Phylogeny, Morphology, Distribution, and Pathogenicity of Botryosphaeriaceae and Diaporthaceae from English Walnut in California

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Abstract

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Species of family Botryosphaeriaceae and genus *Diaporthe* (anamorph: genus *Phomopsis*, family Diaporthaceae) were reported and caused diseases on various fruit and nut trees in California. In the last several years, diseases on English walnut (*Juglans regia*) caused by species of Botryosphaeriaceae and *Diaporthe* were observed frequently in California. Disease symptoms include stem canker; shoot canker and blight; twig, leaf, and fruit blight; and necrotic leaf lesions. Isolates of the pathogen were collected from English walnut in 13 counties in California. The aims of this study were to identify these isolates and to test their pathogenicity to English walnut cultivars. In total, 159 California isolates were identified based on comparisons of DNA sequence data of the internal transcribed spacer, translation elongation factor 1-α, and β-tubulin gene regions, and combined with the morphological features of the cultures and conidia. Research results revealed that isolates represent 10 species of Botryosphaeriaceae and two species of *Diaporthe*. These species include *Botryosphaeria dothidea*, *Diplodia mutila*, *D. seriata*, *Dothiorella iberica*, *Lasiodiplodia citricola*, *Neofusicoccum mediterraneum*, *N. nonquaesitum*, *N. parvum*, *N. vitifusiforme*, *Neoscytalidium dimidiatum*, *Diaporthe neotheicola*, and *D. rhusicola*. Pathogenicity on three English walnut cultivars ('Chandler', 'Tulare', and 'Vina') using a mycelium plug inoculation method revealed that all these species are pathogenic to all the tested cultivars, with *L. citricola* and *N. parvum* being the most pathogenic species, followed by *N. mediterraneum*, *N. dimidiatum*, and *B. dothidea*. Chandler was more tolerant to infection than Tulare and Vina. Results in this study determined that multiple numbers of the Botryosphaeriaceae fungi and two *Diaporthe* spp. cause cankers and blights of English walnut and vary in their virulence from highly to slightly virulent, respectively.

Walnut seed are a high-density source of nutrients, particularly proteins and essential fatty acids. The two most common major species of walnut are grown for their seed—the Black walnut (*Juglans nigra*) and the English walnut (*J. regia*; 9). The Black walnut is of high flavor but, due to its hard shell and poor hulling characteristics, it is not grown commercially for nut production (9). The commercially produced walnut varieties are nearly all hybrids of the English walnut (9). The worldwide production of walnut seed has been increasing rapidly in recent years. In 2011, the world's three largest producers of inshell walnut seed were China (1.65 million metric tons [t]), Iran (0.49 million t), and the United States (0.42 million t) (21). In California, there are approximately 89,000 ha of English walnut with >30 cultivars planted, producing 99% of English walnut seed in the United States (9,57). Almost all production takes place in the two major Sacramento and San Joaquin Valleys of California (9).

Members of the family Botryosphaeriaceae (Botryosphaeriales) have a worldwide distribution and cause disease on a wide variety of woody plants (8,54). In California, Botryosphaeriaceae fungi have been reported and caused diseases on many important fruit and nut trees, such as almond (*Prunus dulcis*; 28), avocado (*Persea americana*; 33,34), grapevine (*Vitis vinifera*; 61–63), olive (*Olea europaea*; 41,65), and pistachio (*Pistacia vera*; 32,36). The disease symptoms caused by Botryosphaeriaceae fungi on fruit and nut trees in California include stem and branch canker, shoot blight, twig and fruit blight, bud blight, and twig dieback and leaf blight (28,32,33,36,41,63–65). For the California fruit and nut trees, some species of Botryosphaeriaceae are considered as important pathogens; for example *Lasiodiplodia citricola* (11,14), *L. theobromae* (13,61) and *Neofusicoccum parvum* (28,34,61) are highly pathogenic to their hosts, *N. mediterraneum* is widely distributed and moderately pathogenic to almond (28).

The genus *Phomopsis* (anamorph of *Diaporthe*) contains >1,000 species names, which comprise important phytopathogenic microfungi from a wide ranges of hosts (24,48,58,59). For species identification in the genera *Diaporthe* and *Phomopsis*, the traditional methods, including morphology, culture characteristics, and host association, are inadequate or unreliable (24,50,58). Recently, molecular technologies have been used to identify species of *Diaporthe* or *Phomopsis* effectively and credibly (24,50,58). We are now able to link anamorph and teleomorph states through molecular sequence data regardless of whether the taxon in question expresses sexual or asexual structures (24,26,27,49,52,69). There is a movement underway to provide all fungal species with a single name (26,27,52,69). In this study, we have chosen to use the sexual name (*Diaporthe*), the older name (*Diaporthe* in 1870 versus *Phomopsis* in 1905), for the species of *Diaporthe* or *Phomopsis*.

In California, species of *Diaporthe* cause stem and branch cankers, shoot blight, twig dieback, fruit rot, leaf spot and blight to a wide range of plant hosts (22,42). *Diaporthe* spp. have been reported on various nut crops and fruits, such as almond (2), avocado (22), fig (37), olive (65), pistachio (16,22,39) and strawberry (*Fragaria* × *ananassa*; 22). Recent inoculation results indicated that species of *Diaporthe* were considered as weak pathogens to olive in California (65).

The limited research about diseases on English walnut in California has resulted in identification of five species of Botryosphaeriaceae, include *Diplodia seriata*, *L. citricola*, *N. mediterraneum, N. parvum* and *Neoscytalidium dimidiatum* (11,28). All these species were isolated from diseased walnut stems, branches or twigs. Pathogenicity tests indicated that they are all pathogenic to English walnut branches (11) and *L. citricola* and/or *N. dimidia-*

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tum were able to kill the walnut graft union in a commercial nursery in California (11). These results suggested that members of the family Botryosphaeriaceae constitute a threat to the English walnut industry in California.

In California, during the last several years, a mass of diseased walnut samples from various counties where English walnut are grown were collected, and the relative fungi with the typical morphological characteristics of family Botryosphaeriaceae or genus *Diaporthe* were isolated. The objectives of this study were to (i) identify the species of Botryosphaeriaceae and *Diaporthe* associated with the English walnut disease based on molecular and morphological methods, (ii) clarify the geographic distribution of these pathogens, and (iii) determine pathogenicity of various fungal species or isolates and compare the tolerance of several English walnut cultivars to these fungi.

Materials and Methods

Collections of fungal isolates. Isolates used in this study were collected from English walnut trees in different counties of California from 1999 to 2011. Isolations were made from diseased stems, diseased shoots and twigs, and decayed fruit, as well as from pycnidia formed on the diseased tissues of English walnut, showing typical morphology of Botryosphaeriaceae or *Diaporthe* fungi. Affected tissues were surface disinfested with household bleach (Clorox Professional Product Company) at 10% (vol/vol) in sterile water for 3 min. Bark tissues (3 to 5 by 2 to 5 mm) were cut with a sterile scalpel and placed in petri dishes containing 2% potato dextrose agar (PDA; 20 g of potato dextrose agar and 1,000 ml of water; Microtech Scientific) acidified with lactic acid (2.5 ml of 25% [vol/vol] per liter of medium; APDA) to minimize bacterial growth. When fungal fruiting structures formed on the diseased tissue, the fruiting structures were removed using a sterile transfer needle and cultured in petri dishes on APDA. Petri dishes were incubated at 25 ± 3°C for 2 to 7 days until fungal colonies were large enough to be examined. To obtain pure cultures, single hyphal tips from the colonies with typical growth characteristics of Botryosphaeriaceae or *Diaporthe* spp. were transferred to fresh PDA and incubated as described above. These isolates are maintained in the culture collection of the Department of Plant Pathology at the University of California, Davis (Kearney Agricultural Research and Extension Center in Parlier). In addition to isolates from English walnut in California, some other isolates in our tree hosts which were isolated from almond, avocado, and pomegranate in California, as well as isolates from English walnut in Greece and Spain, were also used in this study.

DNA extraction, polymerase chain reaction amplification, and DNA sequencing. For DNA extraction, single-hyphal cultures were grown on 2% PDA for 7 to 10 days at 25 ± 3°C to obtain pure single-genotype cultures. Mycelium used for DNA extraction was scraped directly from the medium using a sterile scalpel. The total genomic DNA was extracted from pure culture mycelia using a FastDNA Kit (BIO 101, Inc.). The internal transcribed spacer (ITS) regions ITS1 and ITS2 and the 5.8S gene of the ribosomal DNA were amplified using primers ITS1 and ITS4 (68). Part of the translation elongation factor 1-α (TEF-1α) gene was amplified using the primers EF1-728F and EF1-986R (10). A portion of the β-tubulin (BT) gene was amplified by the primers Bt2A and Bt2B (23). The polymerase chain reaction (PCR) of ITS, TEF-1α, and BT was conducted according to previous studies (15,53). The PCR products were purified using an Ultra Clean PCR Clean-Up Kit (MO BIO Laboratories, Inc.).

The resulting amplicons were sequenced in both directions using the same primers that were used for PCRs. Sequence reactions were run by using an automated sequencer by the Division of Biological Sciences sequencing facility, University of California–Davis. The nucleotide sequences were read and edited using MEGA4 software (55). Sequences obtained in this study were deposited in GenBank.

Phylogenetic analyses. The ITS, TEF-1α, and BT sequence of the strains isolated in this study were subjected to BLAST search using the National Center for Biotechnology Information (National Center for Biotechnology Information [NCBI]; http://www.ncbi.nlm.nih.gov) nucleotide database to obtain preliminary identifications. Sequences of the type specimen isolates or strains for closely related Botryosphaeriaceae and *Diaporthe* spp. were obtained from GenBank (http://www.ncbi.nlm.nih.gov) to compile datasets for phylogenetic analyses. The sequences for each of the three gene regions were aligned using the online interface of MAFFT v. 5.667 (29), with the iterative refinement method (FFT-NS-i settings). The sequence alignments were edited manually in MEGA4 (55). The sequence alignments for each of the datasets were deposited in TreeBASE (http://treebase.org/treebase-web/).

Maximum-likelihood (ML) phylogenetic analyses were conducted for each of the ITS, TEF-1α, and BT datasets. The best models of nucleotide substitution were established with Modeltest 3.7 (46). The ML analyses were conducted with PhyML 3.0 (25). For the ML analyses, additional ML parameters in PhyML included the retention of the maximum number of 1,000 trees and the determination of nodal support by nonparametric bootstrapping with 1,000 replicates. MEGA4 was used to construct consensus trees (55).

Culture and conidia morphological characterization. In order to characterize species of Botryosphaeriaceae and *Diaporthe* using the morphological characteristics and depending on the amount of isolates identified by the phylogenetic analyses, one to six representative isolates of each species were selected to study their culture and conidia characteristics. A 7-mm plug of a colony for each selected isolate was removed from these cultures and transferred to the center of 90-mm petri dishes containing 2% PDA. These cultures were grown at different incubation temperatures of 0 to 40°C at 5°C intervals for 2 to 4 days. Five replicate plates of each isolate were used at each temperature. The plates were incubated in the dark and two measurements of colony diameter, at right angles to each other, were taken daily until the fastest-growing culture had covered the plate, and the averages were then calculated for each of the nine temperatures. The experiment was repeated once. Colony morphological characteristics were observed and colony color was determined using the color charts of Rayner (47).

To study conidial morphology of the selected representative isolates, pycnidia formation was induced by growing isolates on a 2% agar medium with pistachio leaves (pistachio leaves were autoclaved at 120°C for 20 min and then placed into petri dishes containing a 2% agar medium (agar; 10 g of agar and 500 ml of water; Microtech Scientific) that were incubated at 25 ± 3°C for 15 days. Pycnidia-containing conidia were mounted in sterile water and squeezed on glass slides, and the length and width of 50 conidia for each isolate were measured at a magnification of ×1,000 with a compound microscope (Zeiss Microscope System Standard 16; Carl Zeiss Ltd.). Average (mean), standard deviation (std. dev.), minimum (min), and maximum (max) measurements were made and are presented as (min–) (average – std. dev.) – (average + std. dev.) (–max) for each isolate. Conidial shape, color, and the presence of septa were also recorded.

Inoculation of walnut branches and hulls with mycelium plugs. To test pathogenicity, one to three isolates of each identified species of Botryosphaeriaceae and *Diaporthe* from English walnut in California were selected for the field inoculation tests. Three cultivars ('Chandler', 'Tulare', and 'Vina') of *J. regia* were selected. Pathogenicity tests were conducted by inoculating 2-year-old branches and the current hulls of the fruit. Inoculations were conducted by using a 7-mm mycelium PDA plug from a 7-day-old culture. Wounding of the bark and hulls was created with a 7-mm cork borer and placing an agar plug bearing mycelia, upper surface down, into the fresh wound. Five 2-year-old branches or hulls of five fruit per isolate were inoculated. Five additional branches or hulls of each of the three cultivars were wounded and inoculated with a sterile 2% PDA plug and served as negative controls. Two experiments (the first in July and the second in August 2012) were conducted using the same methodology. Infection data were recorded 3 weeks after inoculation by measuring length of branch canker and fruit blight.

Effect of inoculation method on infection of walnut. To test the effect of inoculation method on infection of English walnut, branches and fruit on walnut trees were inoculated with either a mycelium plug or a conidial suspension. Isolates in the species which were dominant in distribution or most pathogenic were selected. The mycelium plug inoculation method was performed as described above. For the conidial suspension inoculation, a suspension of 1.0×10^6 conidia/ml was used. Pathogenicity tests were conducted by inoculating five 2-year-old branches or hulls of five fruits per isolate for each inoculation method. Five additional branches or hulls of each of the three cultivars were inoculated with a sterile 2% PDA plug or water and served as negative controls. All the inoculations were conducted on Chandler, Tulare, and Vina walnut. Two inoculation experiments (the first in July and the second in August) were conducted. Three weeks after each inoculation method, length of developed cankers and blight of fruits were recorded.

Statistical analyses. Results of experiments of this study were analyzed with SAS (51) using the PROC general liner model. Analysis of variance was performed on the data and treatment means were compared using Tukey's least significant difference test at $P = 0.05$.

