber*	Spain	J. Luque	AY259109		
CDFA519	*D. corticola*	*Quercus chrysolepis L.*	CA, USA	J.R. Úrbez-Torres	GU799472	GU799466	GU799469

TABLE II. Continued

Isolate	Identity	Host	Origin	Collector	GenBank accession no. ITS[a]	β-tubulin	EF1-α[b]
UCD1260So	*D. corticola*	*Quercus chrysolepis L.*	CA, USA	J.R. Úrbez-Torres	GU799470	GU799464	GU799467
UCD2398TX	*D. corticola*	*Vitis vinifera*	TX, USA	J.R. Úrbez-Torres	FJ790843		
PD46	*D. mutila*	"Christmas tree"	USA		GU251116	GU251776	GU251248
PD61	*D. mutila*	*Persea americana*	CA, USA	TJM, PI	GU251117	GU251777	GU251249
PD73	*D. mutila*	*Ilex sp.*	CA, USA	TJM, PI	GU251118	GU251778	GU251250
PD75	*D. mutila*	*Ilex sp.*	CA, USA	TJM, PI	GU251119	GU251779	GU251251
CBS 115041	*Do. iberica*	*Quercus ilex*	Spain		AY573202	AY573222	AY573222
CBS113188	*Do. iberica*	*Quercus suber*	Spain		AY573198	EU673278	EU673278
CBS 115038	*Dothiorella sarmentorum*	*Malus pumila*	the Netherlands		AY573206	AY573223	AY573223
IMI 63581b*	*Do. sarmentorum*	*Ulmus sp.*	United Kingdom		AY573212	AY573235	AY573235
PD280	*Do. sarmentorum*	*Ulmus sp.*	Great Britan	E.A. Ellis	GU251171	GU251831	GU251303
PD78	*Do. sarmentorum*	*Prunus dulcis*	CA, USA	TJM, PI	GU251169	GU251829	GU251301
PD79	*Do. sarmentorum*	*Prunus dulcis*	CA, USA	TJM, PI	GU251170	GU251830	GU251302
CMW28333	*Lasiodiplodia parva*	*Terminalia superba*	Cameroon, Africa		GQ469961	GQ469892	GQ469903
CMW28309	*L. parva*	*Terminalia ivorensis*	Cameroon, Africa		GQ469962	GQ469894	GQ469904
CMW28292	*L. parva*	*Terminalia ivorensis*	Cameroon, Africa		GQ469963	GQ469893	GQ469905
CBS447.68	*Guignardia philoprina*	*Taxus baccata*	the Netherlands		FJ824768	FJ824779	FJ824773

[a] Internal transcribed spacer.
[b] Elongation factor.
[c] Isolates used for pathogenicity study.
* Holotype.
* Lectotype.

tetracycline (Fisher Scientific®; PDA-tet). Plates were incubated at 25 C. After 7–10 d, fungal growth was subcultured for identification and long-term storage.

DNA extraction, polymerase chain reaction amplification and phylogenetic analysis.—Total genomic DNA was extracted from the mycelium of pure culture from each isolate (TABLE II) with a modification of the method described by Cenis (1992). Oligonucleotide primers ITS4 and ITS5 were used to amplify the ITS1, ITS2 and 5.8 regions of rDNA (White et al. 1990). Oligonucleotide primers Bt2a and Bt2b were used to amplify a portion of the β-tubulin (*tubB*) gene (Glass and Donaldson 1995). Oligonucleotide primers EF1F and EF1R were used to amplify a portion of the elongation factor (*EF1-α*) gene (Jacobs et al. 2004). Each 30 μL polymerase chain reaction (PCR) contained 20.25 μL PCR-grade water, 3 μL ThermoPol® Reaction buffer, 0.6 μL dNTPs, 2.25 μL each primer, 0.15 μL NEB *Taq* DNA polymerase (Taq PCR core kit; QIAGEN, Valencia, California) and 1.5 μL template DNA. PCR was carried out in a thermal cycler (Bio-Rad Laboratories, Inc., Hercules, California) with the temperature profiles for ITS and BT from McDonald and Eskalen (2011). Temperature profiles for *EF1-α* were modified from those of Slippers et al. (2004). PCR amplification products were separated by electrophoresis in 1.5% agarose gels in 1.0 × Tris-boric acid-EDTA (TBE) buffer and photographed under UV light after staining with SYBR® Green (Invitrogen, Eugene, Oregon). PCR products were purified with ExoSAP-IT® kit (Affymetrix, Santa Clara, California). ITS, *btub* and *EF1-α* regions were sequenced in both directions at the Institute for Integrative Genome Biology facility of the University of California, Riverside. Sequences were edited and assembled with Sequencher 4.6 (Gene Codes Corp., Ann Arbor, Michigan), locally aligned with Clustal X 2.1-Mac OSX (Conway Institute, UCD, Dublin, Ireland) (Thompson et al. 1997) and manually aligned with MacClade 4.08 OSX (Sinauer Associates, Sunderland, Massachusetts) (Maddison and Maddison 2001).

Phylogenetic analysis of Botryosphaeriaceae species was conducted with PAUP 4.0b10 (Swofford 2002). Alignment gaps were treated as missing data. Sequences for each species recovered in this study in addition to those available in GenBank were used (TABLE II), with *Guignardia philoprina* (CBS447.68) as an outgroup. Separate and combined analyses were run for the ITS, *tubB* and *EF1-α* sequences after a partition homogeneity test for Botryosphaeriaceae species determined that ITS, *tubB* and *EF1-α* data could be combined. Maximum parsimony analyses were performed in PAUP with the heuristic search option (NNI branch swapping). Bootstrap values were calculated with 1000 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. In preliminary analyses, additional sequences of other Botryosphaeriaceae species for phylogenetic comparison from a thorough BLAST query in GenBank had been included, but these additional sequences were not included in final reported trees because inclusion did not affect tree topology. The complete alignment of the ITS1/5.8S/ITS2 region, *tubB* and *EF1-α* genes are available in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S12370). ITS, *tubB* and *EF1-α* sequences were deposited in GenBank.

Morphological characterization.—Pure cultures were obtained by transferring fungal tips to PDA-tet. Isolates were characterized based on colony and conidial morphology and compared to those reported in studies (Phillips et al. 2002, 2005; Alves et al. 2004; Taylor et al. 2005; Úrbez-Torres et al. 2006; McDonald and Eskalen 2011). Fungal tips were transferred to PDA-tet to ensure homogeneity of cultures. Conidial morphology was determined after 3 wk incubation on 3% oatmeal agar (OMA) at 25 C under intermittent florescent light (12 h) to induce sporulation (Gooding and Lucas 1959, Phillips et al. 2005, Amponsah et al. 2008). For each isolate, the lengths and widths of 50 conidia from pycnidia were measured with a compound microscope (Olympus BX40 with a Leica DFC420 camera) and SPOT imaging software 4.7.0.35 (Diagnostic Instruments Inc., Michigan). Summary statistics (minimum, mode, maximum, mean, standard deviation) for lengths and widths of conidia were calculated in SAS 9.3 (SAS Institute, Cary, North Carolina). Conidial shape, color and presence or absence of septa also were recorded.

