Title

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Journal

Plant Disease, 90(12)

ISSN

0191-2917

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Publication Date

2006-12-01

Peer reviewed

Identification and Distribution of *Botryosphaeria* spp. Associated with Grapevine Cankers in California

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ABSTRACT

Úrbez-Torres, J. R., Leavitt, G. M., Voegel, T. M., and Gubler, W. D. 2006. Identification and distribution of *Botryosphaeria* spp. associated with grapevine cankers in California. Plant Dis. 90:1490-1503.

Botryosphaeria spp. recently have been identified as important grapevine pathogens worldwide. To date, *Botryosphaeria rhodina* has been the only species associated with cankers on *Vitis vinifera* in California. A field survey of 166 vineyards in 21 counties was conducted in order to determine the occurrence of other *Botryosphaeria* spp. in California. In all, 1,735 samples of cankered trunks, cordons, and spurs were collected. *Botryosphaeria* spp. were the most common fungi isolated from grapevine cankers in California. Morphological identification along with phylogenetic analysis of the internal transcribed spacer region (ITS1-5.8S-ITS2) of the nuclear ribosomal DNA (rDNA) and a partial sequence of the β-tubulin gene showed that at least seven *Botryosphaeria* spp. occur on grapevines in California: *B. australis*, *B. dothidea*, *B. lutea*, *B. obtusa*, *B. parva*, *B. rhodina,* and *B. stevensii*. *Botryosphaeria* spp. were found in grapevine cankers in all grape-growing regions surveyed in California, whereas incidence and distribution varied with location. Grapevine cankers in California have been associated mainly with Eutypa dieback. However, the frequent recovery of *Botryosphaeria* spp. from cankers in this study indicates that the role of these fungi in grapevine health needs to be more carefully considered.

Additional keywords: esca, *Eutypa lata*, *Phomopsis viticola*, trunk diseases

The California grapevine industry (wine, raisin, and table grapes) presently comprises over 345,200 ha and is the third most important agricultural commodity in the state, producing an annual crop valued at over $2.3 billion (40). Fungal trunk diseases have become a growing threat to grapevines in California and throughout the world. Trunk diseases of grapevine are caused by fungal pathogens that invade through pruning wounds located on the woody parts of the plant. Symptoms include a slow decline and dieback of the vine as a result of interruption of xylem conductivity or toxin production (19). Trunk diseases principally include black measles (esca), Eutypa dieback, young vine decline, and other diseases caused by *Botryosphaeria* spp. (19). Trunk diseases reduce yields and increase production costs. Production costs are increased as a result of cultural and chemical preventive measures as well as removing diseased wood from the vine after infection (9). In California, the overall loss in net income for wine grape caused by Eutypa dieback and Botryosphaeria canker disease was estimated to be over $260 million per annum (32).

Botryosphaeria spp. occur on a large number of hosts and have a wide geographical distribution (26,42). Since this genus was introduced in 1863 (Ces. & De Not.), different species of *Botryosphaeria* have been well established as canker-causing agents in several woody plants (26,27). However, their association with dieback in grapevine has been overlooked for many years because *Botryosphaeria* spp. have been largely considered saprophytes or secondary colonizers in grapevines (25). To date, 13 *Botryosphaeria* spp. have been reported to cause disease symptoms in grapevines in different production regions worldwide, but only in the last decade has the significance of these fungi as grapevine pathogens been recognized (25,37,41). *Botryosphaeria* spp. known to occur in grapevine include *B. australis* Slippers, Crous, & M. J. Wingf., *B. dothidea* (Moug.:Fr.) Ces. & De Not., *B. lutea* A. J. L. Phillips, *B. obtusa* (Schwein.) Shoemaker, *B. parva* Pennycook & Samuels, *B. rhodina* von Arx, *B. ribis* Gross. & Duggar, *B. stevensii* Shoemaker, *B. viticola* A. J. L. Phillips & Luque, and the anamorphs *Diplodia sarmentorum* (Fr.:Fr.) Fr., *D. porosum* Niekerk & Crous, *Fusicoccum viticlavatum* Niekerk & Crous, and *F. vitifusiforme* Niekerk & Crous (16,25,41).

Botryosphaeria spp. frequently have been isolated from grapevines showing decline or dieback symptoms in Egypt (5), California (9,14), Arizona, Mexico (13), Hungary (15), France (12), Italy (29), Portugal (23–25), Spain (16,39), South Africa (41), Chile (1), and Australia (2,37). Pathogenicity and epidemiology of *Botryosphaeria* spp. in vines have been the subject of confusion for many years (25,41). Virulence and symptoms caused by *Botryosphaeria* spp. on grapevines have been reported to be different depending on cultivars and countries. For example, *B. dothidea*, *B. obtusa,* and *B. stevensii* were found to be the cause of "black dead arm" in France. The disease was characterized by wood streaking and red patches at the margin of the leaves, with large areas of chlorosis and deterioration between the veins (12). In contrast, no symptoms of black dead arm were found associated with the same species on grapevines in Portugal (23,25).

The best example of controversy concerning pathogenicity of *Botryosphaeria* spp. occurs with *B. obtusa*. Although this species has been reported as virulent on grapevine in Chile (1), New South Wales region in Australia (2), and South Africa (41), it has been considered weakly pathogenic in Portugal (25). In another study, *B. obtusa* did not cause any symptom on inoculated grapevine cuttings in Western Australia (37). Several vascular and foliar symptoms attributed to *Botryosphaeria* spp. in grapevine, such as perennial cankers, trunk dieback, wood necrosis, vascular streaking and mild chlorosis, or wilting of the leaves, have been described (12,25,41), but they often are difficult to distinguish from symptoms caused by other fungal pathogens, such as *Eutypa lata* (Pers.:Fr.) Tul. & C. Tul. (2), *E. leptoplaca* (Mont.) Rappaz (38), and *Phomopsis viticola* (Sacc.) Sacc. (2,24). Thus, the relationships between *Botryosphaeria* spp. and grapevine disease symptoms have been difficult to determine.

To date, grapevine trunk diseases in California have been associated mainly with *E. lata*, *Phaeomoniella chlamydospora,* and different species of *Phaeo-*

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This work was partially funded by grants from the American Vineyard Foundation and the Viticultural Consortium.

Accepted for publication 20 June 2006.

DOI: 10.1094/PD-90-1490

acremonium causing Eutypa dieback, Petri disease, and esca (black measles), respectively (6,19,31,32). New findings also have shown two species of *Cylindrocarpon* to be the cause of black foot disease of Californian grapevines (21). However, the role of *Botryosphaeria* spp. on grapevines in the state has remained largely unknown. Of the *Botryosphaeria* spp. that are recognized to occur on grapevine worldwide, *B. rhodina* was the only species reported from Californian grapevines (14,35,43). It is associated with wedge-shaped cankers and grapevine dieback in California and the disease it causes has been known locally as "Bot canker." *B. rhodina* is now considered an endemic pathogen in arid climate regions of southern California (10).

The objectives of this study were to (i) identify the species of *Botryosphaeria* associated with canker symptoms in Californian grapevines based on morphological and molecular characteristics and (ii) determine their geographic distribution in California vineyards.

MATERIALS AND METHODS

Field survey, fungal isolation, and data analysis. Between 10 and 15 perennial cankers from spurs, cordons, or trunks were collected from different grapevines in each surveyed vineyard (Fig. 1a–c). In all, 1,735 samples were collected from 166 vineyards in 21 counties throughout California between 2003 and 2005. Samples were obtained from 111 and 55 vineyards from the predominant wine and table-raisin grape cultivars of California, respectively. Grapevine samples were cleaned of loose bark and surface disinfested in 10% sodium hypochlorite for 10 min. After air drying, the surface tissue was cut away to expose both sides of the canker. Small pieces of tissue from both sides of the canker were placed on 85-mm-diameter petri dishes containing 4% potato dextrose agar (PDA) (Difco Laboratories, Detroit) amended with tetracycline hydrochloride (0.01%) (Sigma-Aldrich, St. Louis) (PDA-tet). Cultures were incubated at room temperature until fungal colonies were observed. Pure cultures of *Botryosphaeria* spp. were obtained by excising a hyphal tip from colony margins emerging from the tissue pieces onto fresh plates of PDA-tet.

Statistical analysis was performed in order to determine the relationship between the incidence of *Botryosphaeria* spp. and grapevine regions, grapevine age, and grapevine cultivar. Data were analyzed using analysis of variance (ANOVA) on the proportion of samples yielding *Botryosphaeria* spp. from each vineyard (ANOVA using PROC GLM, SAS System, version 8.1; SAS Institute, Cary, NC). To satisfy the assumptions of the ANOVA, the arcsine transformation of the proportion was used ($Y = 2 \times \arcsin \sqrt{p}$). Homogeneity of variance was assessed using Levene's test. Tukey's test was used for treatments means comparisons. Vineyards from 21 counties were split into eight grapevine regions (Table 1).

Morphological characterization. *Botryosphaeria* spp. isolated from cankers were identified tentatively based on colony morphology and conidial characteristics reported in previous studies (20,25,33,37). *Botryosphaeria* isolates first were divided based on colony morphology. The color and growth from these isolates were recorded during 15 days of growth on PDA-tet at 25ºC in a 12-h light-and-dark cycle, illuminated by fluorescent and near-ultraviolet light (366 nm). Characteristics of conidial morphology in 30 *Botryosphaeria* isolates also were observed after placing cultures on 2% water agar (Difco Laboratories) containing autoclaved grapevine wood chips. Isolates were incubated at 25ºC under intermittent light (12 h) for 5 weeks to induce sporulation (41). The length and width of 60 conidia per isolate were measured with the aid of a compound microscope (Nikon Inc. Instruments Group, Melville, NY) using the imaging device SPOT RT software (v3.5.1, SPOT; Diagnostic Instruments Inc., MI). Mean and standard deviation of the conidial measurements were obtained using summary statistics in SAS and compared with those previously reported for *Botryosphaeria* spp. Conidial color, shape, and presence or absence of septation also were recorded.