Results

Collection of fungal isolates. In California, 159 fungal isolates from English walnut showing typical morphology of Botryosphaeriaceae or *Diaporthe* fungi were collected from 13 counties. These counties included Butte, Colusa, Fresno, Glenn, Kings, Merced, San Benito, Stanislaus, Sutter, Tulare, Ventura, Yolo, and Yuba (Table 1; Fig. 1). In California, in addition to English walnut, 19 more isolates with typical characteristics of family Botryosphaeriaceae were collected: 15 from pomegranate, 2 from almond, 1 from *Umbellularia californica* (California Bay Laurel), and 1 from avocado. Furthermore, nine and two isolates of

Table 1. Isolates sequenced and used for phylogenetic study and pathogenicity tests in this study

Identity	Type[v]	Number Isolate	Host	Location[w]	Date[x]	GenBank accession number[u] ITS	TEF-1α	BT2
Botryosphaeriaceae								
Botryosphaeria dothidea	AAA	2E55[y,z]	*Juglans regia*	Orland, Glenn, CA	4/4/2000	KF778783	KF778973	KF778878
B. dothidea	AAA	2K23	*J. regia*	California	1/10/2010	KF778784	KF778974	KF778879
B. dothidea	BAA	2D20[y,z]	*J. regia*	Chico, Butte, CA	3/2/2009	KF778785	KF778975	KF778880
B. dothidea	BAA	6I19[y]	*J. regia*	Yuba, CA	9/16/2011	KF778786	KF778976	KF778881
B. dothidea	CBA	5A02	*J. regia*	Greece	7/2/2010	KF778787	KF778977	KF778882
B. dothidea	CBA	5A03[z]	*J. regia*	Greece	7/2/2010	KF778788	KF778978	KF778883
Diplodia mutila	AAA	4D33	*Persea americana*	Ventura, CA	6/15/2004	KF778789	KF778979	KF778884
D. mutila	AAA	5B64	*J. regia*	Cordoba, Spain	8/12/2010	KF778790	KF778980	KF779885
D. mutila	AAA	6B99[y,z]	*J. regia*	Yolo, CA	5/31/2011	KF778791	KF778981	KF778886
D. mutila	AAA	6F64	*Punica granatum*	Tulare, CA	7/16/2011	KF778792	KF778982	KF778887
D. mutila	AAA	6F65[y,z]	*P. granatum*	Tulare, CA	7/16/2011	KF778793	KF778983	KF778888
D. mutila	AAA	6F66	*P. granatum*	Tulare, CA	7/16/2011	KF778794	KF778984	KF778889
Diplodia seriata	AAA	2K33	*P. granatum*	Parlier, Fresno. CA	10/2/2009	KF778795	KF778985	KF778890
D. seriata	AAA	3H18[y,z]	*J. regia*	Parlier, Fresno. CA	1/15/2004	KF778796	KF778986	KF778891
D. seriata	AAA	3K67	*J. regia*	Tulare, CA	3/18/2010	KF778797	KF778987	KF778892
D. seriata	AAA	3K69	*J. regia*	Tulare, CA	3/18/2010	KF778798	KF778988	KF778893
D. seriata	AAA	4D23	*J. regia*	Merced, CA	4/1220/05	KF778799	KF778989	KF778894
D. seriata	AAA	5C52	*J. regia*	Kings, CA	9/20/2010	KF778800	KF778990	KF778895
D. seriata	AAA	5F07	*J. regia*	Tulare, CA	10/2/2010	KF778801	KF778991	KF778896
D. seriata	AAA	5F12	*J. regia*	Hollister, CA	9/22/2010	KF778802	KF778992	KF778897
D. seriata	BBB	3K70[z]	*J. regia*	Tulare, CA	3/18/2010	KF778803	KF778993	KF778898
D. seriata	BCB	5E99	*J. regia*	California	10/7/2010	KF778804	KF778994	KF778899
D. seriata	BCB	5F10	*J. regia*	Hollister, CA	9/22/2010	KF778805	KF778995	KF778900
D. seriata	BCB	5F11[y,z]	*J. regia*	Hollister, CA	9/22/2010	KF778806	KF778996	KF778901
D. seriata	BCB	5F13	*J. regia*	Hollister, CA	9/22/2010	KF778807	KF778997	KF778902
Dothiorella iberica	AAA	5G97[y,z]	*J. regia*	Davis, Yolo, CA	12/13/2010	KF778808	KF778998	KF778903
Lasiodiplodia citricola	AAA	6I34[y,z]	*J. regia*	Stanislaus, CA	10/6/2011	KF778809	KF778999	KF778904
L. citricola	AAA	6I35[y,z]	*J. regia*	Stanislaus, CA	10/6/2011	KF778810	KF779000	KF778905
Neofuscicoccum mediterraneum	AAA	1H96	*J. regia*	Selma, Fresno, CA	9/15/2006	KF778811	KF779001	KF778906
N. mediterraneum	AAA	1L86	*J. regia*	Stanislaus, CA	8/27/2004	KF778812	KF779002	KF778907
N. mediterraneum	AAA	4D13	*J. regia*	Parlier, Fresno. CA	11/18/1999	KF778813	KF779003	KF778908
N. mediterraneum	AAA	4K69[z]	*J. regia*	Stanislaus, CA	9/21/2004	KF778814	KF779004	KF778909
N. mediterraneum	AAA	5D03	*J. regia*	Yuba or Sutter, CA	5/6/2010	KF778815	KF779005	KF778910
N. mediterraneum	AAA	6F33	*J. regia*	Tulare, CA	7/6/2011	KF778816	KF779006	KF778911
N. mediterraneum	AAA	6F35	*J. regia*	Tulare, CA	7/6/2011	KF778817	KF779007	KF778912
N. mediterraneum	BAB	1L85	*J. regia*	Butte, CA	11/4/2005	KF778818	KF779008	KF778913
N. mediterraneum	BAB	3K72	*J. regia*	Sutter, CA	3/24/2010	KF778819	KF779009	KF778914
N. mediterraneum	BAB	3K77	*J. regia*	Stanislaus, CA	3/30/2010	KF778820	KF779010	KF778915
N. mediterraneum	BAB	4C52	*J. regia*	Tulare, CA	9/10/2003	KF778821	KF779011	KF778916
N. mediterraneum	BAB	4D04	*J. regia*	Durham, Butte, CA	11/4/2005	KF778822	KF779012	KF778917
N. mediterraneum	BAB	5A05	*J. regia*	Yuba, CA	7/2/2010	KF778823	KF779013	KF778918
N. mediterraneum	BAB	5C88	*J. regia*	Yuba or Sutter, CA	5/6/2010	KF778824	KF779014	KF778919
N. mediterraneum	BAB	5C92	*J. regia*	Yuba, CA	6/14/2010	KF778825	KF779015	KF778920
N. mediterraneum	BAB	5G96	*J. regia*	Davis, Yolo, CA	12/13/2010	KF778926	KF779016	KF778921
N. mediterraneum	BAB	5G100	*J. regia*	Davis, Yolo, CA	12/13/2010	KF778827	KF779017	KF778922

(*continued on next page*)

[u] ITS = internal transcribed spacer, TEF-1-α = translation elongation factor 1-α, and BT2 = β-tubulin-2 gene regions.
[v] Genotype within each identified species, determined by sequence of ITS, TEF-1α, and BT2 gene regions.
[w] Hollister = Hollister, San Benito, CA; Santa Nella = Santa Nella, Merced, CA; and Maxwell = Maxwell, Colusa, CA.
[x] Collection date: month/day/year.
[y] Isolates used in field pathogenicity tests on *J. regia* in this study.
[z] Isolates used for colony growth, culture morphology, and conidia morphology in this study.

Botryosphaeriaceae were isolated from English walnut in Spain and Greece, respectively.

Phylogenetic analyses. Isolates which represented different genotypes determined by the ITS, TEF-1α, and BT gene regions and covering all the hosts and sampled regions (counties) were selected for phylogenetic analyses (Table 1), and sequences of these selected isolates were deposited in GenBank (Table 1). All the isolates can be separated into two groups by the BLAST searches using the NCBI nucleotide database: the family Botryosphaeriaceae and genus *Diaporthe*. The phylogenetic analyses for the sequences of isolates in this study (Table 1) and sequences obtained from GenBank (Table 2) were conducted within Botryosphaeriaceae and *Diaporthe*, respectively.

Phylogenetic analyses of species of Botryosphaeriaceae. For the species of Botryosphaeriaceae, PCR of isolates resulted in amplicons of approximately 550 bp for the ITS, 300 bp for the TEF-1α, and 450 bp for the BT gene regions. The aligned sequences of each dataset of ITS (83 taxa, 571 characters), TEF-1α (83 taxa, 354 characters), and BT (71 taxa, 421 characters) gene regions were deposited in TreeBASE (number 14888). For ML analyses, model test analysis recommended a TIM+G model (Lset base = [0.2213, 0.2830, 0.2554]; number of substitution rate categories [Nst] = 6; rate matrix = [1.0000, 3.9000, 2.0980, 2.0980, 7.0404]; rates = γ; shape = 0.1938] for the ITS gene region, a TVM+G model [Lset base = [0.1706, 0.3223, 0.2695]; Nst = 6; rate matrix = [2.4163, 5.7914, 1.5365, 1.2889, 5.7914]; rates = γ; shape = 0.8288] for the TEF-1α gene region, and a TrN+G model [Lset base = [0.1974, 0.3288, 0.2506]; Nst = 6; rate matrix = [1.0000, 1.7842, 1.0000, 1.0000, 5.3879]; rates = γ; shape = 0.3339] for the BT gene regions.

Phylogenetic analyses based on three gene regions were unable to resolve the order of divergence of the genera within the family Botryosphaeriaceae; however, each genus is strongly supported (Figs. 2–4). The only exceptions include the unsupported position of *Dothiorella* spp. which clustered among species of *Neofusicoccum* in the ITS analysis (Fig. 2). The combined analysis (*results*

Table 1. (*continued from preceding page*)

Identity	Type[v]	Number Isolate	Host	Location[w]	Date[x]	GenBank accession number[u] ITS	TEF-1α	BT2
N. mediterraneum	BAB	5H01	*J. regia*	Fresno, CA	1/15/2000	KF778828	KF779018	KF778923
N. mediterraneum	BAB	5H53[y,z]	*J. regia*	Colusa, CA	12/13/2010	KF778829	KF779019	KF778924
N. mediterraneum	BAB	5H91	*J. regia*	Santa Nella, CA	1/31/2011	KF778830	KF779020	KF778925
N. mediterraneum	BAB	5L44	*J. regia*	Colusa, CA	2/24/2011	KF778831	KF779021	KF778926
N. mediterraneum	BAB	6F30	*P. granatum*	Tulare, CA	7/6/2011	KF778832	KF779022	KF778927
N. mediterraneum	BAB	6F62	*P. granatum*	Tulare, CA	7/16/2011	KF778833	KF779023	KF778928
N. mediterraneum	BAB	6I24	*J. regia*	Stanislaus, CA	10/6/2011	KF778834	KF779024	KF778929
N. mediterraneum	BAB	6I32	*J. regia*	Stanislaus, CA	10/6/2011	KF778835	KF779025	KF778930
N. mediterraneum	CAA	2E54	*J. regia*	Orland, CA	4/4/2000	KF778836	KF779026	KF778931
N. mediterraneum	CAA	3K78	*J. regia*	Stanislaus, CA	3/30/2010	KF778837	KF779027	KF778932
N. mediterraneum	CAA	3K80	*J. regia*	Stanislaus, CA	3/30/2010	KF778838	KF779028	KF778933
N. mediterraneum	CAA	4C26	*J. regia*	Tulare, CA	9/10/2003	KF778839	KF779029	KF778934
N. mediterraneum	CAA	4L59	*J. regia*	Winters, Yolo, CA	6/10/2010	KF778840	KF779030	KF778935
N. mediterraneum	CAA	4L77	*J. regia*	Winters, Yolo, CA	6/24/2010	KF778841	KF779031	KF778936
N. mediterraneum	CAA	5H09[y,z]	*J. regia*	Colusa, CA	12/13/2010	KF778842	KF779032	KF778937
N. mediterraneum	DAB	4C06	*J. regia*	Colusa, CA	7/23/2004	KF778843	KF779033	KF778938
N. mediterraneum	DAB	5C86	*J. regia*	Yuba, CA	7/1/2010	KF778844	KF779034	KF778939
N. mediterraneum	DAB	5C87[y,z]	*J. regia*	Yuba, CA	7/1/2010	KF778845	KF779035	KF778940
N. mediterraneum	DAB	5H89	*J. regia*	Maxwell, CA	1/31/2011	KF778846	KF779036	KF778941
N. mediterraneum	DAB	6F52	*P. granatum*	Tulare, CA	7/16/2011	KF778847	KF779037	KF778942
N. mediterraneum	DAB	6I27	*J. regia*	Stanislaus, CA	10/6/2011	KF778848	KF779038	KF778943
N. mediterraneum	DAB	6I29	*J. regia*	Stanislaus, CA	10/6/2011	KF778849	KF779039	KF778944
N. nonquaesitum	AAA	4E64	*Umbellularia californica*	Colusa, CA	4/1/2004	KF778850	KF779040	KF778945
N. nonquaesitum	AAA	4L78[z]	*J. regia*	Sutter, CA	6/24/2010	KF778851	KF779041	KF778946
N. nonquaesitum	AAA	5A04[y,z]	*J. regia*	Yuba, CA	7/2/2010	KF778852	KF779042	KF778947
N. nonquaesitum	AAA	5H50	*J. regia*	Colusa, CA	12/13/2010	KF778853	KF779043	KF778948
Neofusicoccum parvum	AAA	1L83[y]	*J. regia*	Butte, CA	11/4/2005	KF778854	KF779044	KF778949
N. parvum	AAA	1L87[y,z]	*J. regia*	Butte, CA	11/4/2005	KF778855	KF779045	KF778950
N. parvum	AAA	2E43[y]	*J. regia*	Durham, Butte, CA	11/4/2005	KF778856	KF779046	KF778951
N. parvum	AAA	4D10	*J. regia*	Durham, Butte, CA	11/4/2005	KF778857	KF779047	KF778952
N. parvum	AAA	5B61	*J. regia*	Spain	8/12/2010	KF778858	KF779048	KF778953
N. parvum	AAA	5B63	*J. regia*	Spain	8/12/2010	KF778859	KF779049	KF778954
N. parvum	AAA	5B65	*J. regia*	Spain	8/12/2010	KF778860	KF779050	KF778955
N. parvum	BBB	5B60[z]	*J. regia*	Spain	8/12/2010	KF778861	KF779051	KF778956
N. parvum	BBB	6B97[z]	*J. regia*	Yolo, CA	5/31/2011	KF778862	KF779052	KF778957
N. parvum	BBB	5B602	*J. regia*	Spain	8/12/2010	KF778863	KF779053	KF778958
N. parvum	BBC	5C95[z]	*J. regia*	Stanislaus, CA	6/17/2010	KF778864	KF779054	KF778959
N. parvum	CAA	2D18[z]	*J. regia*	Spain	5/26/2009	KF778865	KF779055	KF778960
N. parvum	CAA	5B67	*J. regia*	Spain	8/12/2010	KF778866	KF779056	KF778961
N. parvum	DBC	5B62[z]	*J. regia*	Spain	8/12/2010	KF778867	KF779057	KF778962
N. vitifusiforme	AAA	5H02[y,z]	*J. regia*	Fresno, CA	5/11/2001	KF778868	KF779058	KF778963
N. vitifusiforme	AAA	5H022[z]	*J. regia*	Fresno, CA	5/11/2001	KF778869	KF779059	KF778964
Neoscytalidium dimidiatum	AAA	2D57[y,z]	*J. regia*	Kings, CA	9/16/2009	KF778870	KF779060	KF778965
Diaporthe								
Diaporthe neotheicola	AAA	6I30[y,z]	*J. regia*	Stanislaus, CA	10/6/2011	KF778871	KF779061	KF778966
D. rhusicola	AAA	6I14[z]	*Prunus dulcis*	Kings, CA	9/12/2011	KF778872	KF779062	KF778967
D. rhusicola	AAA	6I15	*P. dulcis*	Kings, CA	9/12/2011	KF778873	KF779063	KF778968
D. rhusicola	AAA	6I31	*J. regia*	Stanislaus, CA	10/6/2011	KF778874	KF779064	KF778969
D. rhusicola	AAA	6I43[z]	*J. regia*	Stanislaus, CA	10/6/2011	KF778875	KF779065	KF778970
D. rhusicola	AAA	6I44[y]	*J. regia*	Stanislaus, CA	10/6/2011	KF778876	KF779066	KF778971
D. rhusicola	AAA	6I54	*J. regia*	Stanislaus, CA	10/20/2011	KF778877	KF779067	KF778972