Pathogenicity tests.—Seedling propagation. *Q. agrifolia* acorns were collected from a single seed source in San Diego County and soaked on moist paper towels in a moist chamber 1 wk or until sprouting occurred. Upon sprouting, acorns were transferred to 1.9 L pots filled with a standard University of California Riverside soil mix (PP-UC) composed of 1:1 peat to sand. Plants were grown in a greenhouse 1 y before fungal inoculations, fertilized with Peters Excel Water Soluble Fertilizer® (21-5-20 multipurpose) at a concentration of 1:100 fertilizer to water through the irrigation system that was administered twice daily for 1 min at 9 am and 4 p.m. Upon inoculation, plants averaged roughly 135 cm tall and 0.5 cm diam.

Fungal inoculations. For each of the four bot canker fungal species tested and for controls, 10 seedlings each were wound-inoculated in a randomized design. Two unique isolates per species were used on each replicate seedling and grown onto PDA-tet 1 wk before inoculations (TABLE II). Tissue from two randomly selected stem internodes for each isolate was excised to the cambium with a 4 mm sterile cork borer. An agar plug was cut at the leading edge of fresh mycelium from each culture and placed mycelium-side down onto the wounded tissue and wrapped with parafilm. Stems of control seedlings received sterile agar plugs.

Inoculations were conducted Jan 2011. Plants were checked weekly and five of the 10 seedlings per treatment were destructively sampled after 3 and 6 mo and examined for canker and pycnidia formation, the extent of vascular discoloration and recovery of fungal isolates.

Statistical analyses.—Statistical analyses were performed with SAS 9.3 (SAS Institute, Cary, North Carolina). Conidial length between *D. corticola* isolates of the present study were compared to the mean from Alves et al. (2004) with a distribution *t*-test. Conidial lengths of *Dothiorella* and *Diplodia* isolates of the present and other studies were compared by analysis of variance (ANOVA). Treatment means were compared with Tukey-Kramer HSD *t*-test. Difference in length of vascular discoloration caused by each fungal pathogen was determined by ANOVA. Log_{10} transformation was chosen to correct heteroscedasticity of variance indicated by visual inspection of residuals. Treatment means were compared with Dunnett's *t*-test to assess significant differences between the control group mean and the remaining treatment group means.

RESULTS

Phylogenetic analysis.—Loci first were analyzed separately with maximum parsimony (trees not shown). Topologies were consistent among all three MP trees. A partition homogeneity test indicated that ITS, *tubB* and *EF1-α* data could be combined ($P = 0.01$). Of the 1331 nucleotides analyzed, 255 were parsimony informative. Maximum parsimony analysis yielded one most parsimonious tree of the combined data (FIG. 1; length = 602, CI = 0.882, RI = 0.980, RC = 0.864, HI = 0.118). Two major clades were resolved, the first of which (100% bootstrap support) corresponded to *Dothiorella* anamorphs and was resolved further into *Do. iberica* (78% bootstrap support) and *Do. sarmentorum* (83% bootstrap support, FIG. 1). The second major clade (100% bootstrap support) resolved into two subclades. The first subclade (95% bootstrap support) corresponded to *Diplodia* anamorphs and this resolved further into *D. corticola* (100% bootstrap support) and another group (100% bootstrap support) that included an undescribed *Diplodia* species (*Diplodia* sp.) (100% bootstrap support) and *D. mutila* (86% bootstrap support).

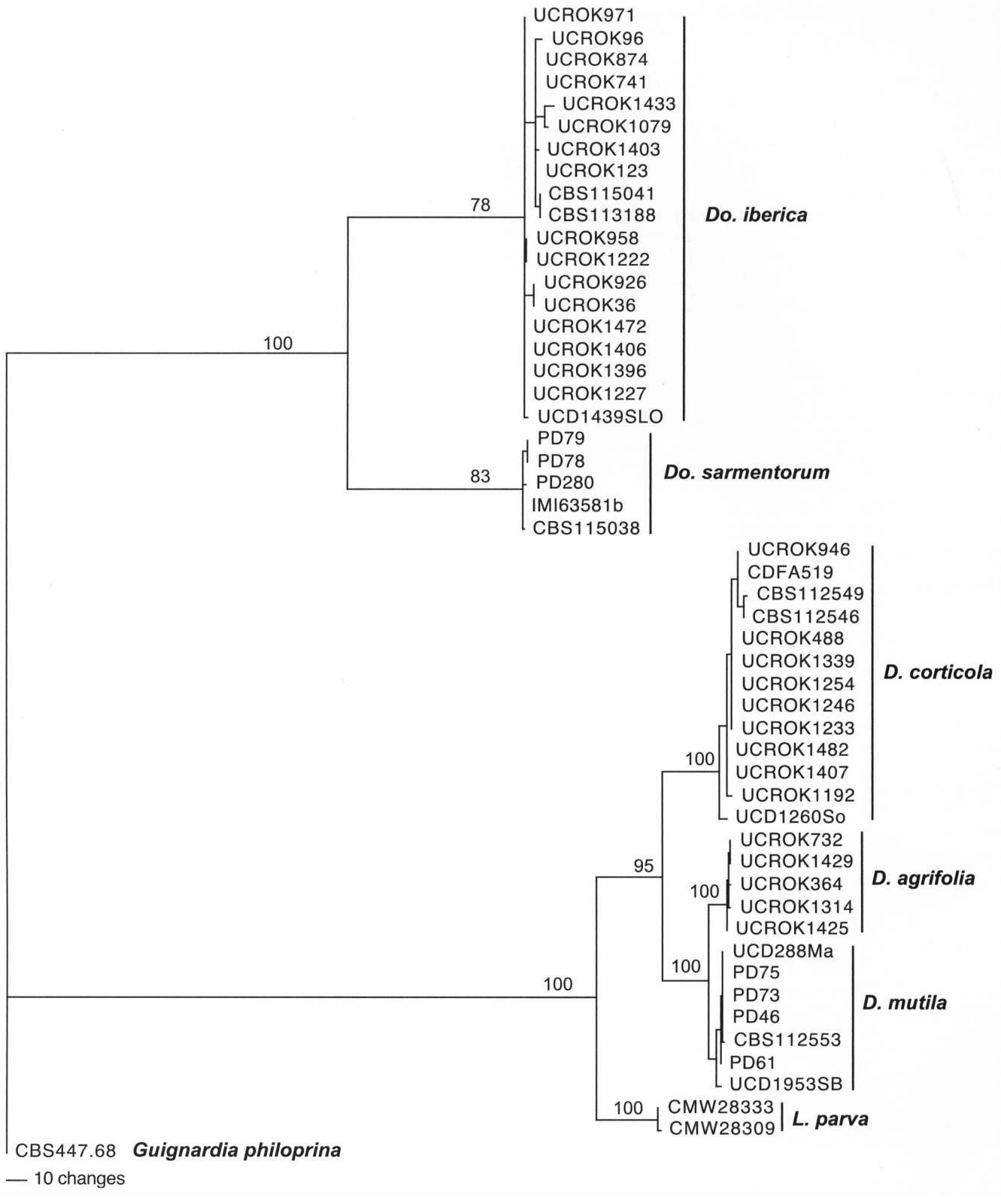

FIG. 1. Maximum parsimony tree with bootstrap values obtained from the combined ITS, β-tubulin and *EF1*-α using 1000 replicates generated in PAUP. The anamorph of each Botryosphaeriaceae member is shown, except for outgroup, which is named by its teleomorph.