Botryosphaeria isolates selected for conidial characterization also were used to determine the effect of temperature on colony growth. A 5-mm-diameter plug from the growing margin of a 3-day-old colony was placed in the center of an 85-mm-diameter PDA-tet petri dish, and three replicates of each isolate were incubated separately at 5, 10, 15, 20, 25, 30, 35, and 40ºC in the dark. Colony diameter was measured after 2, 3, and 4 days of incubation, and data were converted to radial growth in millimeters (30). The experiment was conducted twice. Radial growth values after 48 h versus temperature were adjusted to regression curves using the Rat-

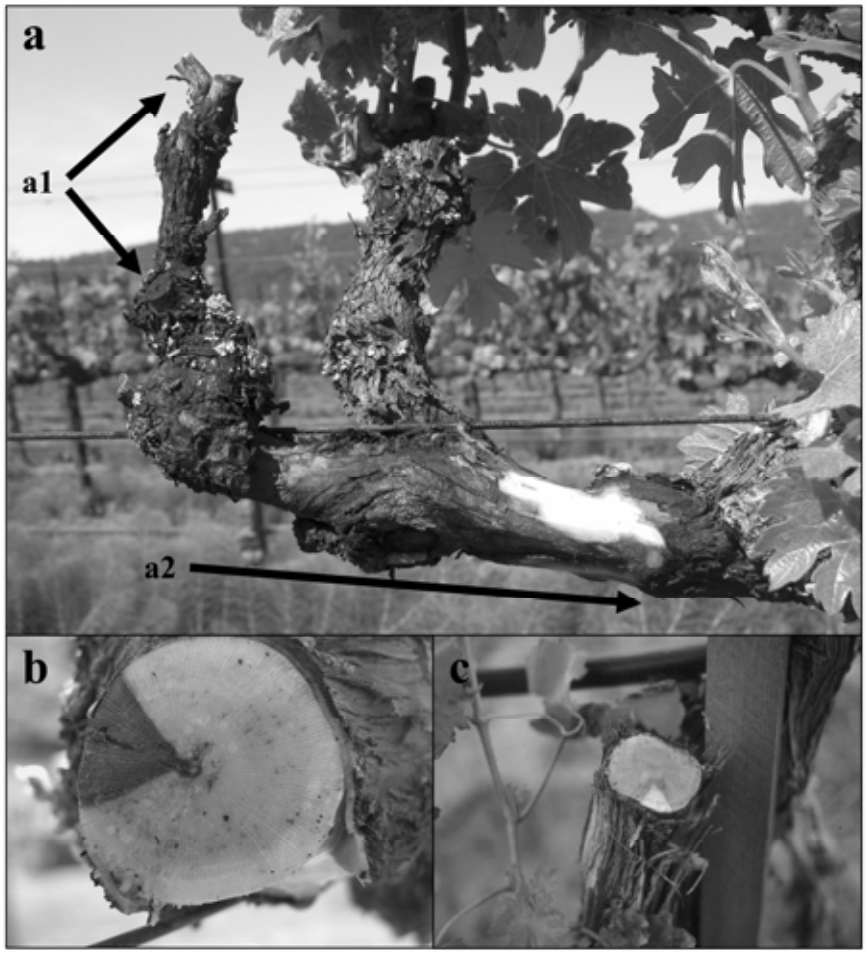

Fig. 1. a, Dead spur position and perennial developing canker in an 18-year-old Merlot grapevine. **a1,** Infection starts when conidia or ascospores colonize fresh pruning wounds. **a2,** Lengthwise canker growth develops slowly from the point of infection basipetally. **b,** Cross section of a 12-year-old Zinfandel grapevine cordon. Wedge-shaped canker is the typical symptom in the earliest stages of the disease. **c,** Mature canker in the trunk of a 17-year-old Cabernet Sauvignon grapevine. Perennial cankers also grow in a lateral direction in the spurs, cordons, and trunks of the vine parts for several years until only a small wedge of tissue remains alive. Death of the vines parts occurs when the remaining tissue is killed by the growth of the fungus.

kowsky model (28); a four-parameter model fit the values in the regression model $\sqrt{r} = b\ (T - T_{min})(1 - \exp\ [c(T - T_{max})])$, where $\sqrt{r}$ is the square root of the radial growth, b is a regression coefficient of the square root of growth rate constant versus degrees Kelvin for temperatures below the optimal temperature, T_{min} and T_{max} are the minimum and maximum temperatures, respectively, at which the rate of growth is zero, whereas c is an additional parameter to enable the model to fit the data for temperatures above the optimal temperature (28). Nonlinear least squares were used to obtain the regression curves and optimum temperatures were calculated using numerical maximization (analyses performed using R version 2.2.0; Free Software Foundation, Inc., MA). Optimum temperature was defined as the temperature that produced maximum radial growth. ANOVA was used to determine the effects of radial growth difference by species at the optimum temperature after 48 h.

DNA isolation, amplification, and phylogenetic analyses. Total genomic DNA from the *Botryosphaeria* isolates selected for morphology study was extracted from pure cultures as described by Cenis (3) (Table 2). Once DNA was obtained, oligonucleotide primers ITS4 and ITS5 were used to amplify and sequence the internal transcribed spacer (ITS) region, including the 5.8S (44). Partial sequence of the β-tubulin (BT) gene, BT2, was amplified using primers Bt2a and Bt2b (8). Polymerase chain reaction (PCR) reactions were carried out in a thermal cycler (PTC 200; M. J. Research Company, Watertown, MA) as follows: an initial preheat for 2 min at 94ºC, followed by 35 cycles of denaturation at 94ºC for 60 s, annealing at 58ºC for 60 s, and extension at 72ºC for 90 s. The PCR amplification products were separated by electrophoresis in 1.2% agarose gels in 1.0× Tris-boric acid-EDTA (TBE) buffer and photographed after staining with ethidium bromide for 10 min. PCR products were purified using the QIAquick PCR purification kit (Qiagen Inc., Valencia, CA). ITS and β-tubulin regions were sequenced in both directions by the University of California, Davis, Division of Biological Sciences (DBS) sequencing facility.

Sequences were edited using the software program Sequencher (version 4.1; Gene Codes, Ann Arbor, MI) and manually aligned using the computer software BioEdit sequencer alignment editor (version 7.0.0; Tom Hall, Isis Pharmaceuticals, Inc., Carlsbad, CA). Alignment gaps were treated as missing data. Whenever possible, sequences of *Botryosphaeria* spp. from grapevine and other hosts from previous studies available in GenBank were included in our analyses (Table 3). Separate phylogenetic analyses were run for the ITS dataset alone, β-tubulin dataset alone, and combined ITS and β-tubulin datasets using PAUP (version 4.0b10; Sinauer Associates, Inc., Sunderland, MA; 36). *Guig-*

Table 1. Incidence of *Botryosphaeria* spp. in the main grapevine-production areas of California

Grapevine region	Vineyards[b]	No. (%) *Bot.*[c]	Total[d]	Number (%) of cankers yielding[a]				
				A[e]	B[f]	A + B[g]	*E. lata*	*P. viticola*
North Coast								
Mendocino Co.	8	8 (100)	91	37 (41)	11 (12)	48 (53)	11 (12)	0
Napa Co.	11	10 (91)	113	30 (26)	13 (11)	43 (38)	54 (48)	0
Sonoma Co.	17	17 (100)	192	85 (44)	13 (7)	98 (51)	41 (21)	0
Total	36	35 (97)	396	152 (38)	37 (9)	189 (48)	106 (27)	0
Central Coast								
Santa Clara Co.	2	2 (100)	23	10 (43%)	7 (30)	17 (74)	4 (17)	0
San Benito Co.	6	6 (100)	65	49 (75%)	9 (14)	58 (89)	6 (9)	1 (1)
Monterey Co.	6	6 (100)	65	44 (68)	4 (6)	48 (74)	13 (20)	0
San Luis Obispo Co.	8	8 (100)	80	37 (46)	7 (9)	44 (55)	15 (19)	0
Total	22	22 (100)	233	140 (60)	27 (12)	167 (72)	38 (16)	1 (0.5)
South Coast								
Santa Barbara Co.	7	7 (100)	81	58 (72)	16 (20)	74 (91)	1 (1)	3 (4)
Mountain Counties								
El Dorado Co.	2	2 (100)	20	9 (45)	2 (10)	11 (55)	2 (10)	0
Amador Co.	3	3 (100)	32	13 (41)	4 (12)	17 (53)	1 (3)	1 (3)
Total	5	5 (100)	52	22 (42)	6 (11)	28 (54)	3 (6)	1 (2)
Sacramento Valley								
Yolo Co.	4	4 (100)	42	10 (24)	11 (26)	21 (50)	9 (21)	0
Solano Co.	5	5 (100)	53	2 (4)	11 (21)	13 (24)	39 (74)	0
Sacramento Co.	7	7 (100)	78	35 (45)	28 (36)	63 (81)	11 (14)	3 (4)
Total	16	16 (100)	173	47 (27)	50 (29)	97 (56)	59 (34)	3 (2)
Northern San Joaquin Valley								
San Joaquin Co.	7	5 (71)	65	4 (6)	6 (9)	10 (15)	34 (52)	0
Stanislaus Co.	5	5 (100)	62	20 (32)	6 (10)	26 (42)	29 (47)	0
Merced	10	10 (100)	116	63 (54)	21 (18)	84 (72)	4 (3)	1 (1)
Total	22	20 (91)	243	87 (36)	33 (13)	120 (49)	67 (27)	1 (0.5)
Southern San Joaquin Valley								
Madera Co.	18	10 (55)	198	27 (14)	5 (2)	32 (16)	17 (9)	16 (8)
Fresno Co.	13	8 (61)	120	10 (8)	3 (2)	13 (11)	1 (1)	63 (52)
Kern Co.	6	4 (67)	42	5 (12)	1 (2)	6 (14)	5 (12)	5 (12)
Tulare Co.	4	4 (100)	41	8 (19)	0	8 (19)	0	19 (46)
Total	41	26 (63)	401	50 (12)	9 (2)	59 (15)	23 (6)	103 (26)
South California								
Riverside Co. (desert area)	17	17 (100)	156	82 (52)	2 (1)	84 (54)	0	3 (2)
Total California	166	148 (89)	1,735	638 (38)	180 (10)	818 (47)	297 (17)	115 (7)

[a] Percentage of the total number of cankers sampled per county.
[b] Number of vineyards sampled.
[c] Number of vineyards (and percentage of the total number of vineyards sampled per county) yielding *Botryosphaeria* spp.
[d] Total number of cankers collected.
[e] A = number of cankers from which a *Botryosphaeria* sp. only was isolated.
[f] B = number of cankers from which a *Botryosphaeria* sp. was isolated together with other grapevine wood fungal pathogens, such as *Eutypa lata*, *Phomopsis viticola*, *Phaeoacremonium* spp., *Phaeomoniella chlamydospora,* and other species of ascomycetes.
[g] Statistical analyses of the data were conducted with the total number of cankers yielding *Botryosphaeria* spp. (A + B) from each vineyard sampled in California.

narda philoprina (Berk. & Curt.) Van der Aa and *Mycosphaerella pini* Rostr. in Monk were used as outgroups for all analyses. In order to determine whether the ITS and β-tubulin sequence data could be combined, the partition homogeneity test was performed with 1,000 replicates in PAUP. Maximum parsimony for all analyses was performed using the heuristic search option (branch swapping NNI), and 1,000 random addition sequences replicates. Bootstrap values were evaluated using 1,000 replicates to test branch strength. Tree length, consistency index (CI), retention index (RI), rescaled consistency index (RC), and homoplasy index (HI) also were recorded for all analyses. Resulting trees were printed in PAUP version 4.0b10. ITS and β-tubulin sequences from *Botryosphaeria* spp. found in California were deposited into GenBank. *Botryosphaeria* spp. isolated from grapevines in California are maintained in the collection in the Department of Plant Pathology at the University of California, Davis, and representative isolates of each species were placed in the American Type Culture Collection (ATCC).

RESULTS

Field survey, fungal isolation, and data analyses. *Botryosphaeria* spp. were found in 148 of 166 vineyards sampled and were isolated from 818 of 1,735 cankers (Table 1). In 638 of 818 cankers yielding *Botryosphaeria* spp., it was the only fungus isolated from decayed wood tissue; whereas, in 180 cankers, *Botryosphaeria* spp. were co-isolated with other known grapevine wood fungal pathogens (Table 1). *Botryosphaeria* was the most abundant fungal genus isolated from grapevine cankers in California, followed by *Eutypa lata*, *Phomopsis viticola,* and another currently unidentified *Phomopsis* spp. *Phaeomoniella chlamydospora* and various species of *Phaeoacremonium* occasionally were isolated from cankers as well. Other sporadically isolated fungi from cankers in California were a *Clonostachys* sp., *Truncatella angustata*, *Alternaria alternata*, a *Pestalotia* sp., and the teleomorphs of *Phomopsis* spp., *Diaporthe helianthi,* and *D. phaseolorum*.

