

Spatiotemporal Changes in the Population Structure of *Botryosphaeria dothidea* from California Pistachio Orchards

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ABSTRACT

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Spatiotemporal changes in the population structure of *Botryosphaeria dothidea*, causal agent of panicle and shoot blight of pistachio, were analyzed by using microsatellite-primed polymerase chain reaction (MP-PCR), partial sequences of the RNA polymerase II (RPB2) gene, and vegetative compatibility groups (VCGs). We examined 390 isolates, 378 recovered from pistachio in seven counties of California from 1990 to

2001 and 12 recovered from peach, apple, and sycamore in Georgia, North Carolina, South Carolina, Illinois, and Pennsylvania. Six microsatellite primers generated 116 polymorphic bands. Based on MP-PCR data, we observed very high (>98%) levels of genetic identity among populations of *B. dothidea* collected from the commercial pistachio orchards in California. The near identity of these populations was supported by VCGs and partial sequences of the RPB2 gene. These findings suggest that populations of *B. dothidea* from commercial pistachio orchards are spatially and temporally stable, at least in the past 5 years.

Panicle and shoot blight of pistachio (*Pistacia vera* L.), caused by the loculoascomycete *Botryosphaeria dothidea* (Moug.:Fr.) Ces & De Not., is characterized by infections of fruit clusters, buds, and twigs, and can result in significant yield reduction (16,19). The disease was first reported in commercial California pistachio orchards from Butte County in 1984 (26). In 1995, panicle and shoot blight was reported in pistachio orchards in the Central San Joaquin Valley and, in 1998, the disease was reported in the southern San Joaquin Valley (13). Since 2001, the disease has been found in all major commercial pistachio orchards of California and represents a significant and growing threat to the pistachio industry.

Control of panicle and shoot blight of pistachio is difficult. The only commercial cultivars in use, the female Kerman and male Peters cultivars, are very susceptible to *B. dothidea* infection (8). Although multiple applications of the fungicides azoxystrobin and tebuconazole provide efficacious control of the disease, the rapid development of fungicide resistance in *B. dothidea* threatens the California pistachio industry (9,12). The use of resistant pistachio cultivars is perhaps the most durable method, but judicious development and deployment of resistant cultivars is predicated on a sound understanding of the population structure of the pathogen. Understanding the genetic diversity of the pathogen and spatiotemporal changes in population structure is vital to the success of any breeding program (5,15).

Research efforts on the population structure of *B. dothidea* sought to determine (i) the current level of genetic diversity in populations of *B. dothidea* from pistachio and alternative plant hosts in California, (ii) how this diversity is distributed in California, (iii) whether the populations are stable over time, and (iv) mechanisms affecting spatiotemporal dynamics of *B. dothidea* populations. Previous studies, using microsatellite-primed polymerase chain reaction (MP-PCR) and sequences of the nuclear

rDNA internal transcribed spacer region (ITS) (8,10), found very low levels of genetic variation in the populations of this pathogen in California. However, these studies surveyed only spatial distribution of genetic variation among a few pistachio orchards and provided no information on the temporal dynamics of population structure for this pathogen.

Vegetative incompatibility is a multilocus system capable of generating large numbers of unique vegetative compatibility groups (VCGs) in many fungi (3,4,20), which has been used extensively to describe fungal population structure and diversity. In most ascomycetes, vegetative incompatibility (*vi*) is controlled by allelic interactions in which two individuals are compatible only if they share the same alleles at all *vi* loci. Conversely, individuals are vegetatively incompatible when alleles are different at one or more *vi* loci (20). Although such a VCG system has been reported on many other fungal species (4), to our knowledge, VCGs have never been reported on *B. dothidea* and other *Botryosphaeria* spp. Molecular markers can provide more detailed information on the genetic diversity and population structure of fungal pathogens (24). In this study, a large number of fungal isolates ($n = 390$) were analyzed by three complementary approaches: MP-PCR (29), partial sequences of the RNA polymerase II (RPB2) gene (6), and VCGs (4). The DNA sequence of RPB2, which has proven to be useful for broad-scale evolutionary studies of a variety of eukaryotic organisms (6), gives the detailed information at one locus and MP-PCR and VCGs give less detailed information but at many loci; therefore, combinations of such complementary approaches show advantages in analysis of fungal population structure. The aim of this study was to document the spatiotemporal dynamics and genetic structure of California *B. dothidea* populations so as to establish a basis for future development and deployment of resistant pistachio cultivars.