not shown) was concordant with the three individual datasets. Isolates in this study consisted of six genera within the family Botryosphaeriaceae, which included *Diplodia*, *Dothiorella*, *Fusicoccum-Botryosphaeria*, *Lasiodiplodia*, *Neofusicoccum*, and *Neoscytalidium*.

In the *Diplodia* phylogenetic lineage, isolates in this study clustered in to two phylogenetic groups, which represented two species, *Diplodia mutila* (Figs. 2–4, BS = 100, 100, and 89% for ITS, TEF-1α, and BT, respectively) and *D. seriata*. Only one isolate in this study clustered in the phylogenetic lineage of *Dothiorella*, which was identified as *Dothiorella iberica* (Figs. 2–4, BS = 95, 99, and 70% for ITS, TEF-1α, and BT, respectively). In the *Fusicoccum-Botryosphaeria* phylogenetic lineage, isolates recovered in this study were identified as *Botryosphaeria dothidea* (Figs. 2–4). Two isolates from California grouped in the *Lasiodiplodia* phylogenetic lineage and were identified as *L. citricola*, which was supported by ITS and TEF-1α gene analyses (Figs. 2 and 3, BS = 74, 96, and 91% for ITS, TEF-1α, and BT, respectively; BT gene sequences were not available for the ex-type specimens of *L. citricola*). Isolates that clustered in the *Neofusicoccum* phylogenetic lineage represented four species, which include *N. mediterraneum*, *N. nonquaesitum*, *N. parvum*, and *N. vitifusiforme*. For the isolates in the *Neofusicoccum* lineage which clustered in the *N. mediterraneum* phylogenetic group, several phylogenetic subgroups existed in ITS gene analyses, which were supported by high bootstrap values (Fig. 2, BS = 77, 72, and 93%, respectively). In contrast, only one phylogenetic subgroup in the TEF-1α gene analyses (Fig. 3) and two phylogenetic subgroups in the BT gene analyses (Fig. 4), all with low bootstrap values (BS < 70%), existed. Because these multiple phylogenetic subgroups by ITS gene analysis were not supported by the TEF-1α or BT genes analyses, indicating that these reflect intraspecific sequence differences rather than interspecific variation, all of these isolates were considered as *N. mediterraneum*. The remaining isolates of *Neofusicoccum* in this study were identified as *N. nonquaesitum*, *N. parvum*, and *N. vitifusiforme*, which were all supported by the phylogenetic analyses of the three gene regions (Figs. 2–4; BT is not available for the ex-type specimens of *N. vitifusiforme*). One isolate in this study clustered in the *Neoscytalidium* phylogenetic lineage and was identified as *Neoscytalidium dimidiatum* by the ITS and TEF-1α gene regions (Figs. 2 and 3; BT gene sequence is not available for the ex-type specimen of *N. dimidiatum*).

Phylogenetic analyses of species of *Diaporthe*. For the species of *Diaporthe*, because the BT gene sequences for most isolates of *Diaporthe* are not available in the GenBank database, only the analyses of ITS and TEF-1α gene regions were conducted in this study. PCR of isolates resulted in amplicons of approximately 550 bp for the ITS and 350 bp for the TEF-1α gene regions. For ML analyses, model test analysis recommended a TrNef+I+G model (Lset base = equal; Nst = 6; rate matrix = [1.0000, 2.2035, 1.0000, 1.0000, 5.3181]; rates = γ; shape = 0.4725) for the ITS gene region

Table 2. Isolates and sequences from other studies used in phylogenetic analyses for this study

Species	Number[y]		Host	Location	Collector	GenBank accession number[z]			Refs
	Isolate	Other				ITS	TEF-1α	BT2	
Botryosphaeriaceae analyses									
Botryosphaeria dothidea	CMW8000^T		*Prunus* sp.	Crocifisso, Switzerland	B. Slippers	AY236949	AY236898	AY236927	53
B. dothidea	CMW7780		*Fraxinus excelsior*	Molinizza, Switzerland	B. Slippers	AY236947	AY236896	AY236925	53
Diplodia cupressi	CBS 168.87^T		*Cupressus sempervirens*	Bet Dagan, Israel	Z. Solel	DQ458893	DQ458878	DQ458861	6
D. cupressi	CBS 261.85		*C. sempervirens*	Bet Dagan, Israel	Z. Solel	DQ458894	DQ458879	DQ458862	6
D. mutila	CBS 112553^T		*Vitis vinifera*	Montemor-o-Novo, Portugal	A. J. L. Phillips	AY259093	AY573219	DQ458850	5,30
D. mutila	CBS 230.30		*Phoenix dactylifera*	California, USA	L. L. Huillier	DQ458886	DQ458869	DQ458849	5,30
D. scrobiculata	CBS 113424		*Pinus greggii*	Mexico	M. J. Wingfield	DQ458900	DQ458885	DQ458868	6
D. scrobiculata	CBS 109944		*P. greggii*	Mexico	M. J. Wingfield	DQ458899	DQ458884	DQ458867	6
D. seriata	CBS 112555^T		*Vitis vinifera*	Montemor-o-Novo, Portugal	A. J. L. Phillips	AY259094	AY573220	DQ458856	6,45
D. seriata	CBS 119049		*Vitis* sp.	Italy	L. Mugnai	DQ458889	DQ458874	DQ458857	6,45
Dothiorella iberica	CBS 115041^T		*Quercus ilex*	Spain	J. Luque	AY573202	AY573222	EU673096	30,44
D. iberica	CBS 113188		*Q. suber*	Spain	M. E. Sanchez	AY573198	EU673278	EU673097	30,44
D. sarmentorum	IMI 63581b^T		*Ulmus* sp.	Warwickshire, England	E. A. Ellis	AY573212	AY573235	EU673102	30,44
D. sarmentorum	CBS 115038		*Malus pumila*	Delft, Netherlands	A. J. L. Phillips	AY573206	AY573223	EU673101	30,44
Fusicoccum fabicercianum	CMW27094^T	CBS 127193	*Eucalyptus* sp.	FuJian, China	M. J. Wingfield	HQ332197	HQ332213	KF779068	15
F. fabicercianum	CMW27121	CBS 127194	*Eucalyptus* sp.	FuJian, China	M. J. Wingfield	HQ332198	HQ332214	KF779069	15
F. ramosum	CMW26167^T	CBS 122069	*Eucalyptus camaldulensis*	Bell Gorge, Western Australia	T. I. Burgess	EU144055	EU144070	N/A	35
Guignardia philoprina	CMW7063	CBS 447	*Terminalia baccata*	Netherlands	H. A. van der Aa	AY236956	AY236905	AY236934	53
Lasiodiplodia citricola	IRAN1522C^T	CBS 124707	*Citrus* sp.	Iran	J. Abdollahzadeh & A. Javadi	GU945354	GU945340	N/A	1
L. citricola	IRAN1521C	CBS 124706	*Citrus* sp.	Iran	A. Shekari	GU945353	GU945339	N/A	1
L. parva	CBS 456.78^T		*Manihot esculenta* field soil	Colombia	O. Rangel	EF622083	EF622063	N/A	7
L. parva	CBS 494.78		*M. esculenta* field soil	Colombia	O. Rangel	EF622084	EF622064	EU673114	7
L. pseudotheobromae	CBS 116459^T		*Gmelina arborea*	Costa Rica	J. Carranza-Velásquez	EF622077	EF622057	EU673111	7

(*continued on next page*)

[y] CMW = Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa; CBS = the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; IMI = CABI Bioscience, Egham, U.K.; IRAN = isolates from 1; PD = isolates from 28; STE-U = Department of Plant Pathology, University of Stellenbosch, South Africa; CPC = Culture collection of Pedro Crous, housed at CBS; T = isolates are ex-type or from samples that have been linked morphologically to type material of the species.

[z] ITS = internal transcribed spacer, TEF-1-α = translation elongation factor 1-α, BT2 = β-tubulin-2 gene regions., and N/A = not available.

and an HKY+G model (Lset base = [0.2212, 0.3438, 0.1762]; Nst = 2; transition/transversion ratio = 2.8057; rates = γ; shape = 1.0599) for the TEF-1α gene region.

The aligned sequences of each dataset of ITS (14 taxa, 627 characters) and TEF-1α (13 taxa, 384 characters) gene regions were deposited in TreeBASE (number 14888). Phylogenetic analyses revealed that isolates in this study grouped into two phylogenetic groups of *Diaporthe*. One isolate was identified as *Diaporthe neotheicola*, which was conserved with the ex-type specimen isolates of *D. neotheicola* (Figs. 5 and 6, BS = 100 and 73% for ITS and TEF-1α, respectively). Six isolates were phylogenetically close to the ex-type specimen of *D. rhusicola* in the ITS phylogenetic analysis (Fig. 5). The TEF-1α sequence is not available for the ex-type specimen isolate of *D. rhusicola*; therefore, these six isolates were considered as *D. rhusicola* in this study.

Culture and conidia morphological characterization. Twenty-nine isolates (Tables 3 and 4), which represent different species of Botryosphaeriaceae and *Diaporthe* identified by the three gene regions in this study, were selected for culture and conidial morphological characterization. All 29 isolates produced anamorphic structures on pistachio-leaf water agar within 2 to 3 weeks, and no teleomorph structures were observed. Based on colony growth characteristics, colony morphology, and conidia morphology, these fungi were grouped into seven distinct groups, which represented 12 species.

The first group was characterized by having slowly growing (colony diameter < 24 mm after 24 h at the optimal growth temperature of 25°C) (*Diaporthe* spp. in Table 3), white to light-gray mycelium. Fungal colonies were suppressed or slightly raised and some developed prominent growth rings, with margins becoming black with age. Colonies produced dark pycnidia over time. Light-cream-colored conidia masses were observed on the pycnidia. Conidia were subcylindrical to fusoid-ellipsoidal, widest in middle, and small (<15 by 5 μm). All these morphological characteristics were consistent with the description of species of *Diaporthe* (24,58). Two species of *Diaporthe*, *D. neotheicola* and *D. rhusicola* were identified in this group. Conidia of *D. rhusicola* (average 8.6 by 3.3 μm) were larger than those of *D. neotheicola* (conidia average 7.9 by 2.5 μm) (Table 4).

The second group of fungi with fast-growing mycelium (colony diameter to 85 mm after 24 h at the optimal growth temperature of 30°C; *N. dimidiatum* in Table 3) had colonies with suppressed, dense mycelium. Colonies were initially white to olivaceous-buff, becoming greenish-olivaceous from the middle of the colonies within 7 days, and light black with age. Three types of conidia were observed: ellipsoidal to oval conidia, muriform conidia (globose, subglobose to obpyriform with muriform), and arthroconidia (conidia occurring in arthric chains, powdery to the touch, disarticulating, cylindrical–truncate) (*Neoscytalidium* spp. in Table 4). These characteristics were consistent with the description of *Neoscytalidium* spp. of Botryosphaeriaceae. This group of fungi were identified as *N. dimidiatum* (Table 4).