Incidence of *Botryosphaeria* spp. varied according to grapevine regions. It was significantly greater in south and central

Table 2. *Botryosphaeria* isolates from *Vitis vinifera* from California used in this study

Species[a]	Origin[b]	Date collected[c]	Isolate	GenBank no.		ATCC no.
				ITS[d]	β-Tubulin	
Botryosphaeria australis	Sonoma Co.	4 September	UCD1314So[e,f,g]	DQ008323	DQ008346	MYA-3698
B. australis	Sonoma Co.	4 September	UCD1467So[e,f,g]	DQ233610	DQ233631	MYA-3699
B. dothidea	Sonoma Co.	4 September	UCD1064So[e,f]	DQ233600	DQ233621	…
B. dothidea	Sonoma Co.	4 September	UCD1065So[e,f,g]	DQ233601	DQ233622	MYA-3707
B. dothidea	Sonoma Co.	4 September	UCD1066So[e,f,g]	DQ008324	DQ008347	MYA-3708
B. dothidea	Mendocino Co.	4 September	UCD1156Me[e]	DQ233602	DQ233623	…
B. dothidea	Mendocino Co.	4 September	UCD1181Me[e,f,g]	DQ008325	DQ008348	MYA-3709
B. dothidea	Mendocino Co.	4 September	UCD1213Me[e]	DQ008326	DQ008349	…
B. dothidea	Sonoma Co.	4 September	UCD1333So[e]	DQ008327	DQ008350	…
B. dothidea	Yolo Co.	5 April	UCD1672Yo[e,f,g]	DQ233603	DQ233624	MYA-3710
B. lutea	Temecula Valley, Riverside Co.	5 May	UCD2057Te[e,f,g]	DQ233604	DQ233625	MYA-3700
B. lutea	Temecula Valley, Riverside Co.	5 May	UCD2089Te[e]	DQ233605	DQ233626	…
B. lutea	Temecula Valley, Riverside Co.	5 May	UCD2090Te[e,f,g]	DQ233606	DQ233627	MYA-3701
B. lutea	Temecula Valley, Riverside Co.	5 May	UCD2098Te[e,f,g]	DQ233607	DQ233628	MYA-3702
B. lutea	Temecula Valley, Riverside Co.	5 May	UCD2103Te[e,f,g]	DQ233608	DQ233629	MYA-3703
B. lutea	Temecula Valley, Riverside Co.	5 May	UCD2118Te[e,f,g]	DQ233609	DQ233630	MYA-3704
B. obtusa	Madera Co.	3 October	UCD244Ma[e,f,g]	DQ008314	DQ008337	MYA-3692
B. obtusa	Monterey Co.	3 November	UCD352Mo[e,f,g]	DQ008315	DQ008338	MYA-3693
B. obtusa	Fresno Co.	3 November	UCD465Fr[e,f]	DQ008316	DQ008339	…
B. obtusa	Kern Co.	3 December	UCD602Kr[e,f,g]	DQ008317	DQ008340	MYA-3694
B. obtusa	Tulare Co.	3 December	UCD614Tu[e]	DQ008318	DQ008341	…
B. obtusa	Sonoma Co.	3 December	UCD645So[e]	DQ008319	DQ008342	…
B. obtusa	Napa Co.	3 December	UCD666Na[e]	DQ008320	DQ008343	…
B. obtusa	San Joaquin Co.	3 December	UCD710SJ[e,f,g]	DQ008321	DQ008344	MYA-3695
B. obtusa	Stanislaus Co	4 January	UCD770St[e]	DQ008322	DQ008345	…
B. parva	Sonoma Co.	3 December	UCD642So[e,f,g]	DQ008328	DQ008351	MYA-3705
B. parva	Sonoma Co.	3 December	UCD646So[e,f,g]	DQ008329	DQ008352	MYA-3706
B. parva	Stanislaus Co	5 January	UCD759St[e,f]	DQ233611	DQ233632	…
B. parva	Napa Co.	3 December	UCD1125Na[e,f]	DQ233612	DQ233633	…
B. parva	Sonoma Co.	3 December	UCD1349So[e,f]	DQ008330	DQ008353	…
B. rhodina	Coachella Valley, Riverside Co.	3 November	UCD191Co[e]	DQ008308	DQ008331	…
B. rhodina	Coachella Valley, Riverside Co.	3 November	UCD196Co[e,f,g]	DQ233592	DQ233613	MYA-3687
B. rhodina	Coachella Valley, Riverside Co.	3 November	UCD197Co[e]	DQ233593	DQ233614	…
B. rhodina	Coachella Valley, Riverside Co.	3 November	UCD202Co[e,f,g]	DQ008309	DQ008332	MYA-3688
B. rhodina	Coachella Valley, Riverside Co.	3 November	UCD205Co[e,f,g]	DQ008310	DQ008333	MYA-3689
B. rhodina	Coachella Valley, Riverside Co.	3 November	UCD206Co[e,f,g]	DQ008311	DQ008334	MYA-3690
B. rhodina	Coachella Valley, Riverside Co.	3 November	UCD220Co[e,f,g]	DQ008312	DQ008335	MYA-3691
B. rhodina	Madera Co.	3 October	UCD256Ma[e]	DQ233594	DQ233615	…
B. rhodina	Kern Co.	3 December	UCD526Kr[e]	DQ233595	DQ233616	…
B. rhodina	Merced Co.	5 April	UCD1814Md[e]	DQ233596	DQ233617	…
B. rhodina	Santa Barbara Co.	5 May	UCD1962SB[e]	DQ233597	DQ233618	…
B. stevensii	Madera Co.	3 October	UCD288Ma[e,f]	DQ008313	DQ008336	…
B. stevensii	Santa Barbara Co.	5 May	UCD1953SB[e,f,g]	DQ233598	DQ233619	MYA-3696
B. stevensii	Santa Barbara Co.	5 May	UCD1965SB[e,f,g]	DQ233599	DQ233620	MYA-3697

[a] *Botryosphaeria* spp. from grapevine from California were determined based on morphology and phylogenetic analyses.
[b] County (Co.) in the state of California in the United States.
[c] Year 2003 (3), 2004 (4), and 2005 (5).
[d] Internal transcribed spacer (ITS).
[e] *Botryosphaeria* isolates used for colony morphology and phylogenetic analyses studies.
[f] *Botryosphaeria* isolates used for conidia morphology and temperature studies.
[g] *Botryosphaeria* isolates deposited at the American Type Culture collection (ATCC).

coast areas than in the other grapevine regions surveyed ($P < 0.001$). High incidence of *Botryosphaeria* infection from cankers also was found in the north coast, mountain counties, Sacramento Valley, northern San Joaquin Valley, and southern California (desert area). The lowest incidence of *Botryosphaeria* spp. in cankers was found in the southern San Joaquin Valley grapevine region (Table 1).

Regions with a high incidence of *Botryosphaeria* spp. (north coast, central coast, south coast, mountain counties, and northern San Joaquin Valley) generally had a low rate recovery of *E. lata* from cankers (Table 1). In the other grapevine region with a high incidence of *Botryosphaeria* infection, Riverside County, *E. lata* was entirely absent (Table 1). In the southern San Joaquin Valley, the incidences of both *Botryosphaeria* spp. and *E. lata* were low

Table 3. *Botryosphaeria* sequences from GenBank used in the phylogenetic analysis[a]

Isolate[b]	Species	Host	Collector	Origin	GenBank no.	
					ITS[c]	β-Tubulin
CMW 3386[d,e]	*Botryosphaeria australis*	*Wollemia nobiles*	M. Ivory	Queensland, Australia	AY615165	AY615149
CMW 6837[e]	*B. australis*	*Acacia* sp.	M.J. Wingfield	Batemans Bay, Australia	AY339262	AY339254
CMW 6853[d,e]	*B. australis*	*Sequiadendron giganteum*	M. J. Wingfield	Canberra, Australia	AY339263	AY339255
CMW 9072[e]	*B. australis*	*Acacia* sp.	J. Roux/D. Guest	Melbourne, Australia	AY339260	AY339252
CMW 9073[e]	*B. australis*	*Acacia* sp.	J. Roux/D. Guest	Melbourne, Australia	AY339261	AY339253
CMW 1110[d,e]	*B. australis*	*Widdringtonia nodiflora*	W. J. Swart	South Africa	AY615166	AY615150
CMW 1112[d,e]	*B. australis*	*W. nodiflora*	W. J. Swart	South Africa	AY615167	AY615151
STE-U 4599[d]	*B. australis*	*Vitis vinifera*	F. Halleen	South Africa	AY343408	n/a
WAC 11075[d]	*B. australis*	*V. vinifera*	A. Taylor	Western Australia	AY727835	n/a
WAC 11337[d]	*B. australis*	*V. vinifera*	A. Taylor	Western Australia	AY727836	n/a
WAC 11346[d]	*B. australis*	*V. vinifera*	A. Taylor	Western Australia	AY727837	n/a
CMW 7020[e]	*B. dothidea*	*Magnifera indica*	G. I. Johnson	Australia	AY615191	AY615178
CMW 7027[d,e]	*B. dothidea*	*M. indica*	G. I. Johnson	Australia	AY615192	AY615179
CMW 7780[e]	*B. dothidea*	*Fraxinus excelsior*	B. Slippers	Switzerland	AY236947	AY236925
CMW 7999[d,e]	*B. dothidea*	*Ostrya* sp.	B. Slippers	Switzerland	AY236948	AY236926
CMW 8000[d,e]	*B. dothidea*	*Prunus* sp.	B. Slippers	Switzerland	AY236949	AY236927
CMW 9075[e]	*B. dothidea*	*Populus* sp.	G. J. Samuels	New Zealand	AY236950	AY236928
STE-U 5045[d]	*B. dothidea*	*V. vinifera*	G. Marta	Argentina	AY343414	n/a
STE-U 5149[d]	*B. dothidea*	*V. vinifera*	A. J. L. Phillips	Portugal	AY343415	n/a
CBS 116743[d]	*B. dothidea*	*Olea europaea*	I. Rumbos	Greece	AY786322	n/a
CMW 9076[d,e]	*B. lutea*	*Malus* × *domestica*	S. R. Pennycook	New Zealand	AY236946	AY236922
CMW10309[d,e]	*B. lutea*	*V. vinifera*	A. J. L. Phillips	Portugal	AY339258	AY339250
CMW10310[d,e]	*B. lutea*	*V. vinifera*	A. J. L. Phillips	Portugal	AY339259	AY339251
STE-U 3088[d]	*B. lutea*	*Bukinghamia* sp.	P. W. Crous	Australia	AF452555	n/a
STE-U 4593[d]	*B. lutea*	*V. vinifera*	A. J. L. Phillips	Portugal	AY343417	n/a
CBS 110299[d]	*B. lutea*	*V. vinifera*	A. J. L. Phillips	Portugal	AY928043	n/a
CMW 7774[d,e]	*B. obtusa*	*Ribes* sp.	Slippers & Hudler	New York, United States	AY236954	AY236932
CMW 7775[d,e]	*B. obtusa*	*Ribes* sp.	Slippers & Hudler	New York, United States	AY236953	AY236902
CMW 8230[d,e]	*B. obtusa*	n/a	n/a	n/a	AY972104	AY972119
CMW 8232[d,e]	*B. obtusa*	n/a	n/a	n/a	AY972105	AY972120
STE-U 4581[d]	*B. obtusa*	*V. vinifera*	P. Larignon	France	AY343439	n/a
STE-U 5037[d]	*B. obtusa*	*V. vinifera*	A. J.. Phillips	Portugal	AY343446	n/a
STE-U 5052[d]	*B. obtusa*	*V. vinifera*	J. M. van Niekerk	South Africa	AY343449	n/a
WAC 11073[d]	*B. obtusa*	*V. vinifera*	A. Taylor	Western Australia	AY727842	n/a
WAC 11074[d]	*B. obtusa*	*V. vinifera*	A. Taylor	Western Australia	AY727843	n/a
CBS 112556[d]	*B. obtusa*	*Pyrus communis*	A. J. L. Phillips	Portugal	AY259096	n/a
CMW 7798[d,e]	*B. parva*	*M. indica*	G. I. Johnson	Australia	AY615183	AY615170
CMW 7799[d,e]	*B. parva*	*Persea americana*	K. G. Pegg	Australia	AY615184	AY615171
CMW 9077[d,e]	*B. parva*	*Actinidia deliciosa*	S. R. Pennycook	New Zealand	AY236939	AY236913
CMW 9079[e]	*B. parva*	*A. deliciosa*	S. R. Pennycook	New Zealand	AY236941	AY236915
CMW 9080[d,e]	*B. parva*	*Populus nigra*	S. R. Pennycook	New Zealand	AY236942	AY236196
CMW 9081[d,e]	*B. parva*	*P. nigra*	G. J. Samuels	New Zealand	AY236943	AY236917
STE-U 5253[d]	*B. parva*	*V. vinifera*	A. J. L. Phillips	Portugal	AY343477	n/a
CBS 110301[d]	*B. parva*	*V. vinifera*	A. J. L. Phillips	Portugal	AY259098	n/a
CMW 9074[d,e]	*B. rhodina*	*Pinus* sp.	B. Slippers	Mexico	AY236952	AY236930
CMW 10130[d,e]	*B. rhodina*	*Vitex donniana*	J. Roux	Uganda	AY236951	AY236929
STE-U 4421[d]	*B. rhodina*	*Vitis vinifera*	F. Halleen	South Africa	AY343479	n/a
STE-U 5051[d]	*B. rhodina*	*V. vinifera*	M. Gatica	Argentina	AY343483	n/a
WAC 7038[d]	*B. rhodina*	*V. vinifera*	P. Wood	Western Australia	AY727848	n/a
WAC 9853[d]	*B. rhodina*	*V. vinifera*	P. Wood	Western Australia	AY727849	n/a
WAC10712[d]	*B. rhodina*	*V. vinifera*	P. Wood	Western Australia	AY727850	n/a
CMW 7060[d,e]	*B. stevensii*	*Fraxinus excelsior*	H. A. van der Aa	Netherlands	AY236955	AY236933
CMW 7776[d,e]	*B. stevensii*	n/a	n/a	n/a	AY972106	AY972121
CMW 7781[d,e]	*B. stevensii*	n/a	n/a	n/a	AY972107	AY972122
STE-U 5038[d]	*B. stevensii*	*V. vinifera*	A. J. L. Phillips	Portugal	AY343484	n/a
WAC 11082[d]	*B. stevensii*	*V. vinifera*	A. Taylor	Western Australia	AY727838	n/a
CBS 112553[d]	*B. stevensii*	*V. vinifera*	A. J. L. Phillips	Portugal	AY259093	n/a