MATERIALS AND METHODS

Fungal isolate collections. From 1990 to 2001, we collected 378 single-spore isolates of *B. dothidea* from commercial pistachio orchards in Butte, Glenn, San Joaquin, Merced, Madera, Fresno, and Tulare counties (two to three orchards per county;

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one isolate per tree), and from pistachio trees at the U.S. Department of Agriculture (USDA) Germplasm Repository in Chico, CA. The representative isolates are presented in Table 1. Isolation and morphological confirmation of *B. dothidea* isolates were performed as previously described (8). In brief, single pycnidia were cut open under a dissecting microscope ($\times 15$) and placed in 500 μ l of sterile water to produce a spore suspension. A 20- μ l aliquot of the spore suspension was spread on a plate of acidified (2.5 ml of a 25% [vol/vol] solution of lactic acid per liter of medium) potato dextrose agar (APDA) (Microtech Scientific, Orange, CA). Plates were incubated at 29°C for 24 h. Colonies arising from single pycnidiospores of *B. dothidea* were recovered from each sample and transferred to fresh APDA plates.

MP-PCR amplifications and data analysis. *B. dothidea* isolates were grown in petri dishes containing 20 ml of potato dextrose broth (Difco Laboratories, Detroit) at 29°C for 4 days. Mycelia were harvested and washed in sterile water, snap frozen in liquid nitrogen, and lyophilized. Fungal genomic DNA was extracted with a FastDNA Kit (QBIogene Inc., Carlsbad, CA). Six microsatellite primers, (AAG)₈, (AG)₈C, (CTC)₄RC, (GTG)₅, M13 (5'-GAGGGTGGCGGTTCT-3'), and T3B (5'-AGGTCGCGGGTTCGAATCC-3'), which were informative in previous studies (8,10), were used for MP-PCR. The primers were synthesized by Invitrogen Life Technologies (Grand Island, NY). PCR was performed with an Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany) in a 50- μ l volume containing: 50 ng of fungal genomic template, 1.0 μ M each microsatellite primer, 0.2 mM each dNTP (Promega Corp., Madison, WI), 2.0 mM MgCl₂, 1 \times Promega *Taq* Polymerase Buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100), and 2 units of Promega *Taq* Polymerase. The following PCR run parameters were used: an initial preheat for 3 min at 95°C, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1.5 min, with a final extension at 72°C for 10 min. PCR was performed twice for each isolate. Amplicons were separated on 1.5% agarose gels in Tris-acetate (TAE) buffer and photographed after staining with ethidium bromide.

Isolates were scored for the presence or absence of each MP-PCR amplicon using Kodak Digital Science ID Image Analysis Software (Eastman Kodak Co., Rochester, NY). MP-PCR markers are considered dominant markers, because homozygotes could not be differentiated from heterozygotes without a progeny test. However, dominance of MP-PCR markers is not an issue for haploid fungi such as *B. dothidea* (14). Genetic similarities (S) were calculated as $S = 2N_{xy}/(N_x + N_y)$, where N_{xy} is the number of bands in common and N_x and N_y are the numbers of bands found in isolate x and y , respectively (7). A phenogram was constructed using the unweighted pair-group method with arithmetic average (UPGMA) by sequential, agglomerative, hierarchical, and nested clustering methods (the program SAHN) of the software package NTSYS-pc 2.1 (Department of Ecology and Evolution, State University of New York, New York). Clade support was assessed by 1,000 bootstrap replicates with PAUP 4.0 β 10 Win (Sinauer Associates, Sunderland, MA). The analyses of differentiation (*Gst*) and Nei's unbiased genetic identity (21,22) among populations were performed using the computer software POPGENE (version 1.32; University of Alberta, Edmonton, Canada). For the interpretation of *Gst*, it has been suggested that a value lying in the range 0 to 0.05 indicates little genetic differentiation, a value between 0.05 and 0.15 indicates moderate differentiation, a value between 0.15 and 0.25 indicates great differentiation, and values above 0.25 indicate very great genetic differentiation (2). Analysis of molecular variance (AMOVA) among the populations was analyzed using the ARLEQUIN software package (L. Excoffier, University of Geneva).