The third group contained *Dothiorella* spp. These species produced moderate mycelium growth (colony diameter of 40.5 mm

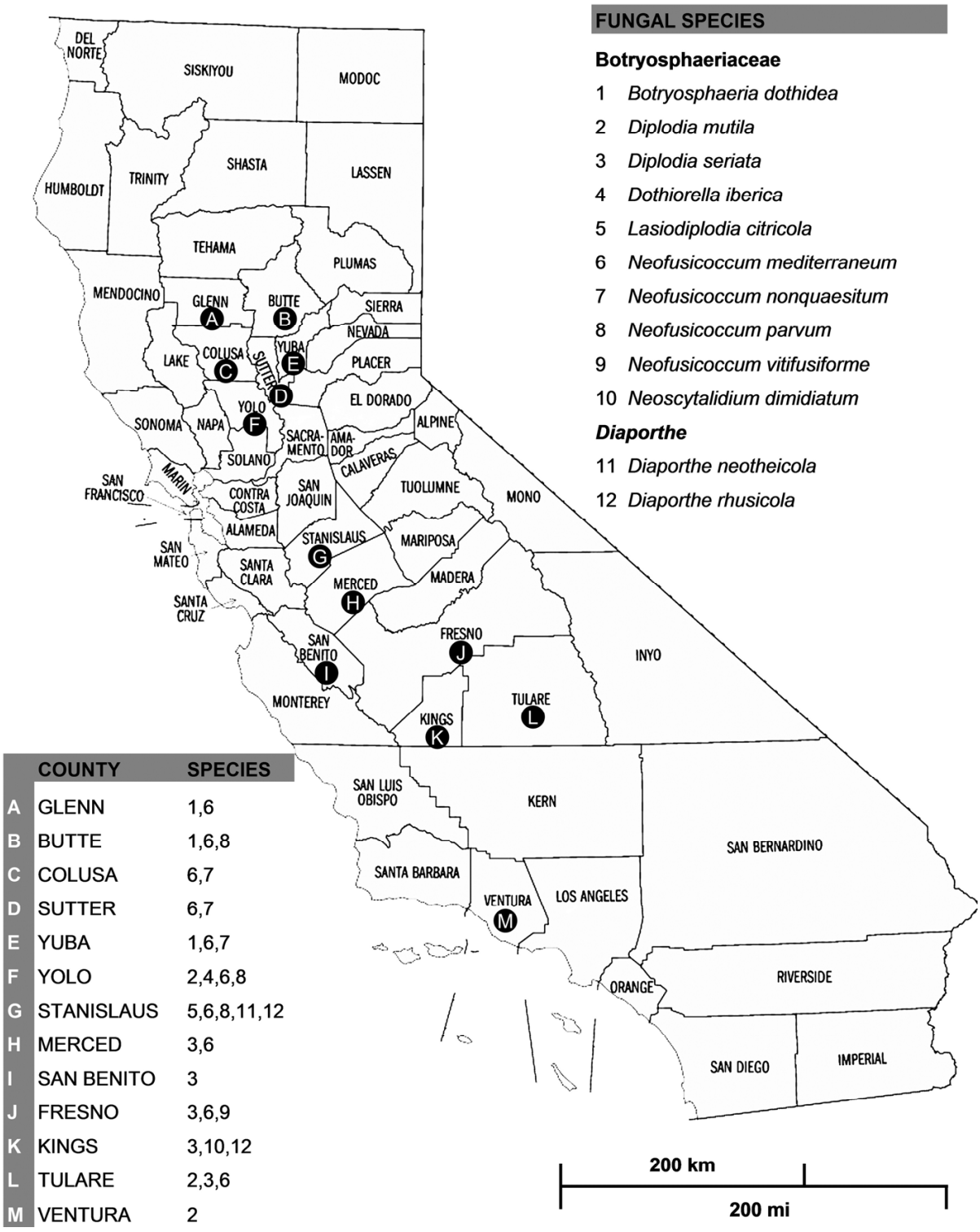
Table 2. (*continued from preceding page*)

Species	Number[y]		Host	Location	Collector	GenBank accession number[z]			Refs
	Isolate	Other				ITS	TEF-1α	BT2	
L. pseudotheobromae	CBS 447.62		*Citrus aurantium*	Suriname	C. Smulders	EF622081	EF622060	EU673112	7
L. theobromae	CBS 164.96^T		Fruit on coral reef coast	New Guinea	A. Aptroot	AY640255	AY640258	EU673110	7,30
L. theobromae	CBS 124.13		N/A	USA	J. J. Taubenhaus	DQ458890	DQ458875	DQ458858	7,30
Neofusicoccum andinum	CBS 117453^T	CMW13455	*Eucalyptus* sp.	Merida state, Venezuela	S. Mohali	AY693976	GU251287	GU251815	40
N. arbuti	CBS 116131^T	PD282	*Arbutus nenziesii*	Washington, USA	M. Elliot	GU251152	GU251284	GU251812	28
N. arbuti	PD281		*A. nenziesii*	Washington, USA	M. Elliot/A. Rossman	GU251151	GU251283	GU251811	28
N. mediterraneum	CBS 121718^T	PD312	*Eucalyptus* sp.	Greece	P. W. Crous, M.J. Wingfield & A. J. L. Phillips	GU251176	GU251308	GU251836	18,28
N. mediterraneum	PD9		*Fortunella* sp.	Colusa, CA	T. J. Michailides	GU251180	GU251312	GU251840	28
N. nonquaesitum	PD484^T		*Umbellularia californica*	Napa, CA	F. P. Trouillas	GU251163	GU251295	GU251823	28
N. nonquaesitum	PD90		*Prunus dulcis*	Butte, CA	T. J. Michailides	GU251157	GU251289	GU251817	28
N. parvum	CMW9081^T		*P. nigra*	New Zealand	G. J. Samuels	AY236943	AY236888	AY236917	53
N. parvum	CMW9080		*P. nigra*	New Zealand	G. J. Samuels	AY236942	AY236887	AY236916	53
N. ribis	CMW7772^T	PD254	*Ribes* sp.	New York, USA	B. Slippers & G. Hudler	AY236935	AY236877	GU251786	53
N. ribis	CMW7773		*Ribes* sp.	New York, USA	B. Slippers & G. Hudler	AY236936	AY236878	AY236907	53
N. viticlavatum	CBS 112878^T	STE-U 5044	*Vitis vinifera*	South Africa	F. Halleen	AY343381	AY343342	N/A	66
N. viticlavatum	CBS 112977	STE-U 5041	*V. vinifera*	South Africa	F. Halleen	AY343380	AY343341	N/A	66
N. vitifusiforme	CBS 110887^T	STE-U 5252	*V. vinifera*	South Africa	J. M. van Niekerk	AY343383	AY343343	N/A	66
N. vitifusiforme	CBS 110880	STE-U 5050	*V. vinifera*	South Africa	J. M. van Niekerk	AY343382	AY343344	N/A	66
Neoscytalidium dimidiatum	CBS 499.66		*Mangifera indica*	Mali	J. Brun	AY819727	EU144063	N/A	20,30,43
N. dimidiatum	CBS 204.33		*Prunus* sp.	Egypt	R. M. Nattrass	AY819728	EU144064	N/A	20,30,43
N. novaehollandiae	CMW26170 ^{T}	CBS 122071	*Crotalaria medicaginea*	Western Australia	T. I. Burgess	EF585540	EF585580	N/A	43
N. novaehollandiae	CMW26169	CBS 122070	*Grevillia agrifolia*	Western Australia	N/A	EF585539	EF585579	N/A	43
Diaporthe analyses									
Celoporthe eucalypti	CMW26908^T	CBS 127190	*Eucalyptus* colony	GuangDong, China	X. D. Zhou & S. F. Chen	HQ730837	HQ730850	N/A	12
Diaporthe alleghaniensis	CBS 495.72^T		*Betula alleghaniensis*	Ontario, Canada	R. H. Arnold	FJ889444	GQ250298	N/A	49
D. hickoriae	CBS 145.26^T		*Carya glabra*	Michigan, USA	L. E. Wehmeyer	FJ889446	GQ250309	N/A	49
D. neotheicola	CBS 123208^T		*Foeniculum vulgare*	Vora, Portugal	A. J. L. Phillips	EU814480	GQ250315	N/A	49,50
D. neotheicola	CBS 123209^T		*F. vulgare*	Vora, Portugal	A. J. L. Phillips	GQ250192	GQ250316	N/A	49,50
D. rhusicola	CPC 18191^T	CBS 129528	*Rhus pendulina*	Western Cape, South Africa	P. W. Crous	JF951146	N/A	N/A	17
Phomopsis viticola	CBS 114016^T		*Vitis vinifera*	Bordeaux, France	P. Larignon	AF230751	GQ250351	N/A	49,67

after 24 h at the optimal temperature of 20°C; Table 3). Mycelia were suppressed and dense. Colonies were initially thin-white to gray, becoming gray-black from the middle within 7 days, and black with age. Conidia were brown to dark walled, one-septate without longitudinal striation within the pycnidia, often before release from the conidiogenous cells; conidia were ovoid or ellipsoid and averaged 26.0 by 9.4 μm (length/width [L/W] = 2.8). *Dothiorella iberica* was identified in this group (Table 4).

The forth group had the typical characteristics of *Lasiodiplodia* spp., with mycelium growing fast (colony diameter to 85 mm after 24 h of growth at the optimal temperature at 30°C; Table 3). Colonies were fluffy with aerial mycelium, initially white to smoke-gray, becoming gray-black from the middle within 7 days, and dark with age. Conidia were initially hyaline, aseptate, ellipsoid to ovoid, becoming pigmented, one-septate with longitudinal striations with age. Conidia averaged 25.4 by 14.7 μm (L/W = 1.8); this group of fungi were identified as *L. citricola* (Table 4).

Each of three isolates of *L. citricola* from English walnut (isolates 7E78, 7E79, and 7E80) (11) and peach (*Prunus persica*; isolates 7E93, 7E94, and 7E95) (14) and three isolates of *L. theobro-*



	COUNTY	SPECIES
A	GLENN	1,6
B	BUTTE	1,6,8
C	COLUSA	6,7
D	SUTTER	6,7
E	YUBA	1,6,7
F	YOLO	2,4,6,8
G	STANISLAUS	5,6,8,11,12
H	MERCED	3,6
I	SAN BENITO	3
J	FRESNO	3,6,9
K	KINGS	3,10,12
L	TULARE	2,3,6
M	VENTURA	2

Fig. 1. Map of California indicating counties where English walnut trees were sampled and the species of Botryosphaeriaceae and *Diaporthe* obtained from each county. The 10 species of Botryosphaeriaceae and 2 species of *Diaporthe* are indicated as numbers 1 to 12.

mae from almond (isolates 7E86, 7E87, and 7E88) (13) were used to compare their morphological characteristics with *L. citricola* in this study (Tables 3 and 4). Results revealed that all *Lasiodiplodia* isolates had the same optimal growth temperature (30°C), with similar culture and conidia morphology (Tables 3 and 4), except that the colonies of *L. citricola* grew faster than those of *L. theobromae* (Table 3) and conidia of *L. citricola* were larger than those of *L. theobromae* (Table 4).

The fifth group of fungi had moderate mycelium growth (colony diameter 34.5 to 67 mm after 24 h at the optimal temperature of

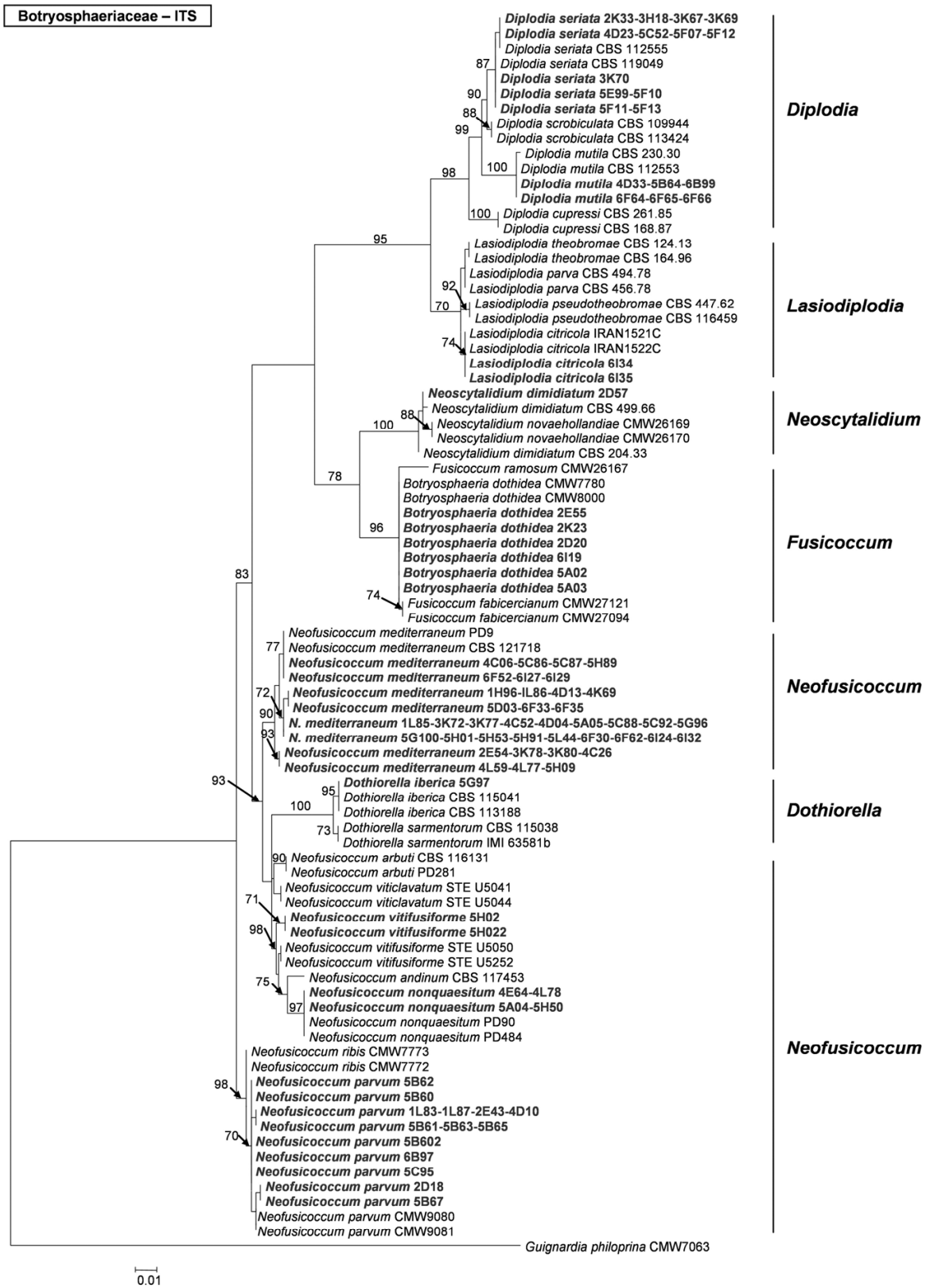


Fig. 2. Phylogenetic tree based on maximum likelihood analysis of internal transcribed spacer (ITS) nuclear ribosomal DNA sequences for various genera in the family Botryosphaeriaceae. Isolates in boldface were sequenced in this study. Bootstrap values > 70% are presented above branches. Bootstrap values < 70% are not shown. *Guignardia philoprina* (CMW7063) represents the outgroup.