[a] n/a = Not available at the time of this publication.
[b] Acronyms of cultures collections: CMW = Culture Collection Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; STE-U = Department of Plant Pathology, University of Stellenbosch, South Africa; WAC = Department of Agriculture Western Australia, Plant Pathogen Collection; and CBS = Centraalbureau Schimmelcultures, Utrecht, Netherlands.
[c] Internal transcribed spacer (ITS).
[d] *Botryosphaeria* isolates from GenBank used in the ITS phylogenetic analysis.
[e] *Botryosphaeria* isolates from GenBank used in the combined ITS and β-tubulin phylogenetic analysis.

but *P. viticola* was isolated at the highest rate of any region in California (Table 1). Only in four counties, Napa, Solano, San Joaquin, and Stanislaus, was the incidence of *E. lata* significantly higher than *Botryosphaeria* spp. ($P < 0.001$; Table 1).

To test for a relationship between vineyard age and the occurrence of *Botryosphaeria* spp., vineyards were divided into three groups: 10 to 20, 21 to 30, and >30 years of age. Based on ANOVA, there was no significant relationship between inci-

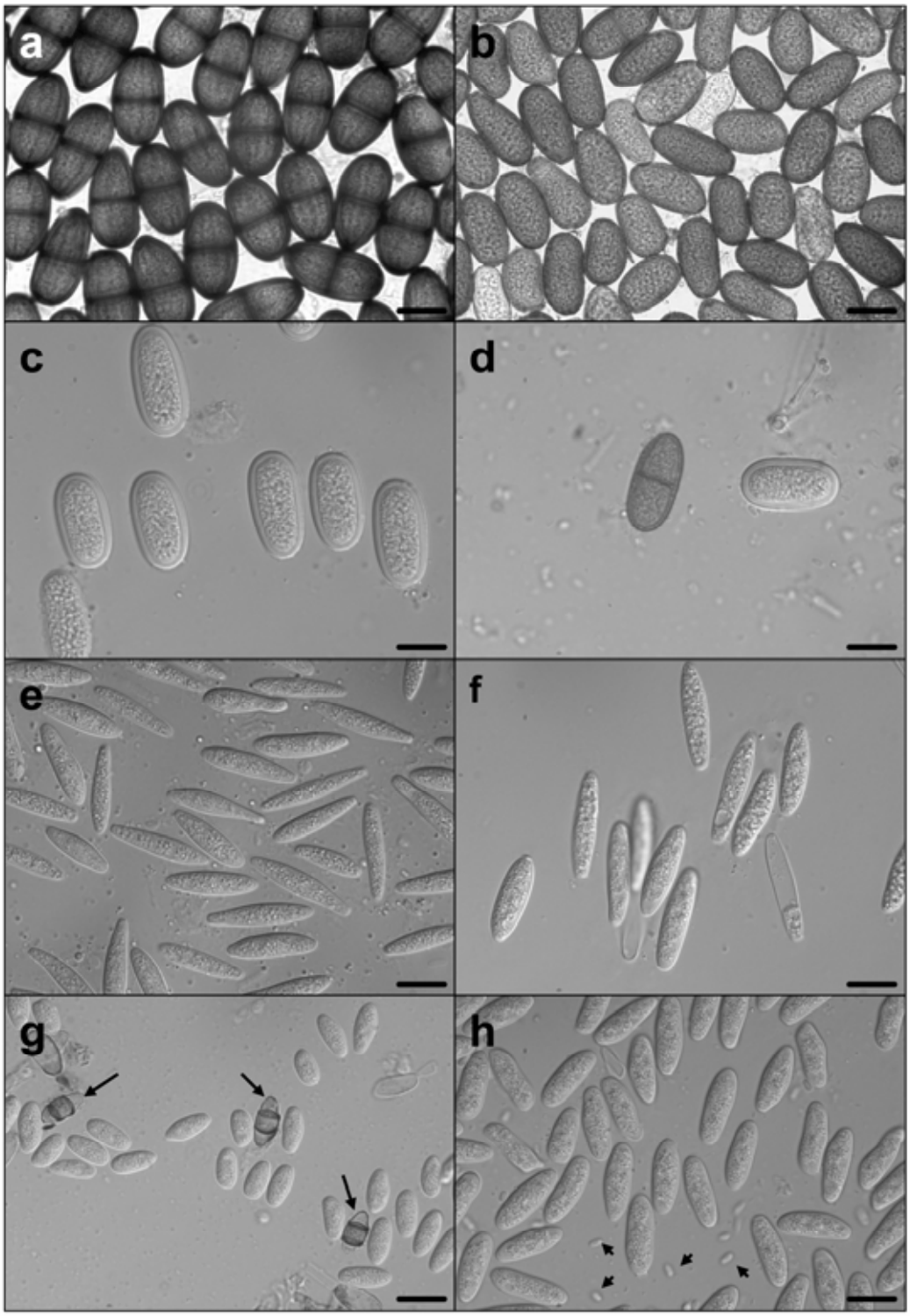

Fig. 2. Conidial morphology of *Botryosphaeria* spp. Photographs were taken at ×100 (immersion oil) from fruiting bodies (pycnidia) formed in cultures on grapevine wood chips and water agar. **a,** Mature Conidia of *Botryosphaeria rhodina* (UCD220Co). *B. rhodina* shape was oval with rounded and pointed ends. Conidia of *B. rhodina* were hyaline, aseptate, and densely granulate when immature, becoming dark brown, with characteristic longitudinal striations with one central septum when mature. **b,** Mature *B. obtusa* conidia (UCD352Mo). Conidia shape of *B. obtusa* was mostly rounded, but some conidia showed a truncate base. Hyaline when immature, becoming light brown when mature. Aseptate conidia. **c,** Young, hyaline, thick-walled, one celled, cylindrical with rounded ends *B. stevensii* conidia (UCD1953SB). **d,** Conidia of *B. stevensii* sporadically turned brown and developed one septum when mature. **e,** Hyaline and aseptate conidia of *B. dothidea* (UCD1065So). *B. dothidea* conidia were ellipsoidal with rounded and flat ends. **f,** Fusiform, hyaline, densely granulate, and aseptate *B. australis* conidia (UCD1314So). **g,** Ellipsoidal with rounded and flat ends conidia of *B. parva* (UCD642So). Conidia of *B. parva* were hyaline, densely granulate, and aseptate when immature. Arrows show two septa conidia of *B. parva*, typical characteristic developed of when mature. **h,** Smooth, fusiform, hyaline, and aseptate conidia of *B. lutea* (UCD2090Te). Arrows show microconidia observed in isolates of *B. lutea* from California. Scale bar = 10 μm.

dence of *Botryosphaeria* spp. and vineyard age ($P = 0.807$). *Botryosphaeria* spp. were isolated from nearly 45% of the cankers collected from both the 10- to 20- and 21- to 30-year-old vineyards and from 50% of cankers in vineyards >30 years old. In addition, the effect of wine grape (Cabernet Sauvignon, Cabernet Franc, Chardonnay, Chenin Blanc, French Colombard, Merlot, Shiraz, Sauvignon Blanc, and Zinfandel) and table-raisin grape (Flame, Thompson Seedless, Red Globe, and Perlette) cultivars on the incidence of *Botryosphaeria* spp. was not significant ($P = 0.9883$).

Morphological characterization. A total of 44 isolates of *Botryosphaeria*, representative of the different regions sampled, were divided into groups based on their appearance in culture (Table 2). Eleven isolates were characterized by abundant aerial and fast-growing mycelium that covered an 85-mm-diameter petri plate in 48 h at room temperature. With age, mycelium became dark green and single or grouped, large (reaching up to 4 to 5 mm wide), black, globose fruiting bodies (pycnidia), with or without stroma, with central ostioles that were observed after 10 days of incubation at room temperature. All these characteristics were consistent with the description of *B. rhodina* (13,37). Conidial morphology and DNA analyses also identified these isolates as *B. rhodina* (see below).