Partial DNA sequences of RPB2 gene. Eighteen *B. dothidea* isolates, derived as representatives from the three groups identi-

fied by MP-PCR, were selected for RPB2 sequence analysis. Primers RPB2-6F (5'-TGG GGT CTT GTC TGC CCG GC-3') and RPB2-7R (5'-CCC ATT GCC TGC TTA CCC AT-3') were designed based on the RPB2 DNA sequences of *B. rhodina* (GenBank accession no. AF107802) (6). RPB2-6F and RPB2-7R are located in motif 6 and 7, respectively. The PCR was performed in 100 μ l containing 60 ng of fungal genomic DNA, 1.0 μ M each primer, 0.2 mM each dNTP, 2.0 mM MgCl₂, 1 \times Promega *Taq* Polymerase buffer, and 1.5 units of Promega *Taq* Polymerase with the following parameters: an initial preheat for 3 min at 95°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 1.5 min, with a final extension at 72°C for 10 min. Amplicons were size verified on 1.5% agarose gels in TAE buffer and purified using the QIAquick gel extraction kit (QIAGEN Inc., Valencia, CA). Both strands of the recovered DNA fragments were sequenced by DBS Sequencing Inc. (Division of Biological Sciences, University of California at Davis) using primers RPB2-6F and RPB2-7R. The GenBank accession numbers for partial sequences of RPB2 gene from *B. dothidea* isolates are AY217046 to AY217063.

The partial sequences of RPB2 from *B. dothidea* were aligned using the computer program Clustal W 1.82 (European Bioinformatics Institute, Cambridge, UK). Phylogenetic relationships among the isolates of *B. dothidea* were determined from the aligned DNA sequences using PAUP 4.0 β 10 Win. *B. rhodina* (GenBank accession no. AF107802) was used as the outgroup isolate. All nucleotides were treated as unordered, unweighted characters. Maximum parsimony trees were inferred by using the heuristic search option with 1,000 random-addition replicates with the tree bisection reconnection (TBR) algorithm. Support for the clades was assessed by 1,000 bootstrap replicates. The graphical tree was displayed using the program TreeView (23).

Vegetative compatibility. Vegetative compatibility tests in *B. dothidea* were carried out in 9-cm petri dishes, each containing 20 ml of pistachio leaf decoction agar (100 g of ground leaves plus 20 g of PDA plus 10 g of agar in a liter of distilled water). For pairing experiments, 3-mm mycelial plugs were taken from the edge of 3-day-old colonies of each isolate and placed 2.5 cm apart on pistachio leaf decoction agar plates. Seven mycelial plugs were paired on each plate. Each isolate was paired with itself (control) and with each of the other isolates. There were two replicate plates for each isolate. After 1 week of incubation at 29°C, pairs were scored as vegetatively compatible when mycelia of two isolates had merged together uniformly. Pairs were scored as vegetatively incompatible when mycelia of two isolates grew to a meeting point on the agar but were separated by a "barrage-like" reaction formed along the line of contact between paired colonies (1,4). The pairing experiment was repeated twice.

RESULTS

MP-PCR. The six MP-PCR primers used in this study consistently generated 116 polymorphic amplicons from the 390 *B. dothidea* isolates surveyed: 378 derived from pistachio in California and 12 derived from apple, peach, and sycamore from other states (Table 1; Fig. 1). Of the 378 isolates from California pistachio, 371 (98.1%) were in group I based on the MP-PCR data (Fig. 2). Within group I, 329 isolates (88.6%) were in subgroup I-1, and were found from pistachio throughout California, whereas subgroup I-2 contained 42 isolates originating from the central-California counties of Madera, Fresno, and Tulare.

A high level of genetic identity (>98%) was found among *B. dothidea* populations obtained from pistachio in different locations of California (Table 2). However, the California populations were significantly different from the isolates recovered from other states (*Gst* = 0.7683). A more detailed hierarchical based on AMOVA partition of the total variance indicated that genetic vari-

ation among populations in California accounted for only 0.65% of the total genetic variation (Table 3). Most of the variation (94.63%) occurred between the population of non-California (non-CA) isolates and the populations from California pistachio. Additionally, very high levels of genetic identity (>99%) were observed for the five subpopulations obtained from the same commercial orchards in Madera County from 1997 to 2001 (Table 4). Similar high levels of genetic identity were observed for subpopulations collected from Fresno and Tulare County from 1999 to 2001. These data indicate that the *B. dothidea* populations from commercial California pistachio orchards were spatially and temporally stable, at least in the past 5 years.