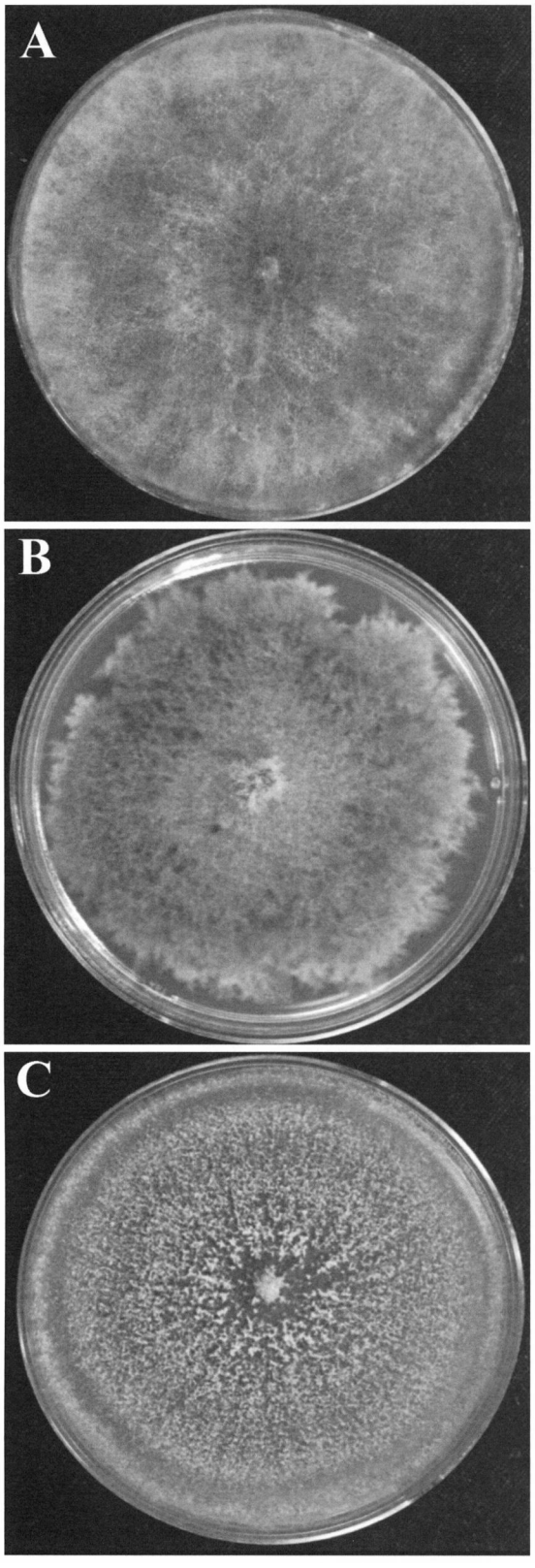

FIG. 2. Upper surface of 2 wk old cultures on PDA-tet of *D. corticola* (A), *D. agrifolia* (B) and *Do. iberica* (C).

The second subclade corresponded to *L. parva* with 100% bootstrap support.

Morphological characterization.—Of the 31 Botryosphaeriaceae examined from 207 trees, isolates separated into two distinct groups distinguishable by colony color and growth characteristics. One group contained *Diplodia* species. Culture morphology in this genus was characterized by abundant, fast growing aerial mycelium that turned dark olivaceous after 1 wk at 25 C (FIG. 2A, B). The reverse side was almost black in older cultures. The second group contained *Dothiorella* species. These had thin-white or gray or brown hyphae that were slightly fluffy to flat (FIG. 2C). The upper surface showed thin to moderately dense, appressed hyphae, with buff edges, while the reverse of the plate showed dark green-black hyphae with buff outer edges.

Average conidial length was slightly greater for *D. corticola* than for isolates of the undescribed *Diplodia* sp. ($P = 0.0001$ at $\alpha = 0.05$) isolates (TABLE III). *D. corticola* conidia were mostly hyaline, smooth and thick-walled, oblong to cylindrical, both ends broadly rounded, rarely becoming brown and septate with age (Alves et al. 2004), and the length and width were consistent with other studies (TABLE III). In contrast to conidia measured and characterized in other studies, conidia of the new *Diplodia sp.* isolates in this study were dark brown and septate with age in equal or greater proportions to those that were hyaline and aseptate and on average were longer than in previous reports ($P = 0.003$ at $\alpha = 0.05$) (FIG. 3, TABLE III) (Alves et al. 2004, Úrbez-Torres et al. 2009). Conidia of *Do. iberica* were pigmented and thick walled, but some isolates in culture produced conidia that were hyaline and aseptate when discharged from pycnidia; those harvested from inoculated seedlings all were pigmented and septate before discharge. There were significant differences in conidial length (at $\alpha = 0.05$) among *Dothiorella* isolates. However, the mean lengths of conidia were not significantly different between *Do. iberica* isolates of the present study and those from other studies ($P = 0.7$–1.0) (TABLE III). The conidial measurements obtained for these Botryosphaeriaceae species from California are provided (TABLE III) together with reported measurements (Phillips 2002, Alves et al. 2004, Phillips et al. 2005, Taylor et al. 2005, Úrbez-Torres et al. 2006, McDonald and Eskalen 2011).