A second group of 12 isolates also produced dark-green colonies, but moderate aerial mycelium growth. In 9 of the 12 isolates, single, small, dark-brown and black pycnidia were observed in young cultures (7 days old). These isolates also were identified as *B. obtusa* based on conidial characteristics. The remaining three isolates, which produced no fruiting bodies, later were identified as *B. stevensii* based on conidial morphology and sequence data.

A third group of eight isolates initially produced white-cream colonies; however, after 4 days of incubation at room temperature, a characteristic yellow pigment at the center of the colony was observed on the reverse of the culture. This is a typical characteristic of *B. lutea* and *B. australis* according to published descriptions (20,25,33,37). Both species were identified based on conidial morphology and sequence data. Six isolates of *B. lutea* became dark green after 10 days of incubation, whereas two isolates of *B. australis* produced light-gray, pale mycelium, but the reverse of the colony turned dark green. Colony morphology of *B. australis* isolates was well differentiated from *B. lutea* colonies due to the formation of a dense, fluffy, aerial mycelium in the center

Table 4. Conidial dimensions of the *Botryosphaeria* species from California used in this study and comparison with those reported from preceding studies

	Conidial dimensions of			
	Selected *Botryosphaeria* spp. in this study		*Botryosphaeria* spp. from previous studies	
Species, isolate	Conidial size (µm)[a]	Mean ± SD (µm)[b]	Conidial size (µm)	Source of data[c]
Botryosphaeria australis				
UCD1314So	(20–)25–30 × (6)7.3–9.8	25.2 ± 1.9 × 7.2 ± 0.7	(20–)23–25(–27) × 4–6[d]	Taylor
UCD1467So	(20.5–)24.3–31 × (5.7)7.3–8.8	24.4 ± 2.3 × 7.3 ± 0.6	(18–)23–26(–30) × 5–6(–7.5)	Slippers
B. dothidea				
UCD1064So	(21–)25.5–32 × (4)6–8	25.4 ± 2.4 × 5.5 ± 0.8	(18–)21–28.5(–30) × (3.5–)4–4.5(–6)[d]	Phillips 2002
UCD1065So	(16–)23.5–30 × (5.5)7–8	23.8 ± 2.9 × 6.6 ± 0.6	17 – 25 × 5 – 7[d]	Larignon
UCD1066So	(20–)24–29 × (5)7–8	24.1 ± 2.1 × 6.6 ± 0.9	(15–)20–26(–32) × (4–)5–6(–9)	Pennycook
UCD1181Me	(19–)24–29 × (5)7.5–9	23.7 ± 2.3 × 6.6 ± 0.7	…	…
UCD1672Yo	(19–)25.5–30 × (4)6–8	25 ± 3 × 6.1 ± 0.9	…	…
B. lutea				
UCD2057Te	(14–)20-25.9 × (4.6)6.7–9	20 ± 2.4 × 6.6 ± 0.8	(15–)18–22.5(–24) × 4.5–6(–7.5)[d]	Phillips 2002
UCD2090Te	(14.3–)19.3–23.3 × (5)6.3–8.8	19.5 ± 2.1 × 6.5 ± 1	(14–)20–24(–32) × (5–)6–7(–9)	Pennycook
UCD2098Te	(15–)20–22.8 × (5.6)7.4–9.5	19.7 ± 1.8 × 7.4 ± 0.8	…	…
UCD2103Te	(15.3–)20–27.4 × (6)7.6–9.4	20 ± 2 × 7.5 ± 0.8	…	…
UCD2118Te	(13.4–)20.3–23.5 × (5.6)7.4–9	20.1 ± 2.3 × 7.3 ± 1	…	…
B. obtusa				
UCD244Ma	(17–)22.5–26 × (8)9.5–14	22.3 ± 2.4 × 9.4 ± 1.1	(20–)21–23(–24) × 8–10[d]	Taylor
UCD352Mo	(16–)21–29 × (9)11.5–17	20.8 ± 2.7 × 11.4 ± 1.4	(13–)22–26 × (9–)10–13(–15)[d]	Phillips 2002
UCD465Fr	(15–)22–29 × (7)9–13	21.5 ± 3.1 × 9.1 ± 1.5	22 – 26 × 10 – 12[d]	Larignon
UCD602Kr	(15–)23–30 × (8)10–14	22.9 ± 3.2 × 10 ± 1.1	20 – 26 × 9 – 12	Punithal 1973
UCD710SJ	(15–)23–29 × (7)10–13	22.3 ± 3.2 × 9.9 ± 1.4	…	…
B. parva				
UCD642So	(10–)14.5–17 × (5)7–9	14.3 ± 1.6 × 7.1 ± 1	17 – 19 × 5 – 6	Slippers
UCD646So	(11–)14–17 × (5)7.5–9	14 ± 1.6 × 6.6 ± 0.8	(12–)15–20(–24) × (4–)4.5–6(–7.5)[d]	Phillips 2002
UCD759St	(11–)13–19 × (5)7–9	13.6 ± 1.5 × 7.8 ± 0.8	(11–)14–18(–23) × 5–7(–10)	Pennycook
UCD1125Na	(11–)14–23 × (6)7–9	14.3 ± 2 × 6.8 ± 0.7	…	…
UCD1349So	(11–)14–18 × (5.7)7–9	14.1 ± 1.7 × 7 ± 0.8	…	…
B. rhodina				
UCD196Co	(18–)20–25 × (9)11–14	20.3 ± 1.5 × 11.3 ± 1	(22–)23–25(–28) × (15–)16–18(–20)[d]	Taylor
UCD202Co	(18–)22.5–26 × (10)12–15	22.2 ± 1.9 × 12.2 ± 1.1	(20–)24–28(–33) × (10–)12–15(–18)	Phillips 2004
UCD205Co	(20–)25–28 × (10)12–16	24.8 ± 1.8 × 12.5 ± 1.4	20 – 30 × 10 – 15	Punithal 1980
UCD206Co	(19–)23–27 × (9)11–14	23 ± 1.7 × 11.5 ± 1.3	…	…
UCD220Co	(18–)23–27 × (9)11–14	22.5 ± 2.5 × 11.3 ± 1.3	…	…
B. stevensii				
UCD288Ma	(21–)25–28 × (10)13–14.6	24.7 ± 1.3 × 12.5 ± 1	(22–)24–26(–30) × 10–14[d]	Taylor
UCD1953SB	(20–)24.7–28 × (10.2)12.2–14	24.3 ± 1.7 × 12.2 ± 1	(21–)23–27(–28) × (10–)11–12(–13)[d]	Phillips 2002
UCD1965SB	(21–)24.4–28 × (9.3)12–14.6	24.3 ± 1.6 × 11.9 ± 1.1	…	…

[a] Minimum size, most repetitive value and maximum size for length and width of 60 conidia were recorded from each *Botryosphaeria* isolate from California selected for conidial dimension.

[b] SD = standard deviation.

[c] Taylor = A. Taylor et al. 2005, Slippers = B. Slippers et al. 2004, Philips 2002 = A. J. L. Phillips 2002, Larignon = P. Larignon and B. Dubos 2001, Pennycook = Pennycook and Samuels 1985, Punithal 1973 = Punithalingam and Waller 1973, Philips 2004 = taken from the website of Centro de Recursos Microbiológicos (Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal), and Punithal 1980 = E. Punithalingam 1980.

[d] Conidial dimension from *Botryosphaeria* spp. from *Vitis vinifera*.

becoming flat around the edges of the colony. Presence of microconidia in *B. lutea* isolates was another characteristic that differentiated this species from *B. australis*.

The last group, comprising 13 isolates, developed a white aerial mycelium which gradually turned green to dark green after a 4- to 5-day incubation period at room temperature. Of the 12 isolates, 8 formed individual, small, black pycnidia after 3 weeks of incubation at room temperature and were determined to be *B. dothidea* based on conidial morphology from those pycnidia and DNA analysis. For the five remaining isolates, dark, globose pycnidia embedded in stroma tissue were observed after 6 weeks of incubation. Conidial characteristics and DNA sequence data identified isolates in this group as *B. parva*.

All isolates of *Botryosphaeria* could be separated into two groups based on conidial characteristics. One group was characterized by production of pigmented and thick-walled conidia, and included *B. rhodina*, *B. obtusa,* and *B. stevensii* (Fig. 2a–d). The second group, characterized by the production of hyaline and thin-walled conidia, comprised *B. dothidea*, *B. australis*, *B. parva,* and *B. lutea* (Fig. 2e–h). Conidial dimensions recorded for *Botryosphaeria* spp. from California are shown in Table 4, along with previously reported values (12,20,25–27,34,37).

Radial growth values were plotted versus temperature (Fig. 3, only representative isolates shown). All isolates grew over a range of temperatures (10 to 40ºC), but the optimal temperature varied depending on the species. *Botryosphaeria* spp. did not grow at 5ºC and, with the exception of *B. rhodina*, they exhibited only very slight growth at 40ºC (Fig. 3). The estimated temperatures at which each *Botryosphaeria* sp. reached the maximum radial growth were 30.8ºC for both *B. rhodina* and *B. dothidea*, 29.4ºC for *B. lutea*, 28.2ºC for *B. parva*, 27.8ºC for *B. australis*, 26.8ºC for *B. obtusa*, and 24.8ºC for *B. stevensii*. Growth rates significantly differed between the species of *Botryosphaeria* at the optimum temperatures ($P < 0.001$) (Fig. 4). Among the *Botryosphaeria* spp. tested, *B. rhodina* had the highest mycelial growth rate at the optimum temperature after 48 h, whereas *B. australis* exhibited the lowest growth at its optimum temperature (Fig. 4). There was no significant variation between isolates of the same species at each specific temperature. All isolates of *B. rhodina* included in this study started to produce a red pigment (chromogenesis) in the agar or the aerial mycelium after 2 weeks of growth at 35ºC. No chromogenesis was observed in the other *Botryosphaeria* spp. examined.

Phylogenetic analyses. ITS sequences of Californian *Botryosphaeria* isolates from grapevine (Table 2) were aligned with GenBank ITS sequences of *Botryosphaeria* isolates from grapevine and other hosts (Table 3). Of the 573 nucleotides analyzed, 107 were parsimony informative. Maximum parsimony analyses of the ITS region resulted in one most parsimonious tree (length = 341, CI = 0.839, RI = 0.978, RC = 0.820, and HI = 0.161; Fig. 5).