RPB2 sequences. The aligned RPB2 gene sequences contain enough phylogenetically informative characters to infer relationships among *B. dothidea* isolates. Of the 694 aligned nucleotide characters, 125 were parsimony informative and 53 were variable and parsimony uninformative. In general, results obtained from

the partial sequences of RPB2 were consistent with data derived from MP-PCR. Ten representatives of group I (371 isolates), which are 98% similar in MP-PCR assays (Fig. 2), had the same DNA sequences of RPB2 gene (Fig. 3). California isolates B72, B73, and B558, which had different MP-PCR fingerprints (Fig. 2), also had different RPB2 DNA sequences (Fig. 3).

Vegetative compatibility. After 1 week of incubation, colonies of vegetatively compatible isolates had merged, forming confluent mycelia (Fig. 4). Incompatible isolates formed a distinct barrage-like reaction along the line of contact between paired colonies because lethal hyphal fusions occurred in the barrage regions (Fig. 4). Most isolates from pistachio were assigned into one of two major VCGs. Among the 390 isolates, 20 VCGs were identified (Table 1). VCG 1 contained 314 isolates of subgroup I-1 (Table 1; Fig. 2), and VCG 2 contained all 42 isolates of subgroup I-2 (Table 1; Fig. 2). In contrast, the 12 fungal isolates from other hosts or locations belonged to nine different VCGs (Table 1).

TABLE 1. *Botryosphaeria dothidea* isolates collected from pistachio ($n = 378$) in California and from apple, peach, and sycamore ($n = 12$) in other states of the United States used for microsatellite-primed polymerase chain reaction (MP-PCR) analysis and vegetative compatibility group (VCG) tests

MP-PCR group	VCG group	No. of isolates	Representative	Host	Location	Year of isolation
I-1	1	4	BC425	Pistachio	Chico, Butte Co., CA	1999
I-1	1	17	BC16	Pistachio	Chico, Butte Co., CA	2000
II	3	1	BC4	Pistachio	Chico, Butte Co., CA	2000
II	3	2	BC15	Pistachio	Chico, Butte Co., CA	2000
I-1	1	4	B71	Pistachio	Durham, Butte Co., CA	1990
I-1	1	5	B171	Pistachio	Durham, Butte Co., CA	1998
I-1	4	1	B175	Pistachio	Durham, Butte Co., CA	1998
III	5	2	B72	Pistachio	Durham, Butte Co., CA	1999
III	6	1	B558	Pistachio	Durham, Butte Co., CA	1999
I-1	1	2	B560	Pistachio	Durham, Butte Co., CA	1999
I-1	1	31	B20	Pistachio	Durham, Butte Co., CA	2000
I-1	7	5	B5	Pistachio	Durham, Butte Co., CA	2000
I-1	1	27	B51	Pistachio	Durham, Butte Co., CA	2001
I-1	1	46	G20	Pistachio	Glenn Co., CA	2000
I-1	8	2	G6	Pistachio	Glenn Co., CA	2000
II	3	1	G24	Pistachio	Glenn Co., CA	2000
I-1	9	1	G40	Pistachio	Glenn Co., CA	2000
I-1	1	22	SJ474	Pistachio	San Joaquin Co., CA	2000
I-1	1	15	Me835	Pistachio	Merced Co., CA	2000
I-1	1	8	Ma59	Pistachio	Madera Co., CA	1997
I-2	2	6	Ma85	Pistachio	Madera Co., CA	1997
I-1	1	11	Ma147	Pistachio	Madera Co., CA	1998
I-1	1	26	Ma68	Pistachio	Madera Co., CA	1999
I-2	2	2	Ma225	Pistachio	Madera Co., CA	1999
I-1	1	9	Ma770	Pistachio	Madera Co., CA	2000
I-1	1	47	Ma1	Pistachio	Madera Co., CA	2001
I-1	10	1	Ma4	Pistachio	Madera Co., CA	2001
I-1	7	4	Ma11	Pistachio	Madera Co., CA	2001
I-2	2	1	Ma14	Pistachio	Madera Co., CA	2001
I-2	2	1	F78	Pistachio	Fresno Co., CA	1997
I-1	1	1	F79	Pistachio	Fresno Co., CA	1997
I-2	2	3	F192	Pistachio	Fresno Co., CA	1999
I-2	2	8	F497	Pistachio	Fresno Co., CA	2000
I-1	1	7	F952	Pistachio	Fresno Co., CA	2000
I-2	2	13	F15	Pistachio	Fresno Co., CA	2001
I-1	1	9	F4	Pistachio	Fresno Co., CA	2001
I-1	11	1	F27	Pistachio	Fresno Co., CA	2001
I-1	1	4	T420	Pistachio	Tulare Co., CA	1999
I-2	2	2	T567	Pistachio	Tulare Co., CA	1999
I-2	2	4	T755	Pistachio	Tulare Co., CA	2000
I-1	1	10	T758	Pistachio	Tulare Co., CA	2000
I-2	2	2	T1187	Pistachio	Tulare Co., CA	2001
I-1	1	9	T1188	Pistachio	Tulare Co., CA	2001
II	12	1	BD14	Apple	Byron, GA	1980
II	13	1	BD18	Apple	Urbana, IL	1980
II	14	1	BDN2	Peach	Byron, GA	2000
II	15	1	BDN3	Peach	Byron, GA	2000
III	16	1	BD926	Sycamore	Maysville, SC	1999
II	17	1	PA1	Apple	Adams Co., PA	1999
II	18	1	PA2	Apple	Adams Co., PA	1999
II	18	1	PA3	Apple	Adams Co., PA	1999
II	19	2	1860	Apple	Fletcher, NC	1992
II	20	2	1881	Apple	Jackson Spgs., NC	1992