Pathogenicity test.—All isolates colonized inoculated stems of 1 y old *Q. agrifolia* seedlings by 3 and 6 mo (FIG. 4). Fungi obtained from re-isolation were identified as the same species as those used in the inoculations based on colony morphology and sequence data. *D. corticola* was recovered from 100%

TABLE III. Conidial measurements of Botryosphaeriaceae fungi harvested OMA

Present study			Other studies		
Species, isolate	Conidial size (μm)[a]	Mean ± SD (μm)[b]	Conidial size (μm)[a]	Mean ± SD (μm)[b]	Source of data
D. corticola					
UCROK271	(24.0–)28.5–36.0 × (10.9–)15.3–17.8	28.3 ± 2.1 × 14.2 ± 1.3	(23.7–)29.6–30.3(–46.1) × (9.1–)13.4–13.8(–20.5)[c]	29.9 ± 2.6 × 13.6 ± 1.4	Alves et al. 2004
UCROK488*	(25.9–)29.8–36.9 × (11.7–)13.0–16.6	30.1 ± 2.6 × 13.9 ± 1.2			
UCROK946	(24.2–)28.6–41.0 × (12.4–)14.9–17.7	28.3 ± 2.9 × 14.8 ± 1.0			
UCROK1233	(22.8–)27.7–39.4 × (10.0–)13.9–17.4	28.8 ± 3.3 × 13.2 ± 1.5			
UCROK1246*	(25.8–)28.7–42.1 × (11.4–)14.6–18.3	31.7 ± 4.0 × 14.1 ± 1.7			
UCROK1254	(25.5–)29.8–40.1 × (12.0–)14.7–19.9	30.5 ± 3.2 × 14.9 ± 1.8			
UCROK1339	(22.9–)27.6–32.7 × (11.8–)15.7–21.9	27.2 ± 2.1 × 16.0 ± 1.9			
D. agrifolia			*D. mutila*		
UCROK364	(25.1–)29.7–33.1 × (12.1–)14.5–16.2	28.7 ± 1.9 × 13.9 ± 1.0	(23.5–)25.1–25.7 × (12.4–)13.2–13.5(–14.3)[d]	25.4 ± 1.0 × 13.4 ± 0.5	Alves et al. 2004
UCROK732*[c]	(21.3–)26.8–31.8 × (12.8–)15.3–16.9	26.5 ± 1.9 × 14.9 ± 0.9	(21–)25–28 × (10.0–)13–14.6	24.7 ± 1.3 × 12.5 ± 1.0	Úrbez-Torres et al. 2006
UCROK1314	(24.6–)28.7–32.1 × (12.4–)13.9–16.3	28.4 ± 1.8 × 14.2 ± 1.0	(20.0–)24.7–28 × (10.2–)12.2–14	24.3 ± 1.7 × 12.2 ± 1.0	Úrbez-Torres et al. 2006
UCROK1425*	(23.7–)30.6–36.5 × (12.6–)14.5–16.2	28.6 ± 2.5 × 14.6 ± 0.9	(21.0–)24.4–28 × (9.3–)12.1–14.6	24.3 ± 1.6 × 11.9 ± 1.1	Úrbez-Torres et al. 2006
UCROK1429	(21.7–)26.7–28.8 × (13.9–)15.6–17.8	26.2 ± 1.6 × 15.8 ± 0.9	(22–)24–26(–30) × 10–14		Taylor et al. 2005
			(21.1–)23–27(–28) × (10.0–)11.0–12.0(–13.0)		Phillips 2002
Do. iberica					
UCROK36	(19.5–)22.8–28 × (8.3–)10.3–13	22.8 ± 1.6 × 10.2 ± 0.9	(17.2–)23.0–23.4(–28..6) × (8.1–)10.8–11.0(–16.0)[c]	23.2 × 10.9	Phillips et al. 2005
UCROK750	(19.1–)22.9–28.8 × (8.8–)10.4–13.2	23.2 ± 2.0 × 10.2 ± 0.8	(17.0–)24.0–30.0 × (11.0–)14.0–19.0	23.8 ± 2.2 × 13.9 ± 1.5	McDonald and Eskalen 2011
UCROK926*	(19.4–)23.5–26.3 × (5.8–)8.6–10.6	23.2 ± 1.4 × 8.8 ± 1.0	(19.0–)24.0–26.0 × (9.0–)10.0–11.0	23.5 ± 1.3 × 9.9 ± 0.6	McDonald and Eskalen 2011
UCROK971*	(19.4–)22.6–25.2 × (7.2–)8.9–11.8	23.0 ± 1.3 × 9.4 ± 1.1	(22.0–)24.0–27.0 × (8.0–)10.0–12.0	24.2 ± 1.2 × 9.8 ± 0.7	McDonald and Eskalen 2011
UCROK1227	(19.1–)20.7–26.2 × (8.1–)10.2–12.9	22.4 ± 1.9 × 10.0 ± 1.5			

TABLE III. Continued

Present study		Mean ± SD	Other studies		
Species, isolate	Conidial size (μm)[a]	(μm)[b]	Conidial size (μm)[a]	Mean ± SD (μm)[b]	Source of data
UCROK1403	(17.6–)21–24.9 × (8.6–)10.1–11.9	21.8 ± 1.5 × 10.3 ± 0.7			
UCROK1472	(20.2–)24.7–27.9 × (7.3–)10.0–12.3	24.5 ± 1.6 × 10.3 ± 1.0			
UCROK1222	(18.8–)23.7–26.0 × (9.0–)10.2–12.1	23.0 ± 1.6 × 10.4 ± 0.7			
UCROK123	(20.0–)21.6–26.1 × (8.7–)11.4–13.5	23.0 ± 1.6 × 11.1 ± 0.9			
UCROK741	(19.7–)24.5–27.8 × (9.1–)9.7–12.4	23.5 ± 1.8 × 10.2 ± 0.8			
UCROK874	(20.0–)25.5–28.5 × (9.2–)10.1–11.3	24.4 ± 1.9 × 10.2 ± 0.5			
UCROK958	(19.6–)22.8–27.3 × (7.9–)9.2–10.8	23.2 ± 1.4 × 9.6 ± 0.5			
UCROK1079	(21.0–)24.3–28.0 × (8.8–)10.1–13.0	24.8 ± 1.4 × 10.5 ± 0.9			
UCROK1433	(22.8–)25.5–29.1 × (8.5–)10.4–12.0	24.8 ± 1.1 × 10.2 ± 0.7			

[a] Minimum size, mode and maximum size in length and width of 50 conidia from each isolate.
[b] SD = standard deviation.
[c] Holotype.
[d] Lectotype.
* Isolates used for pathogenicity test.