25°C; species of *Diplodia* in Table 3). Colonies were moderately dense with slightly aerial mycelium, initially white and turning dark olivaceous from the middle after 7 days. Conidia initially were hyaline, aseptate, oblong to ovoid, becoming dark brown, thick-walled, rarely one-septate without longitudinal striations after being released from the pycnidia with age. Two species of *Diplodia*, *Diplodia mutila* and *D. seriata*, were identified in this group. Colonies of *D. mutila* grew in a denser and more flat pattern than those of *D. seriata*, and conidia of *D. mutila* (average of 29.0 by 13.1 μm) were larger than those of *D. seriata* (average of 24.2 by 11.7 μm) (Table 4).

Fungal groups six and seven produced moderate to fast-growing mycelia (colony diameter of 26.5 to 82 mm after 24 h at the optimal temperature of 25 to 30°C; species of *Botryosphaeria* and

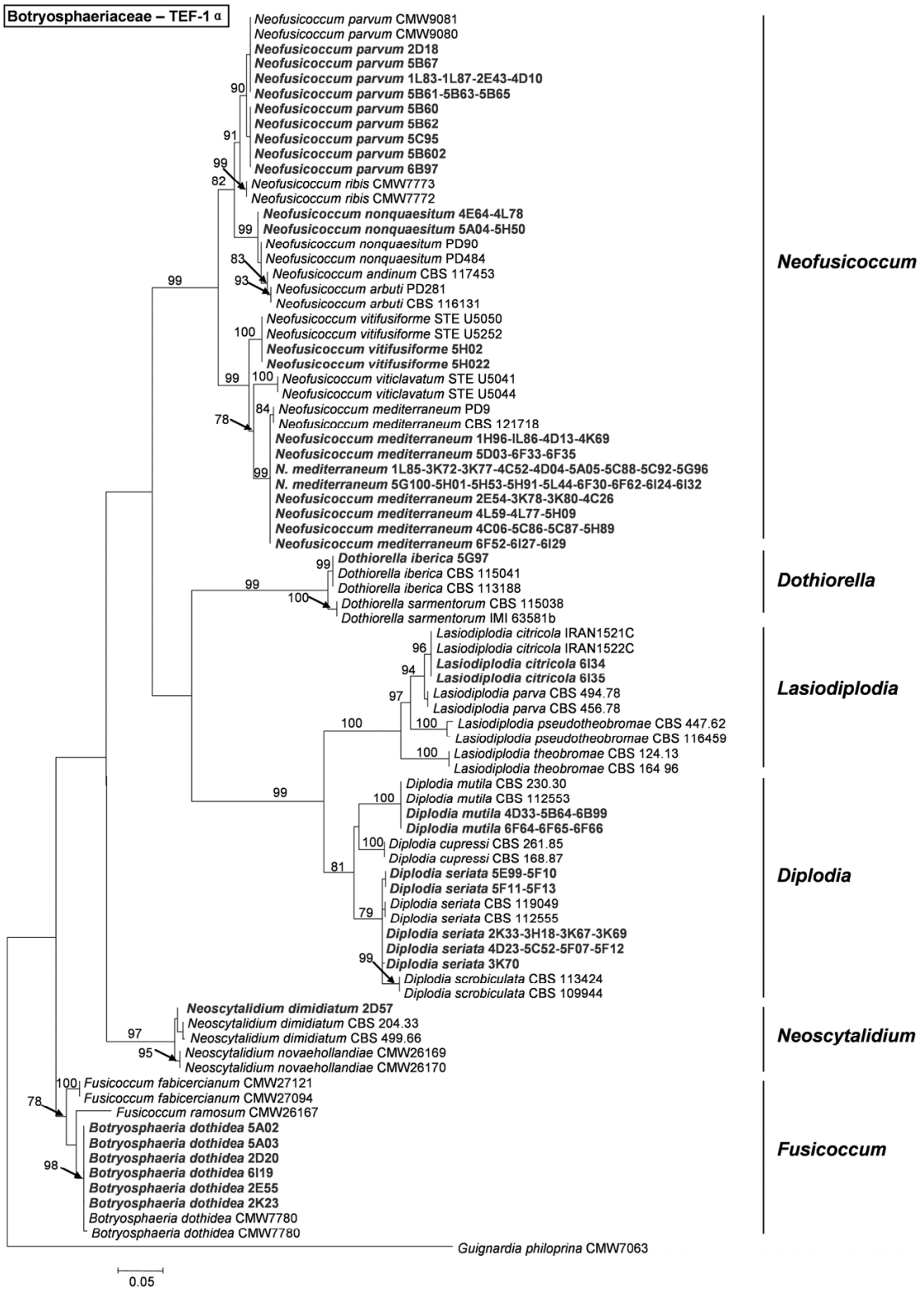


Fig. 3. Phylogenetic tree based on maximum likelihood analysis of translation elongation factor 1-α (TEF-1-α) gene sequences for various genera in the family Botryosphaeriaceae. Isolates in boldface were sequenced in this study. Bootstrap values > 70% are presented above branches. Bootstrap values < 70% are not shown. *Guignardia philoprina* (CMW7063) represents the outgroup.

Neofusicoccum in Table 3). Colonies were moderately dense, with slight aerial mycelium, initially white, then turning gray-black from the middle after 7 days. Fungal group six and seven can be distinguished by conidia morphology. Conidia of group six were fusiform to ellipsoidal, with the average L/W smaller than 4.0, while fungi in group seven were narrow fusiform, with an average L/W of approximately 5.0.

Three species of *Neofusicoccum* were identified in fungal group six: *Neofusicoccum nonquaesitum*, *N. parvum*, and *N. vitifusiforme*. The conidial shapes of these three species are similar to each other, whereas they can be distinguished from each other by their conidial sizes; conidia of *N. nonquaesitum* (average of 28.8 × 7.7 μm) are larger than those of *N. vitifusiforme* (average of 22.6 × 6.5 μm), and the conidia of *N. parvum* (average of 18.0 × 5.9 μm) are the smallest (Table 4).

B. dothidea and *N. mediterraneum* were identified in fungal morphological group seven. Colonies of *B. dothidea* (colony diameter larger than 55 mm after 24 h at the optimal temperature

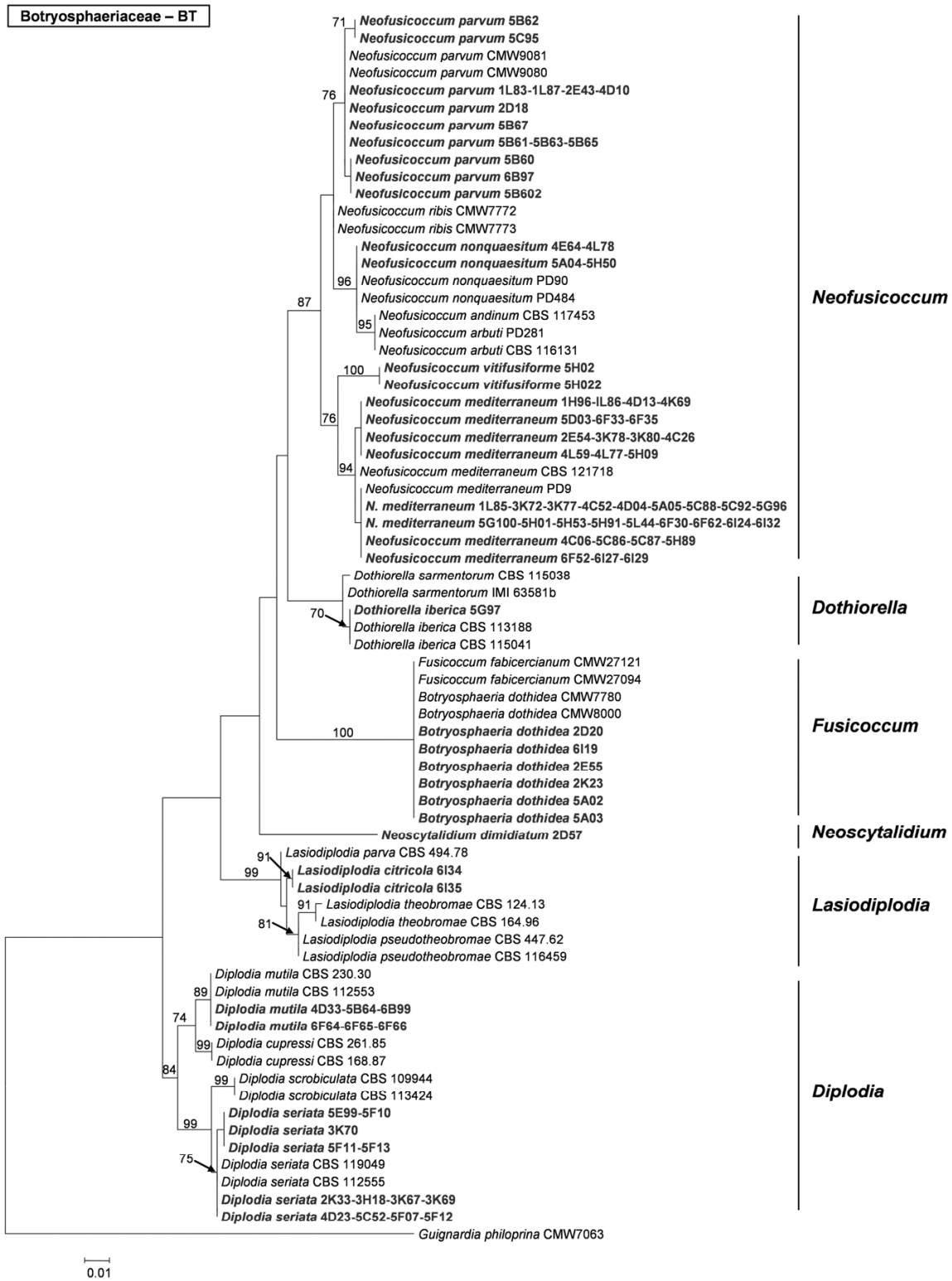


Fig. 4. Phylogenetic tree based on maximum likelihood analysis of β-tubulin (BT) gene sequences for various genera in the Botryosphaeriaceae. Isolates in boldface were sequenced in this study. Bootstrap values > 70% are presented above branches. Bootstrap values < 70% are not shown. *Guignardia philoprina* (CMW7063) represents the outgroup.

of 30°C) grew faster than *N. mediterraneum* (colony diameter <55 mm after 24 h at the optimal temperature of 25 to 30°C; Table 4).

Distribution of Botryosphaeriaceae and *Diaporthe* spp. By the sequence comparisons of ITS, TEF-1α, and BT gene regions, and combined with the colony growth characteristics, culture morphology, and conidia morphology, the 159 fungal isolates collected from diseased English walnut in California belonged to 10 different species of Botryosphaeriaceae and two species of *Diaporthe*. These include *B. dothidea* (4 isolates), *D. mutila* (1 isolate), *D. seriata* (12 isolates), *Dothiorella iberica* (1 isolate), *L. citricola* (2 isolates), *N. mediterraneum* (121 isolates), *N. nonquaesitum* (3 isolates), *N. parvum* (6 isolates), *N. vitifusiforme* (3 isolates), and *Neoscytalidium dimidiatum* (1 isolate). In addition, two species of *Diaporthe*, *Diaporthe. neotheicola* (1 isolate) and *D. rhusicola* (4 isolates), were recovered from infected California English walnut (Table 1). *Neofusicoccum mediterraneum* was dominant; 76% (121 of the 159) of the isolates were identified as this fungus. *N. mediterraneum* was widely distributed and was recovered from 10 different counties (Table 1; Fig. 1). In California, in addition to English walnut, one isolate (4D33) from avocado was identified as *Diplodia mutila*, one isolate (4E64) from *U. californica* as *N. nonquaesitum*, and two isolates from almond (6I14 and 6I15) as *Diaporthe rhusicola* (Table 1). Among 15 isolates from pomegranate, 3 (6F64, 6F65, and 6F66) were identified as *Diplodia mutila*, 1 (2K33) as *D. seriata*, and 11 (6F30, 6F52, 6F60, and 8 other isolates which are not shown) as *N. mediterraneum* (Table 1). In addition to California, two isolates (5A02 and 5A03) from walnut in Greece were identified as *B. dothidea* and, among nine walnut isolates from Spain, eight (2D18, 5B60, 5B61, 5B62, 5B63, 5B65, 5B67, and 5B602) were identified as *N. parvum* and one (5B64) as *D. mutila* (Table 1).