DISCUSSION

B. dothidea populations from California pistachio orchards have remarkably low levels of genetic variation, with more than 98% of the isolates belonging to a single MP-PCR group. The homogeneity in the populations derived from pistachio orchards at diverse locations in California, in some cases separated by more than 370 km, suggests that *B. dothidea* populations have undergone little genetic change since the disease was first detected in 1984 (26). The near identity of these populations was demonstrated further with partial sequences of the RPB2 gene and VCGs. All representative isolates of group I, defined by MP-

PCR, had the same RPB2 DNA sequences. Among 378 isolates from California pistachio, 356 (94%) were assigned to two major VCGs. This genetic homogeneity also persisted over time, because subpopulations of *B. dothidea* collected in the past 5 years from the same locations in Madera, Fresno, or Tulare County were very similar (>99% genetic identity). Additionally, the MP-PCR band profile, partial sequences of RPB2, and VCG of four isolates collected from Durham, Butte County, CA, in 1990 were the same as those of the isolates collected in 2001. These results strongly suggested that *B. dothidea* populations from California commercial pistachio orchards have been spatially and temporally stable, at least during 1997 to 2002.

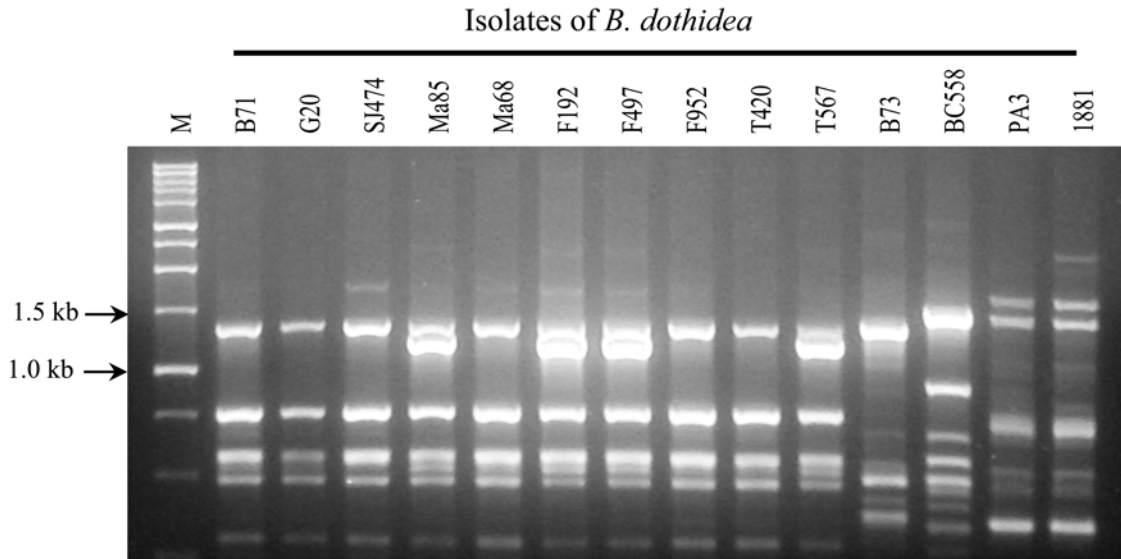


Fig. 1. An example of electrophoretic separation of polymerase chain reaction amplicons of *Botryosphaeria dothidea* isolates obtained from primer T3B. M is a molecular weight marker (1-kb DNA ladder; Promega Corp., Madison, WI).