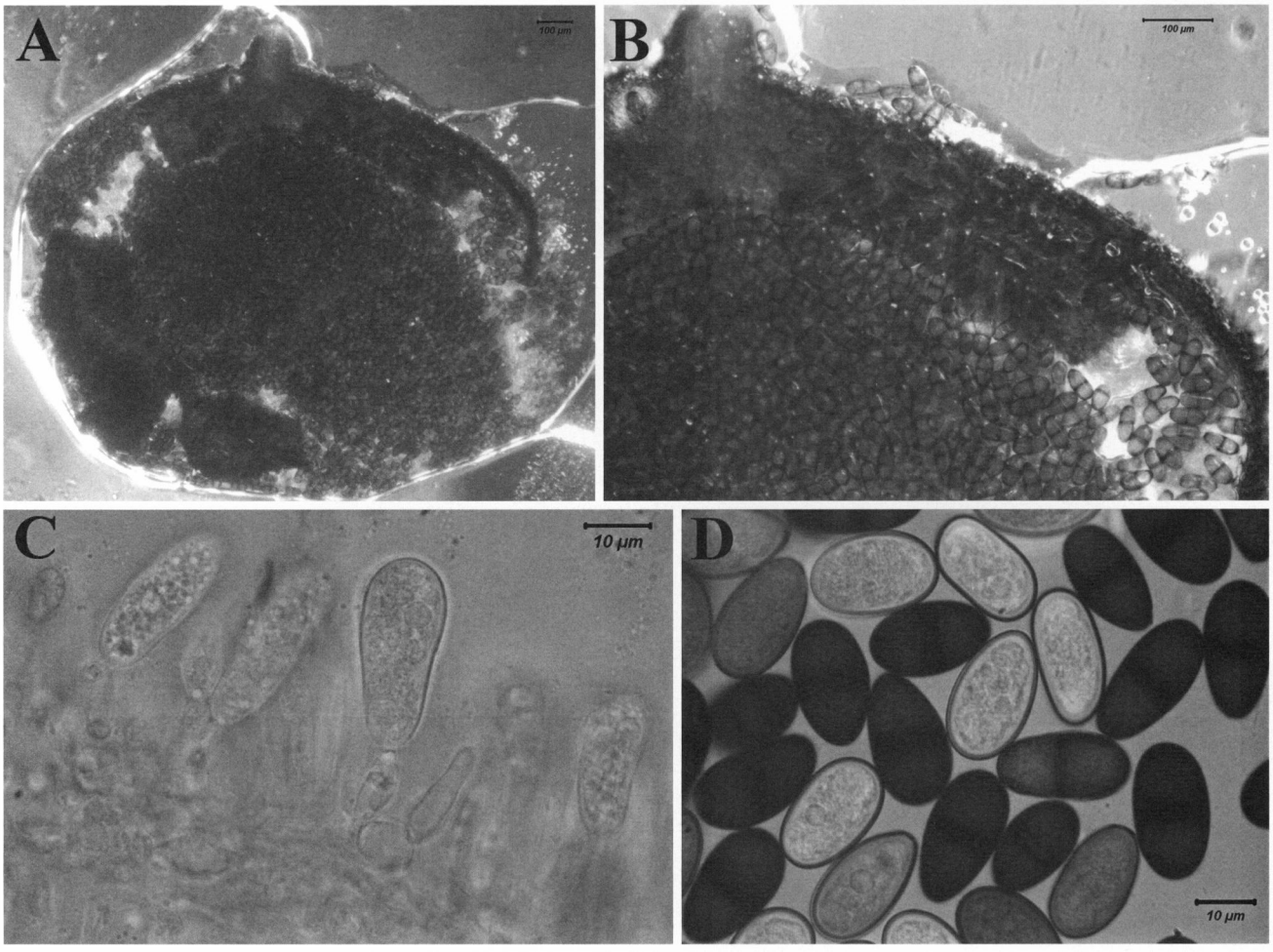

FIG. 3. Vertical section of a *Diplodia agrifolia* UCROK732 pycnidium (A) with a close-up view of dark, one-septate conidia (B). C. Conidiogenous cells in culture of UCROK 1314 with annelation marked by arrow and conidia (D) that are both hyaline and one-septate.

seedlings after 4 wk. At 3 mo, recovery frequency was lowest for *Do. iberica* (80%). Recovery frequency was 90% for *Diplodia sp.*

At 6 mo, recovery frequency was lowest for *Do. iberica* (60%) and highest for the new *Diplodia sp.* (80%). Recovery of *Do. iberica* was 33.3% lower at 6 mo than at 3 mo. The average distance of growth in wood was significantly different between fungal inoculation treatments and controls, while magnitude of colonization varied by fungal species ($P < 0.0001$ at $\alpha = 0.05$), which was 136.32 cm for *D. corticola* (the entire plant, including roots), 18.1 cm for the new *Diplodia* sp., and 2.2 cm for *Do. iberica.*

Symptomology.—After 3 wk, bleeding and pycnidia formation were observed on all stems inoculated with *D. corticola*; bleeding was observed at and as far down as 10 cm from the inoculation point, occurring at the nodes (FIG. 5). All seedlings exhibited leaf desiccation, and 50% of the trees displayed epicormic shoot formation. These seedlings were harvested ahead of other treatments at week four because by this time all but three plants had died. The three remaining plants had 3% of live tissue left just above the soil line. Lesions caused by *D. corticola* were observed throughout the plants and conidia embedded in the plant tissue were observed on dead seedlings. *D. corticola* killed the xylem in advance of the living phloem and moved into the taproot on 70% of inoculated seedlings and two of the 10 seedlings exhibited necrosis throughout the taproot (FIG. 6). Taproots on average were 9.0 cm long and the average vascular extent of *D. corticola* into the taproot was 6.0 cm.

Seedlings inoculated with other Botryosphaeriaceae species were alive at the end of 3 and 6 mo. Plants inoculated with the new *Diplodia* sp. exhibited bleeding and epicormic shoot formation. All Botryosphaeriaceae species produced pycnidia around the inoculation point on every seedling.