The ITS phylogenetic tree included two well-separated clades. *Botryosphaeria* spp. having pigmented and thick-walled conidia formed a strongly supported clade, with a bootstrap value of 98%. These species have *Diplodia* anamorphs and included *B. rhodina*, *B. obtusa*, and *B. stevensii*. *B. rhodina* formed a strongly supported clade (90%) that was a sister clade to the one containing *B. obtusa* and *B. stevensii* (Fig. 5). *B. obtusa* isolates from California had nearly identical sequences and were grouped together with Australian, French,

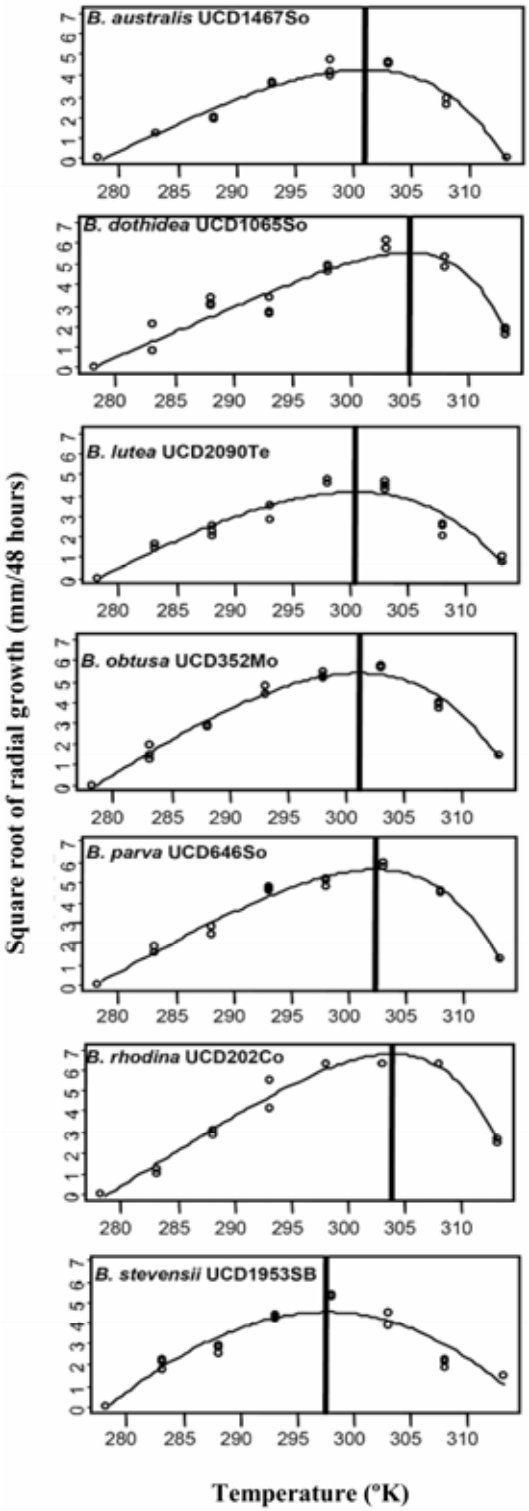

Fig. 3. Temperature effect on colony growth of the most representative isolates of *Botryosphaeria* spp. from California. Black straight lines indicate the estimated optimum growth temperature.

and Portuguese grapevine isolates (Fig. 5). *Botryosphaeria* spp. with hyaline and thin-walled conidia resided in a separate clade supported at 60%. These species included *B. dothidea*, *B. lutea*, *B. australis,* and *B. parva*, which all have *Fusicoccum* spp. anamorphs (Fig. 5). Within this group, there was no variation in the DNA sequences of *B. dothidea*, which formed a well-supported (100%) sister group to one comprising *B. lutea*, *B. australis,* and *B. parva* (Fig. 5). *B. australis* showed a close relationship to *B. lutea*. Isolates of *B. lutea* and *B. australis* from California and other areas of the world did not show variation in ITS sequences, except for the *B. australis* isolate (CMW3386) from Queensland, Australia. Californian isolates of *B. parva* contained no variation in the DNA sequences and were grouped with one isolate from *Vitis vinifera* from Portugal (Fig. 5).

β-Tubulin sequences from our grapevine isolates were aligned with 31 GenBank sequences of representative *Botryosphaeria* spp. (Table 3). Of the 415 nucleotides analyzed, 95 were parsimony informative. The maximum parsimony analyses yielded one most parsimonious tree (length = 320, CI = 0.85, RI = 0.965, RC = 0.82, and HI = 0.15). β-Tubulin tree topology (Fig. 6) differed from the topology of the ITS tree (Fig. 5) by showing a polytomy at the most basal node of the tree, which separates *Botryosphaeria* spp. from the outgroup. The β-tubulin phylogenetic tree included *Botryosphaeria* spp. with pigmented and thick-walled conidia in a well-separated clade with a bootstrap value of 74% (Fig. 6). *Botryosphaeria* spp. with hyaline and thin-walled conidia were separated into two different clades. *B. dothidea* isolates formed a separate clade with bootstrap value of 100% from all other species with *Fusicoccum*-type conidia, which resided in a different clade supported at 66% (Fig. 6). *Botryosphaeria* isolates with *Fusicoccum* anamorphs from California had identical β-tubulin sequences and grouped together with the Culture Collection Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa (CMW) collection isolates included in the analysis (Fig. 6).

A partition homogeneity test indicated that the ITS and β-tubulin datasets could be combined (P = 0.83). Of the 987 nucleotides analyzed, 208 were parsimony informative. The maximum parsimony analyses yielded one most parsimonious tree (length = 678, CI = 0.854, RI = 0.971, RC = 0.829, and HI = 0.146). The topology of this tree (Fig. 7) was consistent with the topology of the ITS tree (Fig. 5). *Botryosphaeria* spp. with *Diplodia*-type conidia (pigmented and thick walled) formed a highly supported clade (95%). Isolates representing individual species within this cluster, *B. rhodina*, *B. obtusa*, and *B. stevensii*, were contained in strongly supported individual clades with bootstrap values of 100, 99, and 83% respectively (Fig. 7). *B. obtusa* isolates from California grouped together, showing DNA sequence variation from the *B. obtusa* isolates from the CMW collection (Fig. 7). *Botryosphaeria* spp. with *Fusicoccum*-type conidia (hyaline and thin walled) composed a separate highly supported clade (86%). Within this clade, *B. australis*, *B. lutea*, *B. parva,* and *B. dothidea* were all well separated, with bootstrap values of 91, 92, 100, and 100%, respectively (Fig. 7). Based on the combined tree, no variation in the DNA sequences of *B. dothidea*, *B. lutea*, and *B. australis* isolates was observed, except for both *B. dothidea* (UCD1156Me) and *B. australis* (CMW3386) isolates from California and Australia, respectively (Fig. 7). *B. parva* isolates from California grouped together, diverging from the *B. parva* isolates from the CMW collection included in the analysis (Fig. 7).

Distribution of *Botryosphaeria* spp. in California. Seven *Botryosphaeria* spp. were found in California: *B. australis*, *B. dothidea*, *B. lutea*, *B. obtusa*, *B. parva*, *B. rhodina,* and *B. stevensii*. At least one *Botryosphaeria* sp. was found in all counties sampled in this study (Fig. 8). *B. obtusa* was the most frequently isolated species from cankers, followed by *B. dothidea*, *B. rhodina*, *B. parva*, *B. lutea*, *B. stevensii*, and *B. australis*. Differences in geographic distribution of *Botryosphaeria* spp. throughout California also were apparent. *B. dothidea*, *B. parva,* and *B. australis* primarily were isolated from vineyards in northern California (Fig. 8). *B. australis* was isolated only from cankers from one vineyard in Sonoma County. None of these species were found throughout the coastal and southern viticulture regions sampled (Fig. 8). *B. stevensii* was found in only two vineyards located in Madera and Santa Barbara Counties. *B. rhodina* was found mainly on grapevines from Coachella Valley in Riverside County. However, some isolates of *B. rhodina* also were found in Merced, Madera, Kern, and Santa Barbara Counties (Fig. 8). *B. rhodina* was not isolated from the northern grapevine areas of California. Isolates of *B. lutea* were only found in five vineyards in Temecula Valley, the other grapevine region sampled within Riverside County.

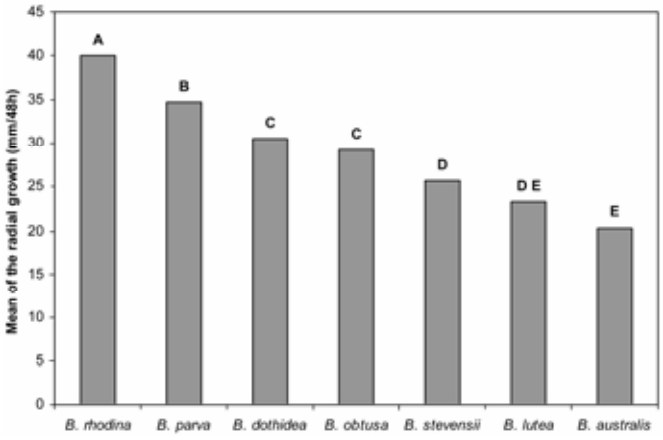

Fig. 4. Mean of the radial growth of the seven *Botryosphaeria* spp. found in California at the optimum temperature. Mean not represented with the same letter differ significantly according to Tukey's test (P < 0.001).

DISCUSSION

This study constitutes the first attempt to assess the presence and diversity of *Botryosphaeria* spp. on grapevine in California. Based on morphological characters and partial sequence analysis of two genes, seven *Botryosphaeria* spp. were isolated from cankers of grapevines in California from all the major grape-production areas. The associations of *B. australis*, *B. dothidea*, *B. lutea*, *B. obtusa*, *B. parva,* and *B. stevensii* with grapevines in California have not been reported before. Only *B. rhodina* previously was recognized as a pathogen of grapevine in southern California (14).

All the *Botryosphaeria* spp. found in California have been reported in other grape-growing areas worldwide, and they are associated mainly with a broad range of grapevine symptoms other than cankers. These symptoms include vascular streaking, cane bleaching, graft failure, bud necrosis, limited or no bud burst, and canes with stunted or shortened internodes (12,25,29,37). However, previous studies also have associated *Botryosphaeria* spp. with cankers. For example, *B. rhodina* and *B. obtusa* frequently have been isolated from cankers in South Africa (41) and Australia (2,37). In Western Australia, *B. australis* and *B. stevensii* also were associated with wedge or half-moon shaped internal lesions of grapevines (37). In Brazil, *B. dothidea* is known to cause "trunk canker" (7) and, in France, *B. dothidea*, *B. obtusa,* and *B. stevensii* also were isolated from sectorial vascular necrosis (12). *B. obtusa* has been reported to cause basal

canker in cv. Red Globe in Chile (1). The present study also confirms the presence of *B. parva* and *B. lutea* in grapevine cankers.

Vascular cankers and subsequent dieback in California grapevines have been attributed mainly to *E. lata* and occasionally to Bot canker disease, caused by *B. rhodina*. However, we recovered *Botryosphaeria* spp. from cankers more often than *E. lata*, indicating that the former may be a more important cause of grapevine dieback than previously recognized. In part, this may reflect difficulties in distinguishing between vascular cankers caused by *Eutypa* spp. from those caused by *Botryosphaeria* spp. (2,13,38). Previous work has indicated that *P. viticola* is not a cause of cankers (18). However, the high incidence of this fungal pathogen isolated from cankers in the southern San Joaquin Valley region underscores its important association with cankers in California. Whether it is acting as a saprobe or is a weak pathogen on highly susceptible grapevine cultivars, causing the same type of cankers, has not yet been clarified.