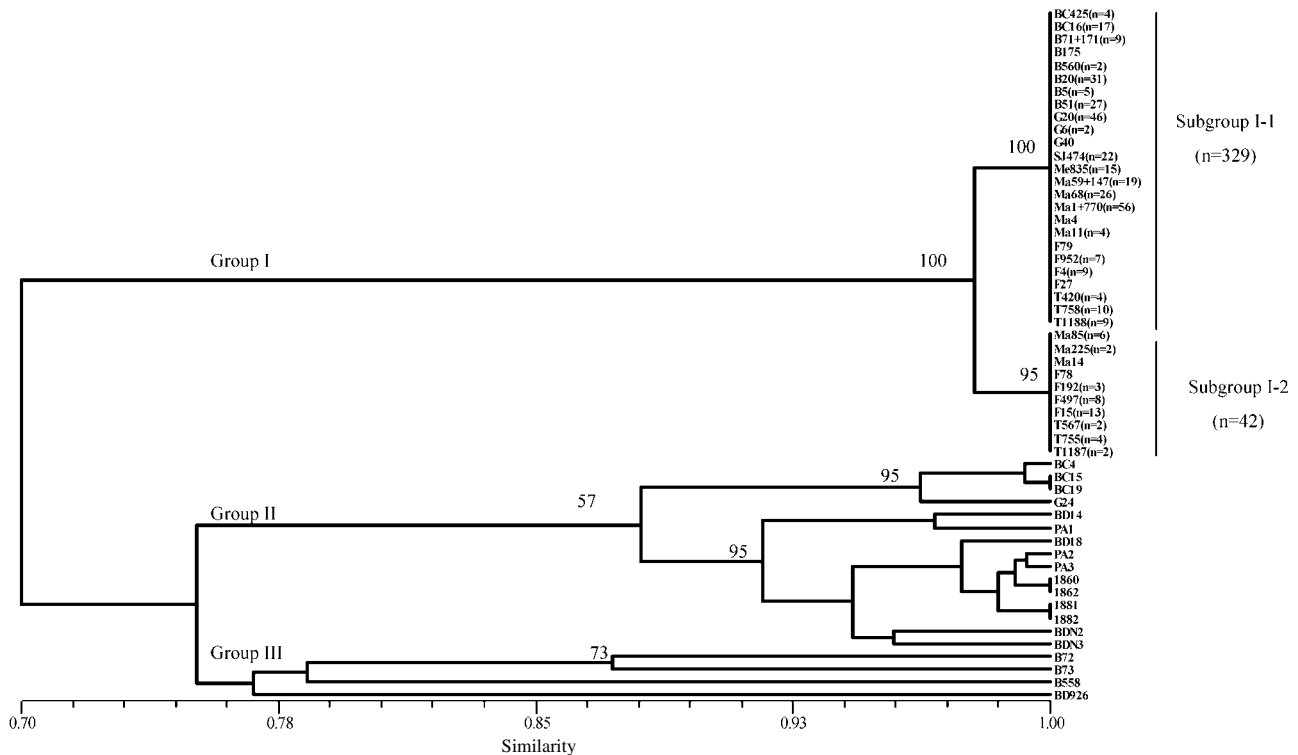


Fig. 2. Unweighted pair group method with arithmetic averages cluster analysis of microsatellite-primed polymerase chain reaction data for 378 isolates of *Botryosphaeria dothidea* from pistachios in California and 12 non-California isolates from other hosts in other states in the United States. Bootstrap frequencies greater than 50% from 1,000 replications are indicated on the branches.