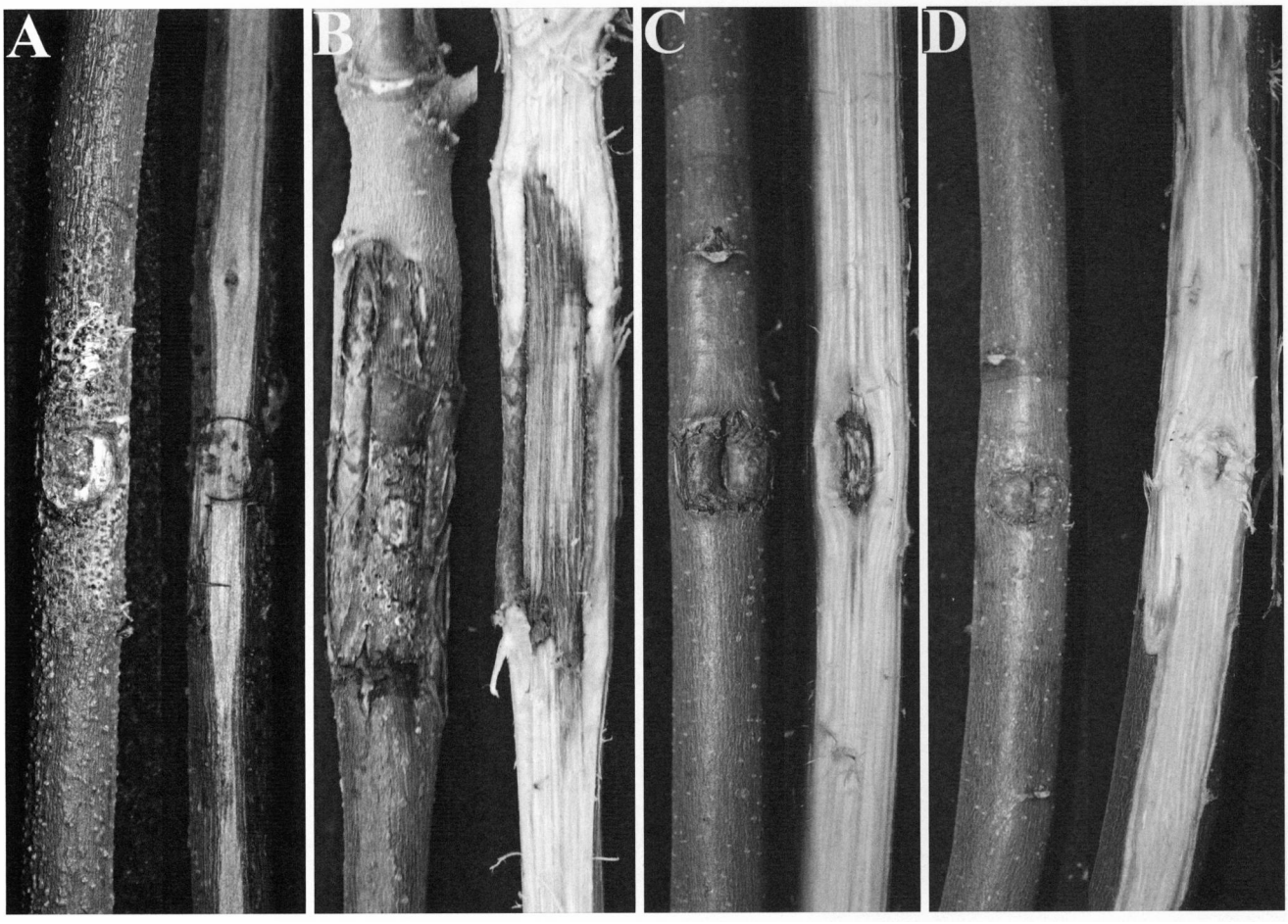

FIG. 4. Outer and vascular extent of necrotic tissue on coast live oak seedlings after 3 mo inoculated with *Diplodia corticola* (A), *D. agrifolia* (B), *Do. iberica* (C) and control (D).

TAXONOMY

Diplodia agrifolia S.C. Lynch, A. Eskalen, sp. nov.

FIGS. 2B, 3A–D

MycoBank MB800443.

No teleomorph observed. Pycnidia single or in groups, immersed to erumpent when mature, black and globose, 189 × 171–836 × 721 µm. Ostiole central, circular, apapillate to papillate. Pycnidial wall composed of three layers. Outer layer dark, cells thick-walled, middle layer with dark brown, thin-walled cells, and an inner layer of thin-walled hyaline cells. Conidiophores absent. Conidiogenous cells 18.0 ± 7.4 × 8.1 ± 2.4 µm, holoblastic, discrete, cylindrical, hyaline, smooth, indeterminate, proliferating at the same level giving rise to periclinal thickenings or proliferating percurrently to form one to two indistinct annelations. Conidia in equal proportions hyaline, aseptate and pale to dark brown and one-septate before and after discharge; smooth, thick-walled, oblong to ovoid; straight, both ends broadly rounded; (21.3–)27.0–36.5 × (12.1–)14.5–17.8 µm, mean and standard deviation = 27.7 ± 2.2 × 14.7 ± 1.2 µm; L:W = 1.90 ± 0.2 (n = 265).

Holotype: UCROK732, United States National Fungus Collection (Herbarium BPI).

Etymology: Derived from the specific epithet of the host from which it was recovered, coast live oak (*Quercus agrifolia*).

Hosts: Q. agrifolia and *Q. kelloggii.*

Host distribution: Coast range of north-central California southward to northern Baja California, with *Q. kelloggii* extending as far north as Eugene, Oregon.

Specimens examined: UNITED STATES. CALIFORNIA: William Heise County Park, San Diego County. Bole stain and canker of *Quercus agrifolia*, 3 Feb 2010, *S.C. Lynch & A. Eskalen, UCROK364, CBS132778* (culture). UNITED STATES. CALIFORNIA: Mataguay Scout Camp, San Diego County. Cankered branch of *Quercus agrifolia*, 23 Feb 2010, *S.C. Lynch & A. Eskalen, UCROK732, BPI 884095* (HOLOTYPE of *D. agrifolia*, dried culture) *CBS132777* (culture) *ATCC MYA-4895* (culture). UNITED STATES. CALIFORNIA: San Timoteo Canyon, Riverside County. Cankered branch of *Quercus agrifolia*, 23 Mar 2010, *S.C. Lynch & A. Eskalen, UCROK1314, CBS132779* (culture). UNITED STATES. CALIFORNIA: Cleveland National Forest, Pine Creek Trailhead, San Diego County, Cankered

