Differentiation of a *Fusicoccum* sp. Causing Panicle and Shoot Blight on California Pistachio Trees from *Botryosphaeria dothidea*

Denise R. Smith, Department of Plant Pathology, University of Wisconsin, Madison 53706; Themis J. Michailides, University of California, Davis, and Kearney Agricultural Center, Parlier, CA 93648; and Glen R. Stanosz, Department of Plant Pathology, University of Wisconsin

ABSTRACT

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A panicle and shoot blight disease of pistachio trees in California is caused by a fungus previously identified as the anamorph of *Botryosphaeria dothidea*. We have compared random amplified polymorphic DNA (RAPD) markers, nuclear rDNA internal transcribed spacer (ITS) region sequences, and conidium morphology of 15 isolates of the pistachio *Fusicoccum* to those of well-characterized isolates of *B. dothidea*, *B. ribis*, and *F. luteum*. Cluster analysis of RAPD markers separated the pistachio *Fusicoccum* isolates from *B. dothidea*, as did parsimony analysis of the ITS region sequences. Conidium size and shape were similar to those of *B. ribis* (*Fusicoccum* sp.) and *F. luteum*, but distinguishable from those of *F. aesculi* (the anamorph of *B. dothidea*). We conclude that the fungus causing panicle and shoot blight of pistachio is distinguishable from *B. dothidea* and is part of a complex containing *B. ribis*, *F. luteum*, and other fungi with *Fusicoccum* anamorphs.

Additional keywords: *Botryosphaeria ribis*, *Fusicoccum aesculi*, *Fusicoccum luteum*, *Pistacia vera*

A disease characterized by panicle and shoot blight was observed on pistachio (*Pistacia vera* L. cv. Kerman) in Butte County, CA in 1984 and later in other parts of the state (17). A pycnidial fungus consistently was isolated from symptomatic plant parts (12). Koch's postulates were completed with production of symptoms on pistachio similar to those observed in the field (12). Subsequent spread of the pathogen throughout California and the severity of epidemics which substantially reduce nut quality and yield have led to consideration of this disease as the greatest threat to the California pistachio industry (11,13).

The pathogen that causes this disease was referred to by Michailides (12) as *Botryosphaeria dothidea*. He reported that isolates of the panicle and shoot blight pathogen were consistent with the description of the pycnidial stage of *Botryosphaeria ribis* (12,15), which had previously been placed in synonymy with *B. dothidea* (1). Identification of the pistachio panicle and shoot blight pathogen as *B. dothidea* also was based on comparison of morpho-

logical and cultural characteristics of several isolates to a fungus identified as *B. dothidea* that causes a bandlike canker of almonds and an isolate of *B. dothidea* from peach (2,8,12). The anamorphs of both the almond bandlike canker pathogen and the pistachio panicle and shoot blight pathogen, which produce hyaline conidia, also have been referred to as *Dothiorella* spp. (8,12). According to Sutton, however, the anamorph of *B. dothidea* (which produces hyaline conidia) is *Fusicoccum aesculi* (28). In addition, the name *Dothiorella* now appears to be inappropriate because it recently was synonymized with *Diplodia*, a genus reserved for fungi with pigmented conidia (6,7).

Identification and classification of *Botryosphaeria* spp. and associated anamorphic fungi can be difficult. Morphological differences, especially among *Fusicoccum* anamorphs, may not be distinctive, and the taxonomic and pathological history of many of these fungi is muddled. For example, although not describing any basis for their decision, von Arx and Müller (1) placed a number of *Botryosphaeria* spp. (and associated anamorphic fungi) in synonymy. Other authors have not accepted the synonymy of taxa such as *B. ribis* with *B. dothidea*, and the former name continues to be used (9,15,16). Another very similar fungus in this group is *F. luteum*, associated with kiwifruit rot (14), but also collected from a variety of native plants in Australia and pistachio in Italy (21). Re-

cently, studies of both morphological characteristics and molecular markers have allowed differentiation of *B. dothidea* and its anamorph from a complex containing *B. ribis*, *F. luteum*, and other fungi with *Fusicoccum* anamorphs (21,32).

During preliminary examination, differences were noted among random amplified polymorphic DNA (RAPD) markers from the pistachio panicle and shoot blight pathogen and those from isolates of *B. dothidea*. Correct identification is important for both disease management and further research on the pistachio panicle and shoot blight pathogen; therefore, studies were undertaken to compare it to previously characterized isolates of *B. dothidea*, *B. ribis*, and *F. luteum*. Differences among RAPD markers and nuclear ribosomal internal transcribed spacer (ITS) sequences were analyzed and the sizes and shapes of conidia were compared.

MATERIALS AND METHODS

Fungal isolates. Isolates used in this study were purchased from culture collections or were obtained from symptomatic host material by the authors or cooperators (Table 1). The isolates of the pistachio panicle and shoot blight pathogen included five from each of three geographic areas of pistachio production in California. Four previously characterized isolates of *Sphaeropsis sapinea* RAPD marker group A (24) were included as an outgroup for comparison in both RAPD marker analyses and ITS sequence analysis (Table 1).