Although little genetic variation was detected in the *B. dothidea* populations in California, we observed significant genetic variation between California populations and the population obtained from other U.S. states. Support for the high genetic diversity among *B. dothidea* isolates collected from different U.S. states also was provided by the analyses of random amplified polymorphic DNA (RAPD) markers. Smith and Stanosz (27) found that isolates of *B. dothidea*, which were collected from Georgia, South Carolina, Washington, D.C., and Wisconsin, had more than 50% dissimilarity by analysis of RAPD markers. Although isolates of non-CA population used in this study were recovered from other hosts, results from a previous study indicated that *B. dothidea* did not show host specificity (8). In previous studies, we also observed high levels of genetic variation between populations from pistachio collected from California and Greece (8,11). Thus, the low diversity in the *B. dothidea* populations from pistachio may result from a founder effect. *B. dothidea* seems to have been introduced into pistachio orchards relatively recently (16), when a limited number of individuals were introduced to pistachio orchards and established a new population; therefore, this limited population might show reduced genotypic diversity compared with original populations.

The distributions of the subgroup I-1 and I-2 genotypes, defined by MP-PCR, were different. Subgroup I-1 isolates were detected throughout California, whereas subgroup I-2 isolates were detected only in central California and were not detected in northern California, including the USDA Germplasm Repository in Chico. Subgroup I-2 isolates have been recovered previously from cedar, redwood, juniper, and other hosts in central California, but not from any host plant surveyed in northern California

(10). Other studies documented the occurrence of *B. dothidea* on native plant species (e.g., cedar and redwood) before the pathogen was detected on pistachio (16,30). Hemipteran insects and birds can vector the pathogen from other hosts to pistachio and between commercial pistachio orchards (18). Thus, the subgroup I-2 isolates might have been introduced into pistachio orchards recently from native plants in central California. These results are consistent with the finding that all subgroup I-2 isolates from pistachio belonged to a single VCG different from VCGs of the isolates in subgroup I-1. The results also suggest that *B. dothidea* on pistachio in central California may have originated from two different sources.

The sexual stage or teleomorph of *B. dothidea* rarely has been observed in nature. Pseudothecia of this pathogen have been found occasionally on giant sequoia, coastal redwood (30), blackberry, olive, avocado, and fire thorn (17) in California, but the sexual stage of *B. dothidea* never has been found on pistachio (8,16). The two major genotypes (I-1 and I-2), defined by multilocus genetic markers (MP-PCR markers and VCGs), were distributed widely across the state and collected over several years. Thus, these isolates presumably were produced clonally (28). The result is consistent with the hypothesis that the populations of *B. dothidea* of California pistachio are primarily asexual in composition.

Vegetative incompatibility has been widely used to provide insights into the genetic structure of fungal populations (4). The extent of polymorphism in vegetative incompatibility phenotypes and the relative ease with which they can be assayed have made these phenotypes popular for studying fungal populations. Vegetative incompatibility in Ascomycetes is heterogenic and allelic (25). Isolates with identical alleles at all of the vegetative com-

TABLE 2. Nei's unbiased measures of genetic identity between *Botryosphaeria dothidea* populations collected from pistachio at different locations in California and non-California (non-CA) populations from other hosts in other states of the United States

Population	Chico (n = 24)	Durham (n = 78)	Glenn (n = 50)	San Joaquin (n = 22)	Merced (n = 15)	Madera (n = 115)	Fresno (n = 43)	Tulare (n = 31)	Non-CA group (n = 12) ^a
Chico	...	0.9966	0.9971	0.9954	0.9954	0.9952	0.9860	0.9935	0.6476
Durham	0.9999	0.9998	0.9998	0.9996	0.9909	0.9981	0.5921
Glenn	0.9999	0.9999	0.9998	0.9911	0.9983	0.5937
San Joaquin	1.0000	0.9998	0.9913	0.9984	0.5824
Merced	0.9998	0.9913	0.9984	0.5824
Madera	0.9935	0.9993	0.5814
Fresno	0.9974	0.5702
Tulare	0.5783
Non-CA group

^a Twelve isolates were recovered from apple, peach, and sycamore in Georgia, South Carolina, North Carolina, Illinois, and Pennsylvania.