Leavitt (13) reported the existence of wedge-shaped cankers caused by *B. rhodina* on 5-year-old vines in the desert area of Coachella Valley in southern California. Four years later, when the vines were 9 years old, dead cordons were observed on most of the vines where cankers

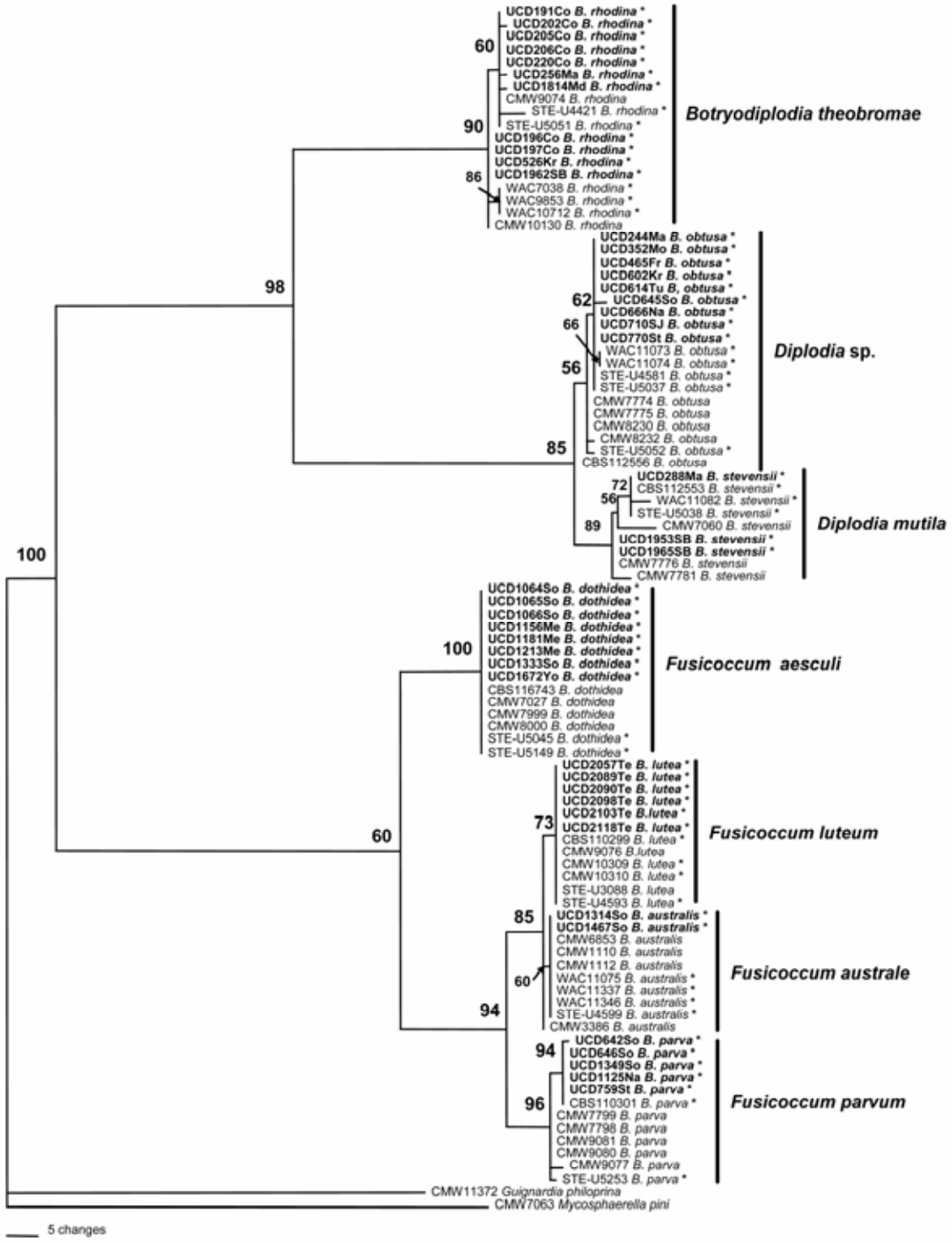

Fig. 5. Most equally parsimonious tree with bootstrap value using 1,000 replicates generated in PAUP 4.0b10 using internal transcribed spacer (ITS)1, 5.8S rDNA region, and ITS2. Asterisks show *Botryosphaeria* spp. from *Vitis vinifera*. Anamorph of each *Botryosphaeria* sp. is shown.

previously were reported. In the current study, *Botryosphaeria* spp. all were isolated from cankers with corresponding spur, cordon, or trunk dieback in grapevines 10 or more years old. However, we would expect a survey of vineyards younger than 10 years old also to reveal cankers induced by diverse species of *Botryosphaeria*.

Susceptibility of grapevine cultivars to some *Botryosphaeria* spp. has been reported in previous studies. For instance, *B. dothidea* has been shown to be a prevalent and destructive pathogen on muscadine grape (*V. rotundifolia*) in the southeast of the United States (17). In the Bordeaux region of France, Sauvignon, Cabernet Sauvignon, and Cabernet Franc were the most susceptible cultivars to the disease known as black dead arm, caused by *B. dothidea*, *B. obtusa,* and *B. stevensii*, whereas cultivars such as Merlot and Sémillon sustained much lower infection rates (12). In the Australian grape-production area of the Hunter Valley, New South Wales, *B. obtusa* was the fungus most commonly isolated from dieback symptoms in cv. Sémillon. In contrast, results from this study have revealed that all surveyed cultivars were associated with *Botryosphaeria* spp. Both cultivar groups analyzed in this work, wine grapes and raisin-table grapes, had a similar incidence of infection by *Botryosphaeria* spp.

Botryosphaeria Ces. & De Not. constitutes a complex fungal genus with many taxonomic and nomenclatural problems. Because teleomorphs of *Botryosphaeria* spp. usually are not found in nature and are difficult to obtain in vitro under laboratory conditions, identification of *Botryosphaeria* spp. in previous studies has been based mainly on morphology of the anamorphs (4,11,25,37). In the present study, none of the teleomorphs of the seven *Botryosphaeria* spp. were found in any of the samples collected. Anamorph characteristics previously reported (13,25), such as colony and conidial morphology, were useful characters for identification of *B. rhodina*, *B. obtusa,* and *B. stevensii* isolates from California. However, it remained difficult to separate *Botryosphaeria* spp. with hyaline conidia, *B. australis*, *B. dothidea*, *B. lutea*, and *B. parva*, based only on conidial morphology.

DNA sequence comparisons allowed us to verify identification of *Botryosphaeria* spp. from grapevines in California. Results of ITS and combined ITS and β-tubulin phylogenetic analyses from this study supported previous work that has classified anamorphs of *Botryosphaeria* in two gen-

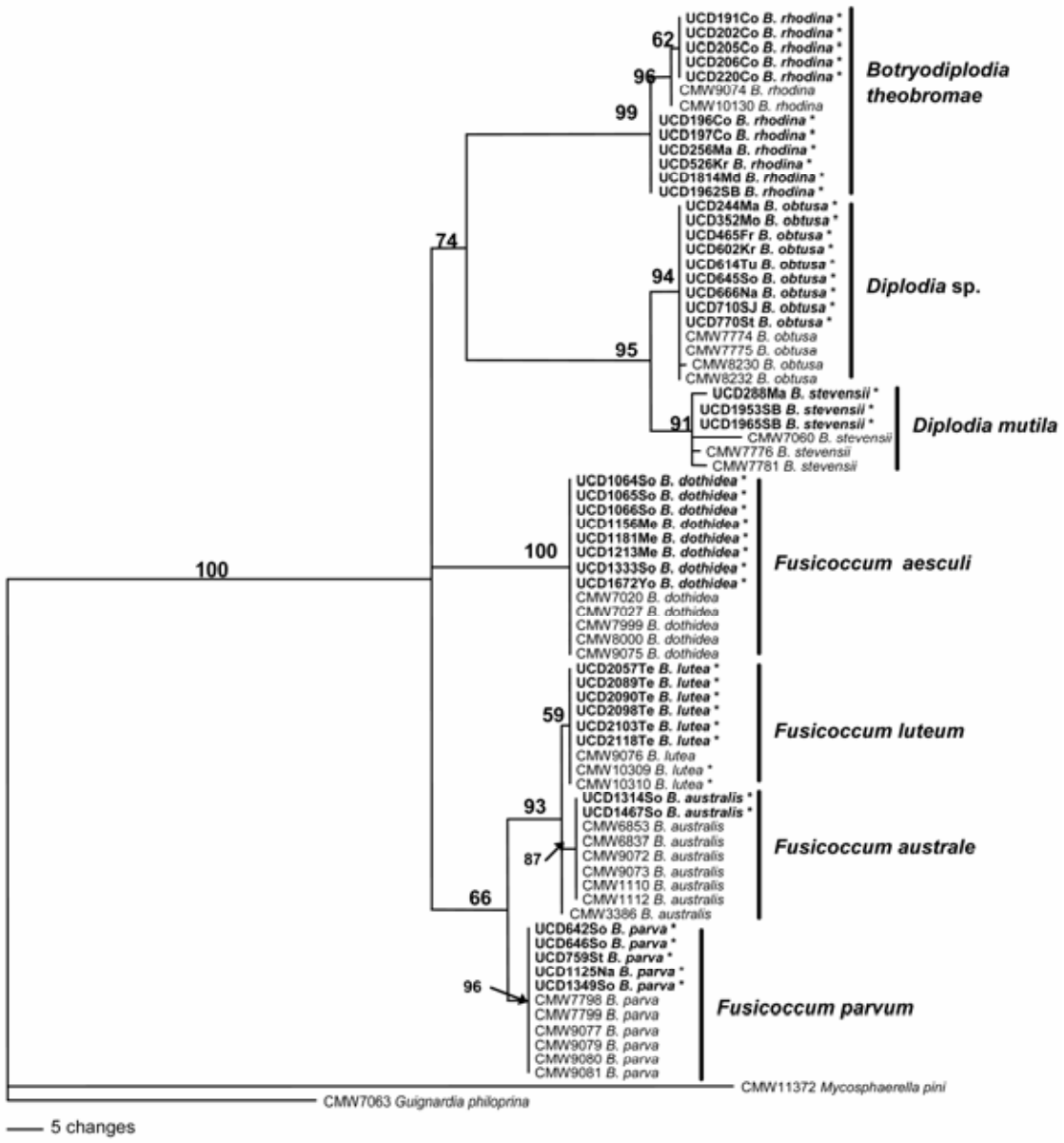

Fig. 6. Most equally parsimonious tree with bootstrap value using 1,000 replicates generated in PAUP 4.0b10 from the partial β-tubulin gene. Asterisks show *Botryosphaeria* spp. from *Vitis vinifera*. Anamorph of each *Botryosphaeria* sp. is shown.

era on the basis of conidium coloration: *Diplodia* Fr. and *Fusicoccum* Corda (4,37,41,45). Although *Botryodiplodia theobromae* is known as the anamorph of *Botryosphaeria rhodina*, previous investigations (4,41,45) have suggested that, due to morphological similarities (pigmented, ornamented, and thick-walled conidia), this species should be included under *Diplodia* Fr. On the other hand, phylogenetic analysis based on β-tubulin gene sequences makes the separation of *Botryosphaeria* spp. between *Diplodia* and *Fusicoccum* unclear. The fact that *B. dothidea* formed a separate clade from the other species with *Fusicoccum* anamorphs indicates uncertainty about phylogenetic relationships within this group.