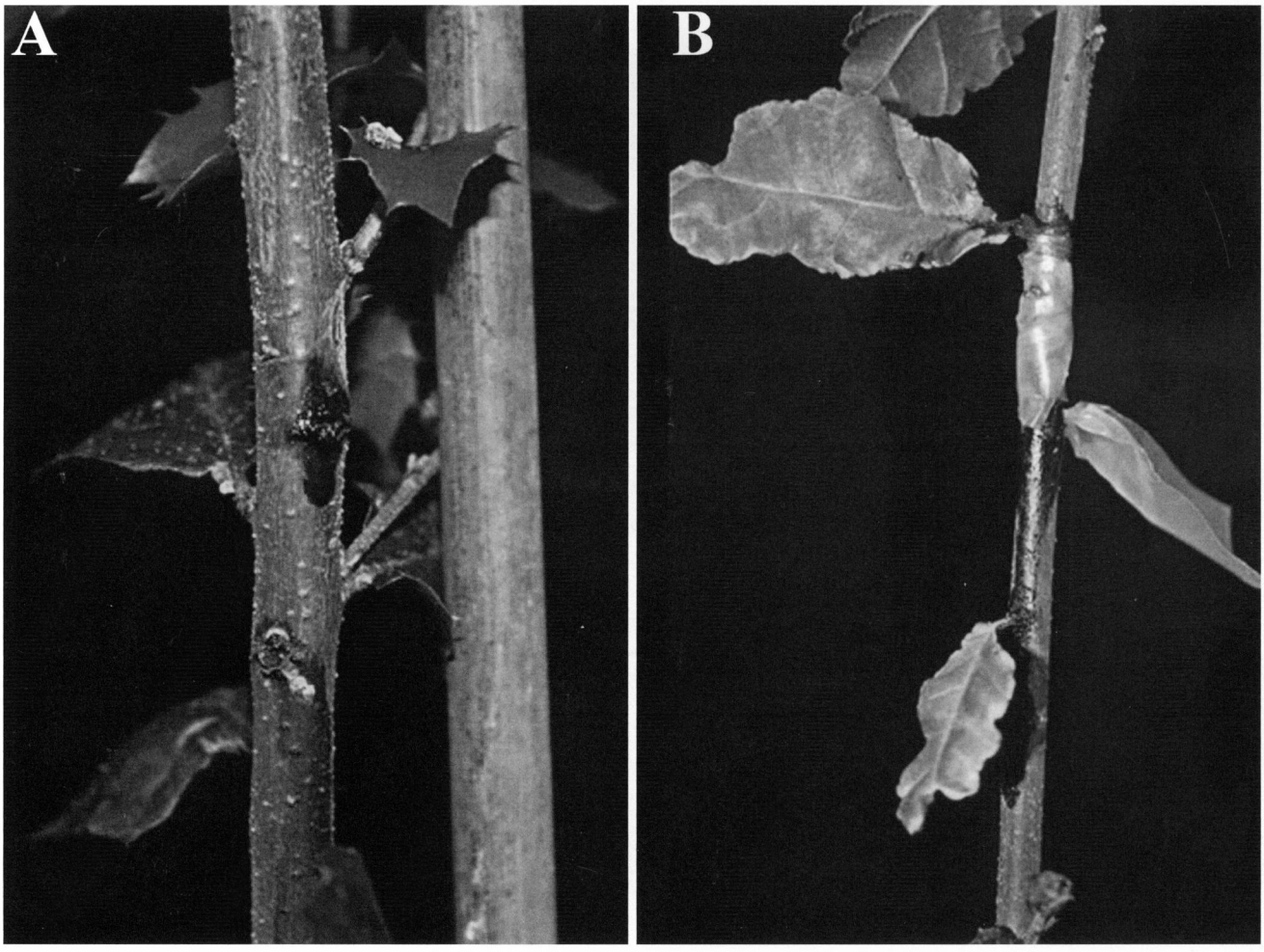

FIG. 5. Bleeding symptom 10 cm below (A) and at the inoculation point (B) on *Q. agrifolia* seedlings inoculated with *D. corticola* after 3 wk. This pattern also was observed on seedlings inoculated with *D. agrifolia*.

branch of *Quercus agrifolia*, 28 Apr 2010, *S.C. Lynch & A. Eskalen, UCROK1425 CBS132841*.

Comments: Phylogenetic analyses show that *D. agrifolia* originating from coast live oak in California is distinct but closely related to *D. mutila* and that these two species consistently group together in the ITS, *tubB*, *EF1-α* and combined phylogenies. *D. agrifolia* differs from *D. mutila* by possessing longer and wider conidia than *D. mutila*. Also, conidia of *D. agrifolia* are hyaline and aseptate, but most become dark brown and one-septate before discharge from pycnidia, whereas conidia of *D. mutila* are hyaline, aseptate, rarely becoming pale brown and one-septate with age.

DISCUSSION

The loss of tens of thousands of acres of oaks throughout California has raised concern over the fate of the important ecosystems they support (Pavlik et al. 1991, Rizzo and Garbelotto 2003, Coleman et al. 2011). The problem of oak die-off throughout the state is multifaceted and contingent on local factors. Pathogenicity tests in the present study and field studies (Lynch et al. 2010) have shown clear evidence that *D. corticola* and the newly discovered species *D. agrifolia* produce bleeding and staining symptoms, cankers, vascular discoloration and dieback on healthy seedlings and trees. This study provides evidence that *Do. iberica* and *D. agrifolia* associated with coast live oak cankers in southern California are pathogenic when inoculated into seedlings. Compared to more opportunistic members of this genus, *D. corticola* is significantly more aggressive and has been recovered at much higher frequencies in the field than *D. agrifolia* (Shannon Lynch unpubl). The ability for *D. corticola* to rapidly move through mature trees (3.7–32.4 cm DBH) and kill healthy seedlings within a short time therefore suggests that this pathogen plays a key role in the die-off of oaks in southern California.