TABLE 3. Hierarchical analysis of molecular variance within and among the populations of *Botryosphaeria dothidea* based on 116 polymorphic microsatellite primed polymerase chain reaction markers

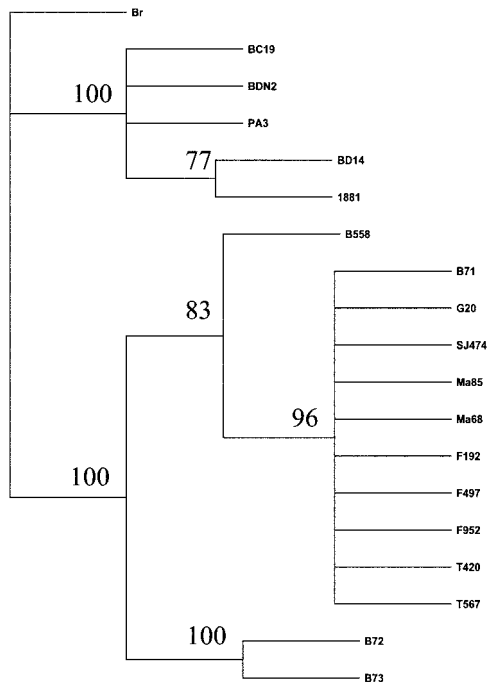
Source of variation	df	Sum of squares	Variance component	Percentage of variation	P value ^a
Between the non-California (non-CA) population and California populations	1	525.11	22.44	94.63	0.000
Among California populations	7	55.50	0.15	0.65	0.000
Within California populations and the non-CA population	381	425.94	1.12	4.72	0.000
Total	389	1,006.55	23.71

^a Probability of having larger values than those observed with 1,000 permutations.

TABLE 4. Nei's unbiased measures of genetic identity between subpopulations of *Botryosphaeria dothidea* collected from the same locations in Madera County during 1997 to 2001

Subpopulation ^a	1997 (n = 14)	1998 (n = 11)	1999 (n = 28)	2000 (n = 9)	2001 (n = 53)
1997 (n = 14)	...	0.9955	0.9970	0.9955	0.9959
1998 (n = 11)	0.9999	1.0000	1.0000
1999 (n = 28)	0.9999	1.0000
2000 (n = 9)	1.0000
2001 (n = 53)

^a Subpopulations were designated by year.



Tree length = 208
 Consistency index = 0.9471
 Retention index = 0.9794
 Rescaled consistency index = 0.9276

Fig. 3. Phylogenetic relations among the isolates of *Botryosphaeria dothidea* based on maximum parsimony analysis of partial sequences of RNA polymerase II (RPB2). A *B. rhodina* isolate (Br) was used as the outgroup isolate. Numbers on branches are bootstrap frequencies of higher than 50% from 1,000 replicates of a heuristic search.

patibility loci form anastomoses, and their mycelia merge. Incompatibility may result in a visible barrage zone when cultures are paired. When the pairing tests were conducted on conventional PDA plates, we observed mycelia growing from one mycelial plug entirely separated from mycelia from a second plug on the same PDA plate; a zone of no mycelial growth developed between the two colonies even when both colonies were initiated from a single isolate. In this study, however, we developed the medium of pistachio leaf decoction agar, which easily allowed recognition of VCGs for *B. dothidea*. We believe that this method will be helpful to study population structures of other species of *Botryosphaeria*.

Presently, the California pistachio industry is based entirely on two pistachio cultivars, Kerman and Peters, both of which are extremely susceptible to *B. dothidea*. The interactions of a genetically uniform yet highly virulent pathogen population and the monoculture of highly susceptible perennial pistachio cultivars may pose a serious threat to the California pistachio industry. The results from three complementary approaches, MP-PCR, sequence of RPB2, and VCGs, showed little genetic variation in *B. dothidea* populations from California pistachio and the lack of the propensity to form a sexual stage on this host, which may suggest that a program to breed resistant pistachio cultivars has a high chance of success.

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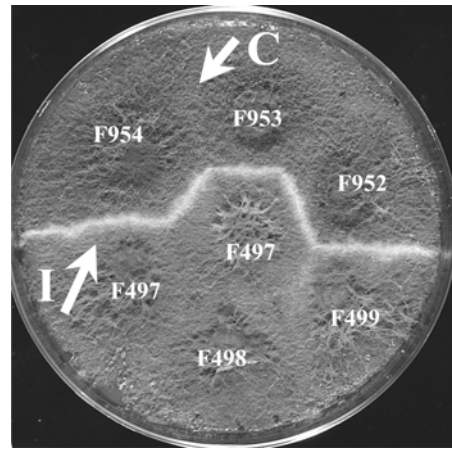


Fig. 4. Vegetative compatibility tests in *Botryosphaeria dothidea* isolates F497, F498, F499, F952, F953, and F954. Compatible isolates merged uniformly along the line of contact (C); and barrage reaction developed along the line of contact of two incompatible isolates (I) because lethal hyphae fused in the barrage regions.

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