Our study showed considerable intraspecific variation in both DNA regions examined in both *B. rhodina* and *B. stevensii*, and it seems that isolates from California are representative of most of the variants found in a worldwide sampling of these species. The presence of isolates of *B. rhodina* and *B. stevensii* included in this study from different hosts as well as from different geographical locations (Table 3) could explain the variation in the DNA sequences between isolates from California and those from other parts of the world. However, the causes of the intraspecific variation observed between Cali-

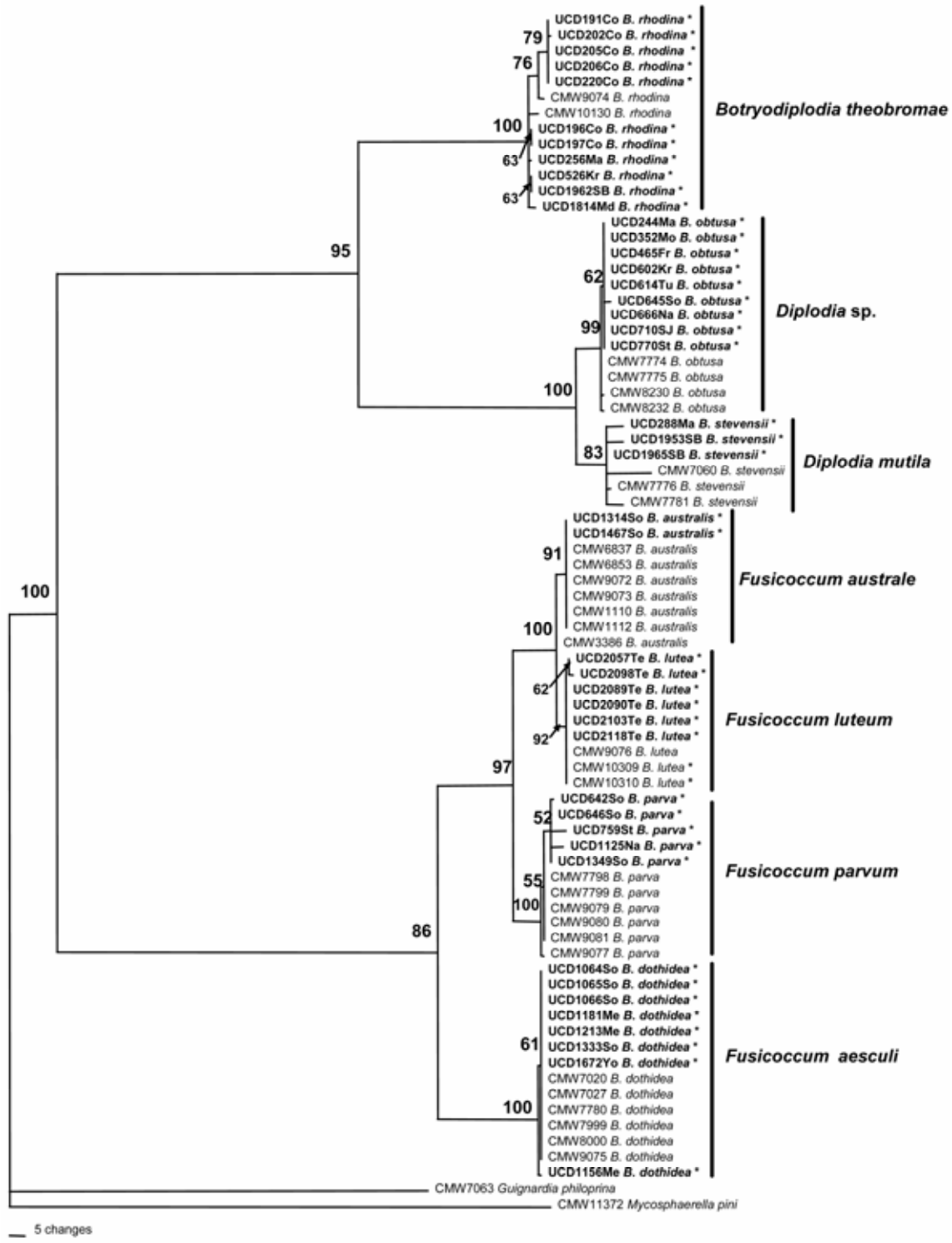

Fig. 7. Most equally parsimonious tree with bootstrap value using 1,000 replicates generated in PAUP 4.0b10 using internal transcribed spacer (ITS)1, 5.8S rDNA region, ITS2, and beta-tubulin partial gene. Asterisks show *Botryosphaeria* spp. from *Vitis vinifera*. Anamorph of each *Botryosphaeria* sp. is shown.

fornia isolates of these species remain unclear. On the other hand, it is interesting to note that, although isolates of *B. dothidea*, *B. lutea,* and *B. australis* have many different geographic origins and a broad range of hosts, no intraspecific variation was observed. However, much more extensive sampling will be required to determine whether this lack of variation is truly representative of these species worldwide.

Radial growth rates and optimum temperatures obtained in this study for *B. lutea*, *B. obtusa*, *B. parva*, *B. rhodina,* and *B. stevensii* are generally in agreement with data from previous literature (11,13,20,30). However, *B. lutea, B. parva,* and *B. stevensii* grew at 35ºC in our temperature study, whereas Pennycook and Samuels (20) reported that *B. lutea* and *B. parva* failed to grow at this temperature, and Jacobs and Rehner (11) made a similar observation for *B. stevensii*. On the other hand, Sánchez et al. reported slow growth of *B. stevensii* isolates from oak at 35ºC (30). The reasons for these discrepancies are unknown, but may simply reflect differences between the isolates studied. The high optimum temperature for *B. dothidea* isolates from California agrees with the data obtained by Jacobs and Rehner (11). However, this result differs with the lower optimum temperatures recorded for *B. dothidea*, 26.1ºC, from the study conducted by Sánchez et al. (30). Chromogenesis, previously reported by other researchers (13,42), was a characteristic observed in the *B. rhodina* isolates from California when grown at 35ºC for 2 weeks. The extremely rapid growth rates along with red coloration were useful characters in separating *B. rhodina* from the other species.

The preponderance of *B. rhodina* in the southern part of California, particularly in the desert area of Coachella Valley (Riverside County), and its nonappearance in the northern part of the state could be a consequence of climate differences. Similar results have been reported from a study recently conducted on grapevines in Western Australia, where *B. rhodina* was isolated only from the warmest area surveyed (37). The geographical distribution of *B. rhodina* observed in this study agreed with the results obtained by Leavitt, in which *B. rhodina* was isolated mainly in the desert area of Coachella Valley and rarely (5% of the total *B. rhodina* isolates) from the northern and southern San Joaquin Valley (13). It is widely known that *B. rhodina* is prevalent in areas with high temperatures, having a broad distribution over tropical and subtropical climates regions (26). The temperatures of the desert production area average 6 to 9ºC higher than temperatures in the San Joaquin Valley.

A similar outcome was observed with *B. lutea*, which was found only in cankers in Temecula Valley (desert area), but there is insufficient information to explain why *B. lutea* was not isolated in other areas surveyed in California. The assumption that climatic conditions can influence the geographical distribution of *Botryosphaeria* spp. in California could be reinforced by the fact that *B. australis*, *B. dothidea,* and *B. parva* were found mainly in the northern part of the state. *B. australis* is a frequent pathogen of native and introduced hosts in the Southern Hemisphere, and may be native to Australia (33). Ours is the first report of *B. australis* on grapevines in the Northern Hemisphere. The frequent recovery of *B. obtusa* and its presence in all grapevine regions in California are consistent with observations in Western Australia, where *B. obtusa* was the most common and cosmopolitan species isolated from grapevines (37).

Other environmental factors, such as wind, rainfall, and humidity, along with different viticulture practices, may influence the statewide distribution of *Botryosphaeria* spp. Factors such as rain, wind, source of inocula, or cultivar susceptibility have been shown to be associated with geographical distribution of other grapevine pathogens, such as *E. lata* and *P. viticola,* in California (13,18,22). However, the effects of these factors on *Botryosphaeria* spp. on grapevines in California have not yet been studied.

Pathogenicity of the seven *Botryosphaeria* spp. found in California has been well established on grapevine in other parts of the world, but their relative importance differs by country (12,37,41). Studies designed to characterize the virulence of *Botryosphaeria* spp. on grapevine in California are now underway.

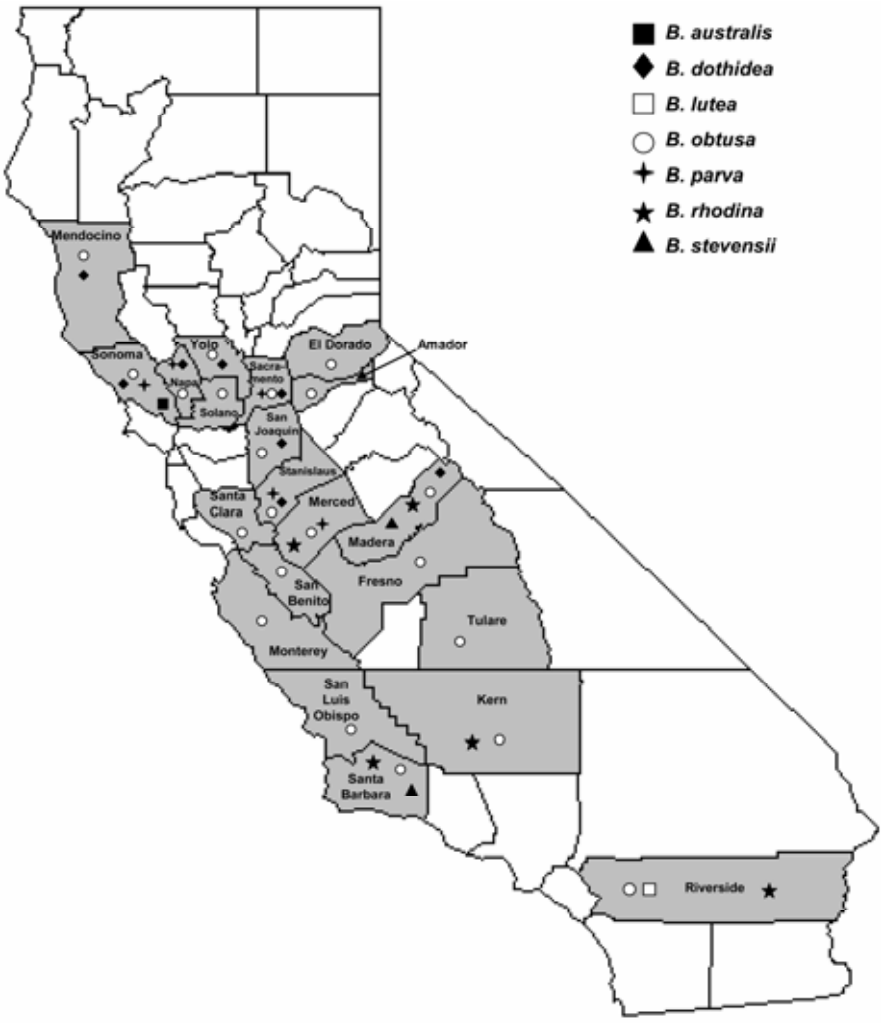

Fig. 8. Geographical distribution of *Botryosphaeria* spp. on grapevines in California.

ACKNOWLEDGMENTS

We thank E. Petit and F. Trouillas for their support in the phylogenetic analysis; J. Braun, Senior Statistician, Department of Statistics, University of California, Davis, for his support in the data analyses; T. Gordon, R. M. Davis, and J. K. Uyemoto (Department of Plant Pathology, University of California, Davis) for their valuable comments on this manuscript; D. Leonard (Department of Linguistics, University of California, Davis); and UCCE Viticulture Farm Advisors C. Gispert, C. Ingels, D. Hirschfelt, E. Weber, G. McGourty, J. Hashim, L. Bettiga, M. Moratorio, M. Battany, M. Norton, P. Verdegaal, R. Smith, R. Duncan, and S. Vasquez for their valuable assistance and guidance in accomplishing the field collections.

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