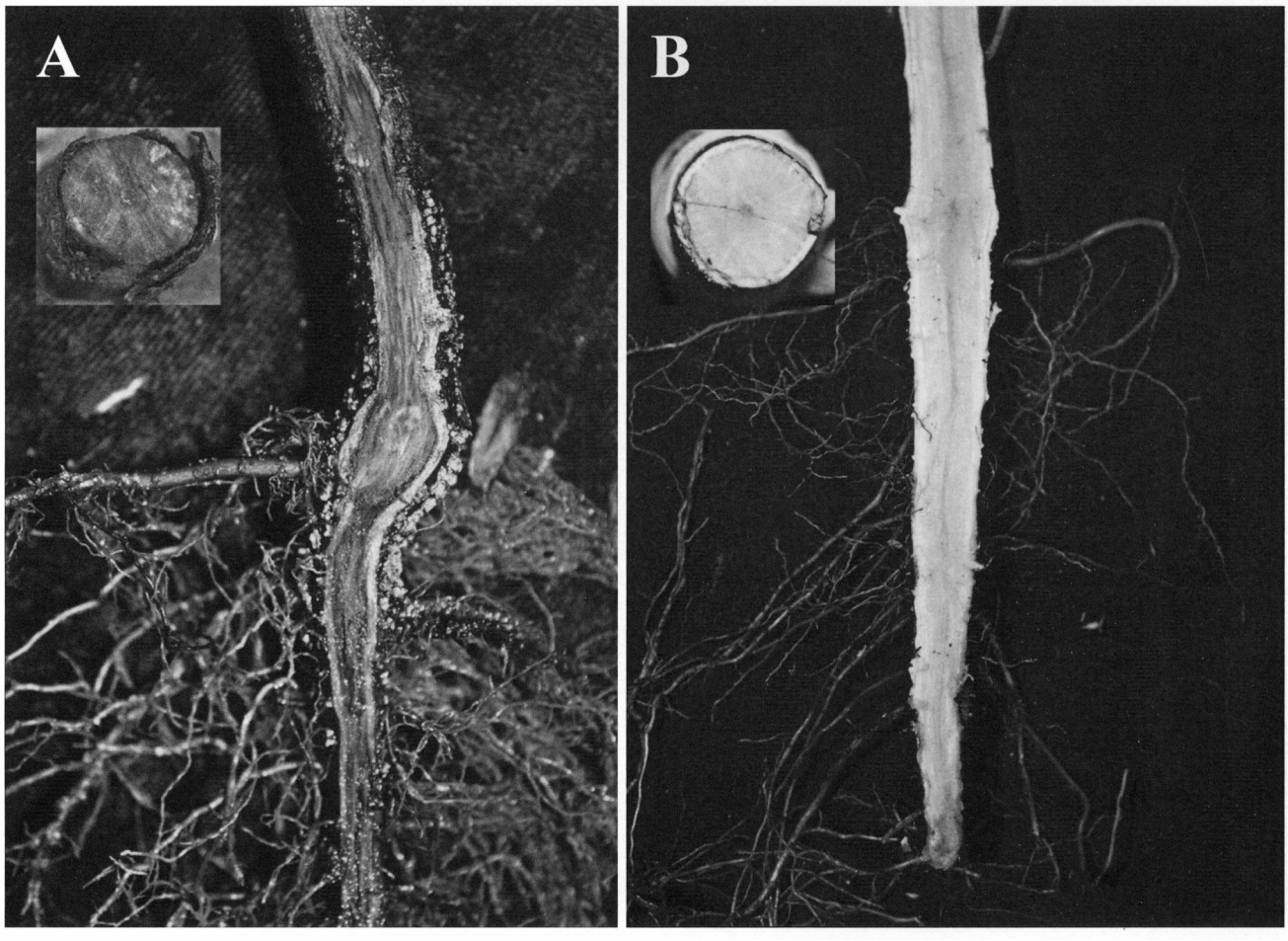

FIG. 6. A. Longitudinal and (left) cross-sectional views of infected root tissue of *Q. agrifolia* seedlings inoculated with *D. corticola*. Dead xylem is surrounded by living phloem. B. Longitudinal and (left) cross sectional views of control.

This study presents evidence that a *Diplodia* species can aggressively colonize root tissues of one of its host species. Apart from a single report describing a teleomorph of *Diplodia tumefaciens* on diseased poplar roots (Zalasky 1973), no Botryosphaeriacae species have been reported to colonize roots. Attempts to inoculate *Q. suber* roots with *D. mutila* had been unsuccessful (Luque 2000). Additional investigations are required to determine whether root colonization observed for *D. corticola* in current inoculation studies has implications for the epidemiology of oak-decline disease in the field, for example by roots acting as an inoculum source for *D. corticola* or for root colonization by Botryosphaeriaceae being involved in symptom development of this or other declines.

Little is known of the origin of *D. corticola* and its status as an exotic or overlooked native pathogen, but there is evidence that suggests the former. *D. corticola* was detected only recently in the United States. It first was reported in 2007 on *Q. agrifolia* in northern California (Erbilgin et al. 2008). It first was reported as a pathogen on grapevine and canyon live oak (*Q. chrysolepis*) in Texas and California in 2009 (Úrbez-Torres et al. 2009), on *Q. agrifolia* in southern California in 2010 (Lynch et al. 2010) and on *Quercus virginiana* in Florida in 2010 (Dreaden et al. 2011). Recent detection of this pathogen throughout ecologically separated regions of the country suggests multiple introductions. In addition, *D. corticola* is now recognized as having been an important contributor to cork oak (*Q. suber*) decline throughout the main cork-producing countries in Europe, including Portugal, Spain, France, Italy and Morocco (Luque et al. 2008), where it also affects *Q. ilex* and *Q. cerris* (Alves et al. 2004). This disease originally was thought to be caused primarily by *D. mutila* (Luque and Girbal 1989, Sánchez et al. 2003). It is now accepted that *D. corticola* was misidentified for many years as *D. mutila* and that *D. corticola* and *D. mutila* are distinct species (Alves 2004, Luque 2008). Given its role in oak decline in Europe, its high pathogenicity on live oaks and early detection in multiple locations throughout the United States, it is likely that *D. corticola* is an

exotic pathogen that needs to be investigated further for effective management.

The anamorph genus *Diplodia* has been characterized as having hyaline conidia that can, but do not invariably, darken and become septate with age after discharge from pycnidia (Phillips et al. 2005). While isolates of *D. corticola* obtained in the present study had conidia morphology consistent with that reported in previous studies, those of *D. agrifolia* were more often dark, septate and longer than described by Alves et al. (2004) for *D. mutila*. Because of this, we propose that coast live oak isolates of *D. agrifolia* are sufficiently distinct from non-oak *D. mutila* isolates to constitute a separate species.

While conidial dimensions were consistent between the present study and those of other studies, septation and pigmentation were not. Not all *Do. iberica* isolates in the present study produced conidia that were consistently pigmented and septate. Instead, conidia were also hyaline and nonseptate after discharge from pycnidia, although conditions used to induce sporulation followed protocols of Phillips et al. (2005). Conidia in *Dothiorella* are brown and one-septate early in their development, while still attached to conidiogenous cells (Phillips et al. 2005, 2008). Differences for *Do. iberica* may indicate differences between California populations and other populations of this species. However, the production of dark, septate conidia by *D. agrifolia*, versus light, aseptate spores of its apparent sister taxon *D. mutila*, calls into question the reliability of using conidia septation and color as a character for differentiating *Dothiorella* from *Diplodia* and suggests the need for further investigation.

In this study *Do. iberica* proved to be weak a pathogen on healthy coast live oak seedlings, which is consistent with tests conducted on grapevine in California (Úrbez-Torres et al. 2007, 2009). However, it is also possible that *Do. iberica* may affect stressed plants more severely than healthy plants. In a pathogenicity study of fungi isolated from *Q. suber* in northeastern Spain, the number and size of vascular lesions was greater when plants were under stress when inoculated with *Biscogniauxia mediterranea* and a *Phomopsis* sp., whereas symptom development with *F. solani* and *D. mutila*—the two most aggressive species tested—was not affected by water restriction (Luque et al. 2000). In the case of native chaparral plants in southern California that were artificially inoculated with *Botryosphaeria dothidea*, lesion length increased with decreasing plant water potential, suggesting that drought stress predisposes plants to disease (Brooks and Ferrin 1994). Drought stress in mature coast live oak trees in natural stands has been shown to increase after GSOB attack (Coleman et al. 2011), so it is likely that *Dothiorella* may play a bigger role in contributing to the demise of GSOB-affected trees under natural conditions than observed in well watered greenhouse seedlings. Investigations on the effect of *Do. iberica* on coast live oak under drought stress are in progress.

Although *Do. iberica* proved to be a weak pathogen by itself, it has potential to operate within a complex of fungal species to weaken and thus shorten the life of coast live oaks. For example, esca disease in mature or old grapevines is a complex of at least two distinct diseases induced by one or more species of *Phaeoacremonium* that is further complicated by wood rot caused by *F. punctata* (Graniti et al. 2000). In Europe, up to 14 pathogenic fungi ranging in intensity of aggressiveness may be recovered from declining cork oak (Luque et al. 2000), and similar diversity of fungi in declining oaks has been observed in southern California (Shannon Lynch unpubl). Occurrence, incidence and association of these fungal species is being assessed stands and trees to determine patterns of fungal colonization and their role in the decline of coast live oak trees in GSOB-infested and-uninfested sites of San Diego and Riverside counties of southern California.

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