Inoculations of walnut branches and hulls with mycelium plugs. For the walnut branches and hulls inoculated with mycelium plugs, 21 isolates (Tables 1, 5, and 6) representing 10 species of Botryosphaeriaceae and 2 *Diaporthe* spp. on English walnut in California were selected. These included *B. dothidea* (2D20, 2E55, and 6I19), *D. mutila* (6B99 and 6F65), *D. seriata* (3H18 and 5F11), *Dothiorella iberica* (5G97), *L. citricola* (6I34 and 6I35), *N. mediterraneum* (5C87, 5H09, and 5H53), *N. nonquaesitum* (5A04), *N. parvum* (1L83, 1L87, and 2E43), *N. vitifusiforme* (5H02), *Neoscytalidium dimidiatum* (2D57), *Diaporthe neotheicola* (6I30), and *D. rhusicola* (6I44) (Tables 1, 5, and 6). There were no significant differences between the results of the two experiments; therefore, these data were combined for statistical analyses. Data of the average lesion length for the combination of two experiments are shown in Tables 5 and 6 and Figures 7 and 8.

On attached branches, all the tested fungi were able to produce longer lesions on the three tested cultivars than the noninoculated control branches (Table 5). All the tested isolates of *L. citricola* and *Neofusicoccum parvum* produced significantly longer lesions than the control (Table 5). Except for isolate 5H09 of *N. mediterraneum* on Chandler, all the tested isolates of *N. mediterraneum* produced significantly longer lesions than the control in all three cultivars (Tables 5). When results of the three cultivars were combined, *L. citricola* and *N. parvum* were clearly the most virulent species, followed by *N. mediterraneum*, *Neoscytalidium dimidiatum*, *B. dothidea*, and *Neofusicoccum nonquaesitum* (Fig. 7). Isolates of *L. theobromae* and *N. parvum* were able to kill the attached branches in all three tested cultivars. Isolates of *N. mediterraneum* killed the attached branches of Tulare and Vina 3 weeks after inoculation. The overall data revealed that Vina and Tulare are more susceptible than Chandler when their branches were inoculated with Botryosphaeriaceae fungi (Fig. 7).

The results from inoculations of fruit hulls on the trees were consistent with those from the branch inoculations. All the tested fungi produced a wider decay area than that in the control fruit (Table 6). With the exception of *Diplodia mutila*, *D. seriata*, *Dothiorella iberica*, *Diaporthe neotheicola*, and *D. rhusicola*, isolates of all the other species produced significantly larger lesions on hulls of the three cultivars (Table 6). After combining the results for the three cultivars, *N. parvum* and *L. citricola* were the most virulent, followed by *B. dothidea*, *N. mediterraneum*, *Neoscytalidium dimidiatum*, and *Neofusicoccum nonquaesitum* (Fig. 8). For Tulare and Vina, all the species of Botryosphaeriaceae were able to rot the entire hull of the fruits in 3 weeks. However, on Chandler, *Diplodia seriata*, *D. mutila*, and *Dothiorella iberica* decayed only part of the hulls 3 weeks after inoculation. Inoculations with the *Diaporthe* spp. revealed that only *Diaporthe neotheicola* was able to decay the entire fruit hull of Vina in 3 weeks. These data revealed that Vina is the most susceptible and Chandler is relatively tolerant (Fig. 8).

Effect of inoculation method on infection of walnut. Five isolates of Botryosphaeriaceae were selected to test the effect of inoculation method on infection of English walnut; these included *B. dothidea* (2E55), *L. citricola* (6I35), *N. mediterraneum* (5C87, 5H53), and *N. parvum* (1L83) (Table 7). The two experiments did not differ significantly and, thus, the two datasets were combined. The average lesion length for the combination of two experiments is shown in Table 7. Inoculations with the mycelium plug showed that all five tested isolates (Table 7) produced diseased lesions on the attached branches and decayed the fruit hulls but no lesions developed in the control branches or hulls (Table 7). However, when a spore suspension was used to inoculate branches and hulls, only *L. citricola* resulted in canker development and decayed the walnut hulls (Table 7); no cankers were produced and the hulls of

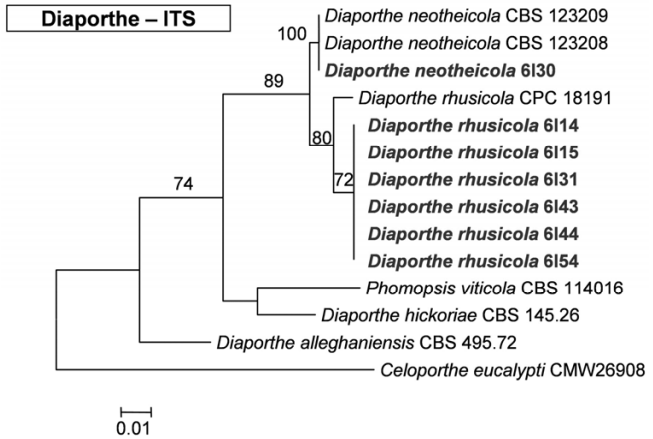


Fig. 5. Phylogenetic tree based on maximum likelihood analysis of internal transcribed spacer (ITS) nuclear ribosomal DNA sequences for various species in the genus *Diaporthe*. Isolates in boldface were sequenced in this study. Bootstrap values > 70% are presented above branches. Bootstrap values < 70% are not shown. *Celoporthe eucalypti* (CMW26908) represents the outgroup.

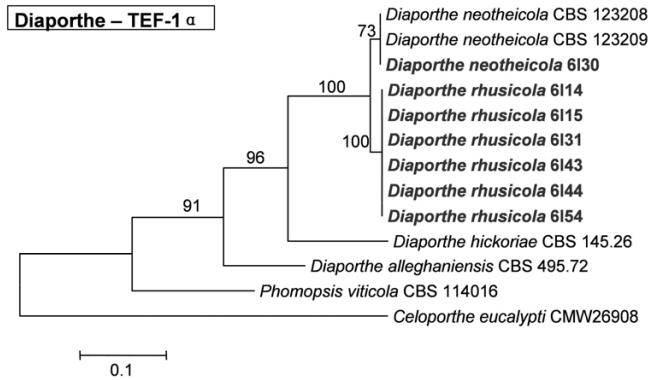


Fig. 6. Phylogenetic tree based on maximum likelihood analysis of translation elongation factor 1-α (TEF-1-α) gene sequences for various species in the genus *Diaporthe*. Isolates in boldface were sequenced in this study. Bootstrap values > 70% are presented above branches. Bootstrap values < 70% are not shown. *Celoporthe eucalypti* (CMW26908) represents the outgroup.

English walnut fruit were not decayed by the spore suspension of *B. dothidea*, *N. mediterraneum*, or *N. parvum*.

Discussion

Research in this study represents the most comprehensive consideration of species of Botryosphaeriaceae from symptomatic English walnut trees affected by stem canker and branch and twig dieback in California to date. Using comparisons of ITS, TEF-1α, and BT sequence data and combined with the morphology and growth of colonies and morphology of conidia, 10 species of Botryosphaeriaceae belonging to six genera were identified from a relatively large collection of isolates. These include *B. dothidea, Diplodia mutila, D. seriata, Dothiorella iberica, L. citricola, N. mediterraneum, N. nonquaesitum, N. parvum, N. vitifusiforme*, and *Neoscytalidium dimidiatum*. In addition, two species of *Diaporthe* (Diaporthaceae), *Diaporthe neotheicola* and *D. rhusicola*, were recovered from infected English walnut. *Diplodia mutila, Dothiorella iberica, N. nonquaesitum, N. vitifusiforme, Diaporthe neotheicola*, and *D. rhusicola* were reported for the first time from English walnut in California.

In previous studies in California, morphological features were used partially to characterize species of Botryosphaeriaceae (28,31,33,65). In this study, the colony growth characteristics, culture morphology, and conidia morphology of the English walnut fungi were tested. These morphological characteristics offered valuable information to support the species identification which was determined by DNA sequence comparisons. Results of morphological comparisons in this study revealed that there is an over-

Table 3. Effect of temperature on mycelium growth of Botryosphaeriaceae and *Diaporthe*

		Temperature and measurements (mm)[y]								
Species	**Isolate**	**5°C**	**10°C**	**15°C**	**20°C**	**25°C**	**30°C**	**35°C**	**40°C**	**Source, OPT[z]**
Botryosphaeriaceae										
Botryosphaeria dothidea	2D20	8	28.5	36.5	66	77.5	**82**	38.5	10	This study
B. dothidea	2E55	8	20.5	24	50.5	64	**72.5**	30	10	This study
B. dothidea	5A03	9	14	17	37	45	**55**	33	11	This study
B. dothidea	…	…	…	…	…	…	…	…	…	53; 25–30°C
Neofuscicoccum mediterraneum	4K69	7	11	15	20	25	**34.5**	12	10	This study
N. mediterraneum	5C87	9	18.5	25	41	**51.5**	44.5	21	12	This study
N. mediterraneum	5H09	9	12	15	23	27.5	**30.5**	12.5	10	This study
N. mediterraneum	5H53	9	12	15.5	18.5	25	**26.5**	10	9	This study
N. mediterraneum	…	…	…	…	…	…	…	…	…	18; N/A
N. nonquaesitum	4L78	8	19	24.5	26	**44.5**	35	15	11	This study
N. nonquaesitum	5A04	8	16.5	24.5	28	**42**	28.5	17	12	This study
N. nonquaesitum	…	…	…	…	…	…	…	…	…	28; N/A
N. parvum	1L87	7	25	34.5	50	**63**	62	28.5	14	This study
N. parvum	2D18	7	22.5	30	55	69.5	**78.5**	25.5	13.5	This study
N. parvum	5B60	7	19	27	41	**52**	50	18	14	This study
N. parvum	5B62	7	17	22	45	55	**59**	20.5	12.5	This study
N. parvum	5C95	8	15.5	20	26.5	33	**39**	17	12.5	This study
N. parvum	6B97	9	14.5	16.5	28.5	37	**37.5**	16	13	This study
N. parvum	…	…	…	…	…	…	…	…	…	53; N/A
N. vitifusiforme	5H02	10	13.5	16.5	38	**48.5**	46	52.5	16	This study
N. vitifusiforme	5H022	9	12	16	35	42	**44**	23	14	This study
N. vitifusiforme	…	…	…	…	…	…	…	…	…	66; 30°C
Dothiorella iberica	5G97	13	17.5	29	**40.5**	40	21	12	7	This study
D. iberica	…	…	…	…	…	…	…	…	…	44; 20–25°C
Diplodia mutila	6B99	10	14	18.5	32	**34.5**	32.5	10	10	This study
D. mutila	6F65	10	11	15	31.5	**38.5**	35.5	10	11	This study
D. mutila	…	…	…	…	…	…	…	…	…	5; 25°C
D. seriata	3H18	10	30	36.5	56	**67**	66	17	11	This study
D. seriata	3K70	7	17.5	22.5	30.5	**37.5**	34.5	13	14	This study
D. seriata	5F11	7	17.5	24.5	37.5	**51**	42.5	21	14	This study
D. seriata	…	…	…	…	…	…	…	…	…	45; 25°C
Lasiodiplodia citricola	6I34	7	19	24.5	59	73	**85**	60	54.5	This study
L. citricola	6I35	7	17	19.5	56.5	73	**85**	60	53.5	This study
L. citricola	778	6	16	22	60	70	**85**	43	41	11
L. citricola	7E79	7	17	20.5	50.5	68.5	**84**	52.5	40	11
L. citricola	7E80	7	17	20.5	63.5	75	**83.5**	51	41.5	11
L. citricola	7F93	7	37.5	50	48.5	59.5	**85**	55	43.5	14
L. citricola	7F94	7	15.5	19	52	63.5	**81**	45.5	41	14
L. citricola	7F95	7	20	30	30	62	**80**	50	42	14
L. citricola	…	…	…	…	…	…	…	…	…	1; 25–30°C
L. theobromae	7E86	7	12.5	15.5	54.5	66	**77.5**	38.5	33.5	13
L. theobromae	7E87	7	13	17	48.5	59	**76.5**	35	23	13
L. theobromae	7E88	7	14	18	50	60	**75**	40	25	13
L. theobromae	…	…	…	…	…	…	…	…	…	7; N/A
Neoscytalidium dimidiatum	2D57	7	29	37.5	50	73.5	**85**	55	50	This study
Diaporthe										
Diaporthe neotheicola	6I30	8	21	26	34.5	**47.5**	26	21	7	This study
D. neotheicola	…	…	…	…	…	…	…	…	…	50; N/A
D. rhusicola	6I14	9	19	25.5	39	**46.5**	35	21.5	7	This study
D. rhusicola	6I43	8	12	17	27	**40**	31	20	7	This study
D. rhusicola	…	…	…	…	…	…	…	…	…	17; N/A

[y] The 7.0-mm culture plugs were removed and transferred to the centers of 90 mm petri dishes containing 2% potato dextrose agar. Measurements of colony diameter were taken after 48 h for isolates of Botryosphaeriaceae and 96 h for isolates of *Diaporthe*. Measurements in bold indicate optimal growth temperature (OPT) of each isolate.

[z] Source of data and OPT of type. OPTs for the isolates have been linked to type material of the species. N/A = not available.

lap between *B. dothidea* and *N. mediterraneum* (in both culture and conidia morphology). Although the culture morphology and conidia morphology of *N. nonquaesitum* with *N. vitifusforme* and *L. citricola* with *L. theobromae* were all very similar, these species could not be distinguished easily only by their morphological characteristics. The morphological overlap has also occurred with species of Botryosphaeriaceae in California in other studies (28,31,33,41). Research results in this study further revealed that morphological features are not solely sufficient to define members within the family Botryosphaeriaceae to species.

Recently, research results indicated that DNA sequence comparisons of ITS, TEF-1α, and BT gene regions can identify species of Botryosphaeriaceae effectively and credibly (30,31,33,41,65). In California, comparing the sequence data of the ITS, TEF-1α, and BT genes successfully clarified and identified species of Botryosphaeriaceae on fruit and nut trees, including isolates from avocado (33), coast live oak (*Quercus agrifolia*; 31), and olive (41,65) trees. In this study, all the species of Botryosphaeriaceae were clearly distinguished by the phylogenetic analyses of each of the three gene regions; phylogenetic analysis results of the three gene regions were consistent with each other as well as with the combined dataset. Genetic variations noted were observed within some species, especially in the ITS gene analyses for the species of *N. mediterraneum*, whereas these variations were not supported by the

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

Species	Isolate[w]	Conidial size (µm) (L × W)[x]	Mean ± SD (µm) (L × W)[y]	L/W[z]	Source of data
Botryosphaeriaceae					
Botryosphaeria dothidea	2D20	(19.5–)21.5–26.5(–28) × (3.5–)4.5–5(–5.5)	24.0 ± 2.4 × 4.8 ± 0.5	5	This study
B. dothidea	2E55	(22.5–)24–28.5(–32) × (4.5–)4.5–6.5(–7.5)	26.4 ± 2.4 × 5.5 ± 0.8	4.8	This study
B. dothidea	5A03	(17–)21.0–27.5(–29) × (3.5–)4–5	24.3 ± 3.0 × 4.5 ± 0.5	5.4	This study
B. dothidea	**Type**	**(20–)23–27(–30) × 4–5(–6)**	**24.7 × 4.9**	**5**	**53**
Neofuscicoccum mediterraneum	4K69	(22–)23–26(–27) × (4–)4.5–5(–5.5)	24.7 ± 1.5 × 4.7 ± 0.2	5.3	This study
N. mediterraneum	5C87	(20.5–)22–26(–27) × (4–)4.5–5.5(–6.5)	24.1 ± 1.9 × 4.9 ± 0.5	4.9	This study
N. mediterraneum	5H09	(19.5–)20.5–24.5(–27) × (3.5–)4.5–5(–5.5)	22.7 ± 2.0 × 4.7 ± 0.3	4.8	This study
N. mediterraneum	5H53	(19.5–)21–26.5(–32) × (4.0–)4.5–5(–6.5)	23.8 ± 2.9 × 4.8 ± 0.8	5	This study
N. mediterraneum	**Type**	**(19–)22–26(–27) × (5.5–)6(–6.5)**	**24 × 6**	**4**	**18**
N. nonquaesitum	4L78	(21.5–)25.5–33(–36.5) × (4.0–)6–9(–9.5)	29.1 ± 3.8 × 8.1 ± 1.2	3.5	This study
N. nonquaesitum	5A04	(24–)26–30.5(–34) × (4.5–)6–8.5(–8.5)	28.4 ± 2.2 × 7.3 ± 1.1	3.8	This study
N. nonquaesitum	**Type**	**(17.2–)20.5–25.9(–29.1) × (5.6–)6.6–8.6(–10.6)**	**23.2 ± 2.7 × 7.6 ± 1.0**	**3.1**	**28**
N. parvum	1L87	(13.5–)14–17(–20) × (4.5–)5.0–7.0(–7.5)	15.6 ± 1.5 × 6.1 ± 0.8	2.6	This study
N. parvum	2D18	15–)15.5–17.5(–19.5) × (5.0–)5.5–6.5(–7.5)	16.7 ± 0.9 × 6.0 ± 0.4	2.8	This study
N. parvum	5B60	(14.5–)17–20(–21)× (4–)5.5–6.5(–7.5)	18.6 ± 1.3 × 6.0 ± 0.8	3.1	This study
N. parvum	5B62	(15–)18–21(–22) × (4.5–)5.5–7(–7.5)	19.6 ± 1.6 × 6.3 ± 0.8	3.1	This study
N. parvum	5C95	(15–)17–21(–23) × (4.5–)5–6.5(–7.5)	19.1 ± 2.1 × 5.7 ± 0.5	3.4	This study
N. parvum	6B97	(14–)16.5–20.5(–22) × (4.5–)5–6(–7.5)	18.5 ± 1.8 × 5.5 ± 0.5	3.4	This study
N. parvum	**Type**	**(12–)15–19(–24) × 4–6**	**16.9 × 5.4**	**3.1**	**53**
N. vitifusiforme	5H02	(17–)19.5–24.5(–27) × (5.5–)6–7.5(–8.5)	22.1 ± 2.5 × 6.8 ± 0.9	3.3	This study
N. vitifusiforme	5H022	(19.5–)21.5–24.5(–27) × (4.5–)5.5–7(–7.5)	23.1 ± 1.6 × 6.2 ± 0.7	3.7	This study
N. vitifusiforme	**Type**	**(18–)19–21(–22) × (4.5–)5.5–6.5(–8)**	**20 × 6.0**	**3.3**	**66**
Dothiorella iberica	5G97	(24–)24.5–27.5(–29.5) × (8.5–)8.5–10.5(–11)	26.0 ± 1.5 × 9.4 ± 0.8	2.8	This study
D. iberica	**Type**	**(17.2–)23.0–23.4(–28.6) × (8.1–)10.8–11.0(–16.0)**	**23.2 ± 1.9 × 10.9 ± 1.2**	**2.2**	**44**
Diplodia mutila	6B99	(26.5–)28–30(–32) × (11.5–)12–13(–13.5)	29.5 ± 1.4 × 12.4 ± 0.5	2.4	This study
D. mutila	6F65	(25.5–)27–30(–32) × (12–)12.5–14.5(–15)	28.6 ± 1.4 × 13.7 ± 0.8	2.1	This study
D. mutila	**Type**	**(23.5–)25.1–25.7(–27.4) × (12.4–)13.2–13.5(–14.3)**	**25.4 ± 1.0 × 13.5 ± 0.5**	**1.9**	**5**
D. seriata	3H18	(19.5–)19.5–22.5(–24.5) × (10.5–)11.5–12.5(–13.5)	21.0 ± 1.6 × 12.0 ± 0.7	1.8	This study
D. seriata	3K70	(23.5–)24–26.5(–29.5) × (9.5–)10.5–12(–12.5)	25.2 ± 1.4 × 11.2 ± 0.7	2.2	This study
D. seriata	5F11	(23.5–)25.5–28(–29.5) × (9.5–)11–12.5(–12.5)	26.5 ± 1.7 × 11.8 ± 0.7	2.2	This study
D. seriata	**Type**	**(21.5–)22–27(–28) × (11–)11.5–14.5(–15.5)**	**24.9 ± 1.9 × 12.9 ± 1.1**	**1.9**	**45**
Lasiodiplodia citricola	6I34	(18.5–)22.5–26(–27) × (12–)12.5–15(–16)	24.8 ± 2.1 × 14.4 ± 0.7	1.7	This study
L. citricola	6I35	(17–)23.5–27(–28.5) × (13–)14–15.5(–16)	26.0 ± 2.2 × 14.9 ± 0.7	1.8	This study
L. citricola	7E78	(25–)26.5–29.5(–31) × (13–)13–14.5(–15)	28.0 ± 1.5 × 13.8 ± 0.5	2	11
L. citricola	7E79	(24–)26–30(–32) × (13–)14–15(–16)	28.1 ± 1.8 × 14.6 ± 0.4	1.9	11
L. citricola	7E80	(26.5–)27–30(–32) × (12–)13–15(–16)	28.5 ± 1.4 × 14.4 ± 0.3	2	11
L. citricola	7F93	(19–)24.5–29(–30) × (13–)14–16(–17)	27.0 ± 2.5 × 14.9 ± 0.9	1.8	14
L. citricola	7F94	(24–)26–28.5(–30) × (14.5–)15–17.5(–17.5)	27.4 ± 1.7 × 16.1 ± 1.1	1.7	14
L. citricola	7F95	(20–)25–29(–30.5) × (13.5–)14–17(–18)	27.0 ± 2.0 × 15.5 ± 0.9	1.7	14
L. citricola	**Type**	**(20–)22–27(–31) × (10.9–)12–17(–19)**	**24.5 ± 0.2 × 15.4 ± 1.8**	**1.6**	**1**
L. theobromae	7E86	(16–)21–26.5(–30) × (9.5–)13–14.5(–16)	23.5 ± 2.0 × 13.8 ± 0.7	1.7	13
L. theobromae	7E87	(17.5–)20–24(–28) × (9–)11.5–13(–15.5)	22.0 ± 1.9 × 12.5 ± 0.4	1.8	13
L. theobromae	7E88	(17.5–)21.5–25(–27) × (10–)12.5–14(–15)	23.3 ± 1.8 × 13.3 ± 0.6	1.8	13
L. theobromae	**Type**	**(19–)21–31(–32.5) × (12–)13–15.5(–18.5)**	**26.2 ± 2.6 × 14.2 ± 1.2**	**1.9**	**7**
Neoscytalidium dimidiatum	2D57				
Ellipsoidal conidia	…	(7–)9–12(–15) × (2.5–)3.5–4.5(–5)	10.9 ± 0.8 × 3.9 ± 0.4	2.8	This study
Muriform conidia	…	(7–)9.5–12.5(–20) × (4–)6.5–10.5(–14.5)	11.1 ± 0.6 × 8.5 ± 0.5		This study
Arthroconidia	…	(3–)5–7(–12) × (2–)3.5–5.5(–7)	6.0 ± 0.3 × 4.0 ± 0.2	1.5	This study
Diaporthe					
Diaporthe neotheicola	6I30	(5.5–)6.5–9.5(–12) × 2–3(–5)	7.9 ± 1.6 × 2.5 ± 0.5	3.1	This study
D. neotheicola	**Type**	**(6.6–)7.6–8(–9.5) × (1.9–)2.2–2.3(–2.6)**	**7.8 ± 0.6 × 2.2 ± 0.2**	**3.5**	**50**
D. rhusicola	6I14	(7.5–)8–9(–10) × (2.5–)3–4	8.5 ± 0.7 × 3.2 ± 0.2	2.7	This study
D. rhusicola	6I43	(7–)8–9.5(–11) × (2.5–)3–3.5(–4)	8.7 ± 0.7 × 3.4 ± 0.3	2.6	This study
D. rhusicola	**Type**	**(7–)8–9(–10) × 3(–3.5)**	**8.5 × 3**	**2.8**	**17**

[w] Type = isolates for measurements are ex-type or from samples that have been linked to type material of the species. The information of the type is in boldface.
[x] L × W = length by width; minimum–(average – standard deviation [SD])–(average ± SD)–maximum.
[y] L × W = length by width.
[z] L/W = average length/average width.

TEF-1α or BT gene analyses, suggesting that these reflect intraspecific sequence difference rather than interspecific variation. Multiple phylogenetic subgroups were also observed within *N. parvum* in the phylogenetic analyses of ITS, TEF-1α, and BT gene regions. Although cryptic species may exist, further genetic analyses with additional genes using a more extensive population sample may help to resolve this intraspecific or interspecific variation.

Results of pathogenicity tests revealed that *L. citricola* is the most pathogenic to the tested English walnut branches and hulls. Before this study, *L. citricola* was isolated from the graft union of English walnut in Tulare County in California (11). This fungus, with *Neoscytalidium dimidiatum*, caused cankers at the graft union and killed the English walnut scion under an open environment in a nursery (11). Species of *Lasiodiplodia* were reported in California from other nut and fruit trees, including *L. citricola* on peach (14), *L. crassispora* on grapevine (64), and *L. theobromae* on almond (13) and grapevine (62). Inoculations with these fungi showed that all are highly virulent to their respective hosts. Although only two isolates of *Lasiodiplodia* were collected in this study from walnut in Stanislaus County, it is recommended that the frequency of these fungi should be monitored to determined their build up and spread in other California counties.

In California, *Neofusicoccum parvum* has been reported from almond (28), avocado (33,34), citrus (*Citrus* sp.) (3), English walnut (28), and grapevine (61). Inoculations of *N. parvum* on almond, avocado, citrus, and grapevine indicated that it is highly virulent (3,28,34,61). In our study, *N. parvum* was second only to *L. citricola* in terms of its virulence, which further supports the view that *N. parvum* is one of the most damaging species of Botryosphaeriaceae on trees (54). Similarly to *L. citricola*, a relatively small

Table 5. Average canker length on branches of *Juglans regia* 'Chandler', 'Tulare', and 'Vina' caused by mycelium plug inoculations with Botryosphaeriaceae and *Diaporthe* fungi

		Canker length (mm)[z]		
Species	**Isolate number**	**Chandler**	**Tulare**	**Vina**
Botryosphaeria dothidea	2D20	24.2 m–u	37 j–t	58.5 i–k
	2E55	32 k–u	34 k–u	30 l–u
	6I19	22.1 m–u	43.9 j–o	30 l–u
Diplodia mutila	6B99	14.8 q–u	19.8 n–u	20 n–u
	6F65	22 m–u	16.9 p–u	20 n–u
D. seriata	3H18	17.3 n–u	20 n–u	17.8 n–u
	5F11	17.6 n–u	19.9 n–u	18 n–u
Dothiorella iberica	5G97	15 q–u	15.2 q–u	18 n–u
Lasiodiplodia citricola	6I34	155 bc	157.5 bc	177 ab
	6I35	86 gh	132.5 cd	196 a
Neofusicoccum mediterraneum	5C87	41 j–q	83 g–i	53 j–l
	5H09	32 k–u	48 j–m	37 j–t
	5H53	40 j–r	43.3 j–p	37.5 j–s
N. nonquaesitum	5A04	43 j–p	27.2 l–u	26 l–u
N. parvum	1L83	114.5 d–f	190 a	157 bc
	1L87	80.5 g–i	82 g–i	177 ab
	2E43	99 fg	130.5 c–e	104 e–g
N. vitifusiforme	5H02	15.6 q–u	25 m–u	19 n–u
Neoscytalidium dimidiatum	2D57	44.5 j–n	30.3 l–u	63.5 h–j
Diaporthe neotheicola	6I30	13.3 r–u	16.2 p–u	15.5 q–u
D. rhusicola	6I44	13.9 q–u	16.5 p–u	15.5 q–u
Control	…	9.9 t–u	10.3 s–u	9 u

[z] Numbers followed by different letters are significantly different ($P = 0.05$).

Table 6. Average rot diameter of fruit hulls of *Juglans regia* 'Chandler', 'Tulare', and 'Vina' caused by mycelium plug inoculations with Botryosphaeriaceae and *Diaporthe* fungi

		Hull rot diameter (mm)[z]		
Species	**Isolate number**	**Chandler**	**Tulare**	**Vina**
Botryosphaeria dothidea	2D20	36.6 j–p	44.7 e–j	56.5 ab
	2E55	37.4 j–o	52.0 a–f	52 a–f
	6I19	35.6 k–p	44.4 f–j	55.6 a–c
Diplodia mutila	6B99	14.8 v–x	39.3 i–m	42.5 g–k
	6F65	17.5 u–w	25 r–u	40.6 h–l
D. seriata	3H18	19.1 t–v	29.7 n–r	41.7 g–l
	5F11	16.4 v–x	33.6 l–q	39.8 h–m
Dothiorella iberica	5G97	13 v–x	21 s–v	26 q–t
Lasiodiplodia citricola	6I34	59.5 a	53 a–e	60 a
	6I35	54 a–d	58.5 a	59 a
Neofusicoccum mediterraneum	5C87	41 h–l	55.5 a–c	50 b–g
	5H09	37.5 j–o	39.0 i–m	41.5 h–l
	5H53	40 h–l	53.9 a–d	38.5 i–m
N. nonquaesitum	5A04	28.5 p–s	41.5 h–l	38 i–n
N. parvum	1L83	58 ab	60 a	60 a
	1L87	54 a–d	48 c–h	60 a
	2E43	57 ab	60 a	60 a
N. vitifusiforme	5H02	20.5 s–v	31.1 o–r	37.5 j–o
Neoscytalidium dimidiatum	2D57	38 i–n	38 j–o	46 d–n
Diaporthe neotheicola	6I30	13.7 v–x	16 v–x	31.5 m–r
D. rhusicola	6I44	14.1 v–x	15.5 v–x	20.5 s–v
Control	…	9.9 w–x	9.6 w–x	9 x

[z] Numbers followed by different letters are significantly different ($P = 0.05$).

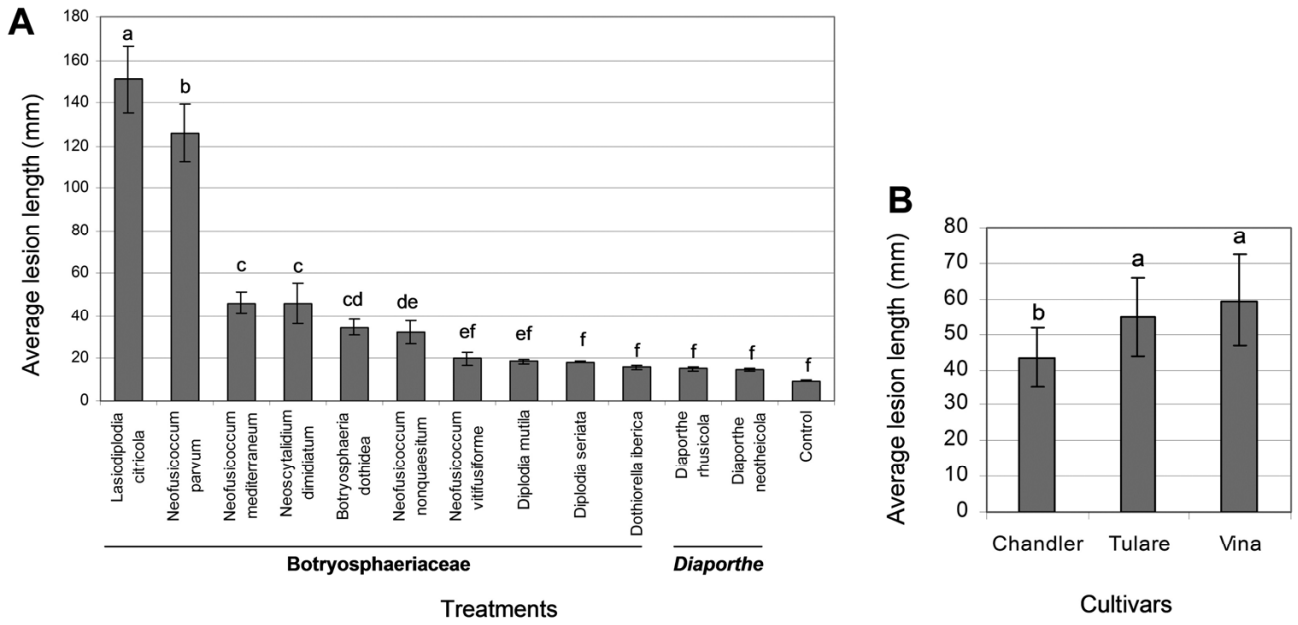


Fig. 7. A, Average lesion length (mm) on attached walnut branches after inoculation with a mycelium plug of 10 Botryosphaeriaceae spp. and 2 species of *Diaporthe*. Inoculations were conducted on three walnut cultivars in two experiments. Bars topped with different letters indicate treatment means that are significantly different (P = 0.05). **B,** Average lesion length (mm) on attached branches of three walnut cultivars after inoculation with a mycelium plug of each of 10 Botryosphaeriaceae spp. and 2 species of *Diaporthe* (average of two experiments). Bars topped with different letters indicate significant differences among cultivars (P = 0.05).

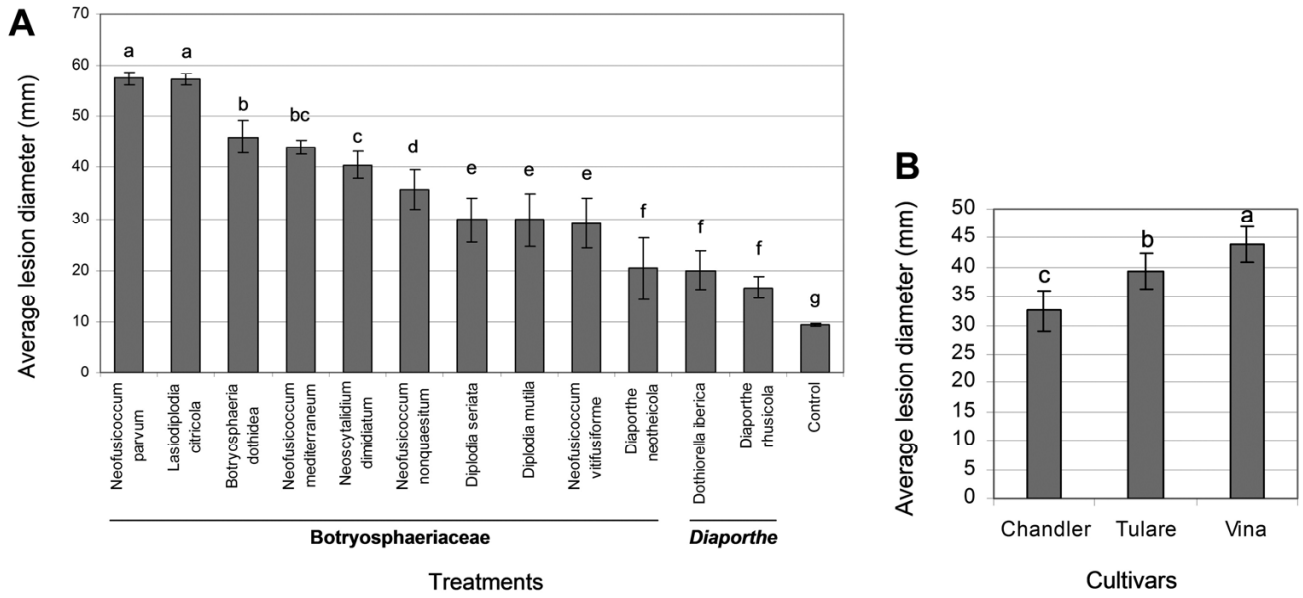


Fig. 8. A, Average lesion diameter (mm) on hulls of attached fruit on the tree after inoculation with a mycelium plug of 10 Botryosphaeriaceae spp. and 2 species of *Diaporthe*. Inoculations were conducted on fruit of three walnut cultivars in two experiments. Bars topped with different letters indicate treatment means that are significantly different (P = 0.05). **B,** Average lesion length (mm) on hulls of attached walnut fruit of three walnut cultivars after inoculation with a mycelium plug of each of 10 Botryosphaeriaceae spp. and 2 species of *Diaporthe* (average of two experiments). Bars topped with different letters indicate significant differences among cultivars (P = 0.05).

amount (six isolates) of *N. parvum* was isolated from relatively few regions (Butte, Stanislaus, and Yolo Counties) but it is necessary to monitor the spread of this species to other regions in California.

N. mediterraneum on English walnut was first reported by Trouillas et al. (57) and caused twig and branch dieback. In their study, pathogenicity tests indicated that this species is highly virulent to English walnut trees (57). In our study, *N. mediterraneum* was the most frequently encountered species among all the species of Botryosphaeriaceae and *Diaporthe*, and was widely distributed in nearly all the counties of California where samples were collected. Inoculations showed that *N. mediterraneum* is intermediately virulent to English walnut among highly and less virulent species. Previous research showed that *N. mediterraneum* was common on other tree hosts in California, such as almond (28) and olive (41); in addition, inoculations indicated that it is intermediately to highly virulent to almond, grapevine, and olive (28,41,60,64). These results suggest that future research on disease management should focus on this widespread and virulent pathogen of English walnut in California.

Botryosphaeria dothidea, *Diplodia mutila*, *D. seriata*, *Dothiorella iberica*, *N. nonquaesitum*, *N. vitifusiforme*, and *Neoscytalidium dimidiatum* were intermediately or weakly virulent to the tested English walnut cultivars in this study and they were not isolated frequently from English walnut in California. These species were also reported from other trees in California. *B. dothidea* has been isolated from almond (28), olive and pistachio (41), and many other hosts (22,38). *Diplodia mutila* was isolated from avocado, *Ilex* sp. (28), grapevine, and *Phoenix dactylifera* (61). *D.*

Table 7. Lesion lengths produced by species of Botryosphaeriaceae using two different inoculation methods; results show the average of three cultivars for all periodic inoculations of branches and fruit hulls on trees

		Treatments[z]			
		Mycelium plugs		Conidia suspension	
Species	Isolate number	On branches	On hulls	On branches	On hulls
Botryosphaeria dothidea	2E55	32.47 d	53.57 bc	12.33 c	12.87 cd
Lasiodiplodia citricola	6I35	149 a	58.58 a	30.57 a	46.97 a
Neofusicoccum mediterraneum	5C87	52.87 c	54.42 b	13.52 b	13.92 c
N. mediterraneum	5H53	39.39 d	52.03 c	12.25 c	12.9 cd
N. parvum	1L83	84.93 b	59.67 a	12.65 c	15.55 b
Control	…	9.57 e	9.77 d	12.08 c	11.93 d

[z] Numbers followed by different letters are significantly different ($P = 0.05$).

seriata was recovered from almond, English walnut, pistachio, *Prunus persica* (28), grapevine (62), and olive (41). *Dothiorella iberica* was recovered from avocado (33), coast live oak (31), and grapevine (61). *Neofusicoccum nonquaesitum* was recovered from almond and *U. californica* (28), *N. vitifusiforme* was recovered from olive (65), and *Neoscytalidium dimidiatum* was recovered from fig (*Ficus carica*; 28). Because all of these species were not highly virulent to their hosts or not widespread among the regions where walnut are grown, they are not considered important pathogens of walnut presently. However, these fungi still need to be monitored carefully, because some of them may spread and act as important pathogens under a suitable environment.

Two species of *Diaporthe* (*Diaporthe neotheicola* and *D. rhusicola*) were identified in this study. *D. neotheicola* (synonym *Phomopsis theicola*) has been reported as a weak pathogen on several hosts, such as almond (19), *Foeniculum vulgare* (50), and kiwifruit (*Actinidia deliciosa*; 56). Furthermore, *D. rhusicola* was recently identified from the leaves of *Rhus pendulina* (17). Inoculation results indicated that these species are weak pathogens of English walnut in California.

Results in this study revealed that inoculation of English walnut branches and hulls with a mycelium plug of Botryosphaeriaceae and *Diaporthe* spp. resulted in much more severe disease than inoculation with a suspension of conidia. In the field, the anamorphic structures (pycnidia) were common, while the teleomorphic structures of Botryosphaeriaceae and *Diaporthe* spp. were rare on English walnut (T. J. Michailides, *unpublished data*). In the natural environment, the conidia of these species need water to exude from pycnidia and for dissemination and deposition on host tissue (4,60). Because the germination of conidia is important for infection of English walnut, the conidia of the various species of Botryosphaeriaceae or *Diaporthe* will differ in their ability to infect walnut. Therefore, it is critical to determine whether there are compounds in walnut tissues that favor or inhibit the germination of Botryosphaeriaceae or *Diaporthe* conidia on the surface of walnut tissues.

In this study, in addition to California, species of Botryosphaeriaceae were also identified from other regions on English walnut, including *Diplodia mutila* and *Neofusicoccum parvum* from Spain and *B. dothidea* from Greece. Pathogenicity of these isolates on English walnut was not tested in this study, while the inoculation results for these species in this study suggest that these fungi in Spain and Greece should be monitored, because blight caused by species of Botryosphaeriaceae has caused significant yield losses in walnut grown in Spain and the disease has been reported in walnut grown in Greece (T. J. Michailides, *unpublished data*).

This study represents the first detailed research about the phylogeny, morphology, distribution, and pathogenicity of species of Botryosphaeriaceae on English walnut in California and a number of significant findings were discovered. A relatively large number of species of Botryosphaeriaceae and two species of *Diaporthe* were identified; species of Botryosphaeriaceae were widely distributed on English walnut in California. Most of the identified species were also reported from other fruit and nut trees; frequently, these tree species are adjacent or in close proximity to English walnut orchards throughout both the Sacramento and San Joaquin Valleys. The occurrence of similar fungal species in all three major nut tree crops in California suggests that cross infection could occur among these crops. Inoculations revealed that all the species identified in this study are pathogenic to the English walnut branches or hulls, with *L. citricola* and *N. parvum* being the most virulent and *N. mediterraneum* being intermediately virulent, despite the fact that is the most widely distributed Botryosphaeriaceae sp. on walnut. It is essential that these pathogens be monitored carefully in order to help make decisions on disease management. Inoculation of the three English walnut cultivars used in this study further suggests that there may be differences in susceptibility or tolerance of the different English walnut cultivars. Therefore, specific control measures need to be developed for the highly susceptible English walnut cultivars in California.

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