RAPD marker data. Data generated for the 15 isolates of the pistachio panicle and shoot blight pathogen were compared with existing data for representative isolates from a more comprehensive study of relationships among *B. dothidea* (94-104, 96-6, 96-15, 97-5, and 97-94), *B. ribis* (94-128, 94-131, 96-12, 96-187, and 97-113), and *F. luteum* (97-38, 97-40, 97-45, 97-78, and 97-88) (21). Procedures, materials, and equipment for genomic DNA extraction and amplification were similar to those previously described (20). Primers DS1 (5'-CTGCGACTGAATCTTGCAG) and DS13 (5'-GGTGCTATAAACGCAAC-AGG), however, were used in place of DS2 and DS4. Eight 17- to 21-nt primers were used. DNA amplification took place in two steps, the first allowing low stringency annealing of primers (40°C) and the second allowing high stringency annealing

Corresponding author: D. R. Smith
dzs@plantpath.wisc.edu

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(55°C). DNA fragments were separated on 0.7% agarose gels in Tris-boric acid-EDTA (TBE) buffer and photographed after staining with ethidium bromide. Each isolate usually was assayed three times by growing a culture, extracting DNA, amplifying the DNA with each primer, and separating the fragments. However, for a few isolate-primer combinations, only two assays were performed.

Each isolate was scored for the presence or absence of each amplification product. Only amplification products that were present throughout all repetitions for at least one isolate were included in the analysis. Because of the large diversity of fragments, Southern blots were not performed to test fragment homology.

Relationships among isolates were determined by calculation of Jaccard's similarity coefficient (S_j) for each pair of isolates. Jaccard's similarity coefficient is calculated by the formula described by Sneath and Sokal (22): $S_j = a/(a + u)$,

where a is the number of matches of positive data points between two isolates and u is the total number of fragments unique to each isolate. A phenogram was constructed after cluster analysis of the similarity coefficients by the unweighted pairgroup method using arithmetic averages (UP-GMA; 22). These calculations were performed with the programs SIMQUAL and SAHN of the software package NTSYS-pc (ver. 2.0; Exeter Software, Setauket, NY).

ITS sequence data. Sequence data from the nuclear ribosome ITS and 5.8S gene were compared for selected isolates (Table 1). These included five isolates of the pistachio panicle and shoot blight pathogen (at least one from each major pistachio-producing area in California). Sequences of *B. dothidea*, *B. ribis*, and *F. luteum* isolates were obtained from GenBank or had been acquired during other recent investigations (21,32). One isolate from *S. sapinea* RAPD marker group A was used as an outgroup.

For sequences acquired in this study, DNA was extracted using the same procedure as for RAPD markers. The ITS region was amplified using fungal universal primers ITS1 or ITS5 and ITS4 (31). Polymerase chain reaction (PCR) was performed in 100- μ l reactions consisting of 1 U of *Taq* DNA Polymerase, 1 $\times$ buffer, 2.0 mM MgCl₂, 150 μ M each dNTP (all purchased from Promega Corp., Madison, WI), 2 μ M each primer (University of Wisconsin-Madison Biotechnology Center), and 2 to 10 ng of DNA. Each reaction mixture was overlaid with mineral oil and the following temperature profile was used in a Perkin-Elmer Thermal Cycler 480 (Norwalk, CT): 1 cycle of 5 min at 95°C; followed by 30 cycles of 1 min at 95°C, 1 min at 60°C, and 1 min at 72°C; followed by 1 cycle of 5 min at 72°C. Fragment sizes were determined on 0.7% agarose TBE gels. The fragments were purified using the QIAGEN QIAquick PCR Purification kit (QIAGEN Inc., Chatsworth,

Table 1. Isolates used to investigate relationships among pistachio *Fusicoccum* isolates and some other *Botryosphaeria* and *Fusicoccum* species

RAPD ^w	Isolate	Host	Location	Collector	Other no. ^x	Acc. no. ^y	References
Fp	99-6	<i>Pistacia vera</i> ^z	Kern Co., CA	T. Michailides	...	...	...
Fp	99-7	<i>P. vera</i>	Kern Co., CA	T. Michailides	...	AF357535	...
Fp	99-8	<i>P. vera</i>	Kern Co., CA	T. Michailides	...	AF357536	...
Fp	99-9	<i>P. vera</i>	Kern Co., CA	T. Michailides	...	...	...
Fp	99-10	<i>P. vera</i>	Kern Co., CA	T. Michailides	...	...	...
Fp	99-11	<i>P. vera</i>	Madera or Merced Co., CA	T. Michailides	...	AF357537	...
Fp	99-12	<i>P. vera</i>	Madera or Merced Co., CA	T. Michailides	...	...	...
Fp	99-13	<i>P. vera</i>	Madera or Merced Co., CA	T. Michailides	...	...	...
Fp	99-14	<i>P. vera</i>	Madera or Merced Co., CA	T. Michailides	...	...	...
Fp	99-15	<i>P. vera</i>	Madera or Merced Co., CA	T. Michailides	...	AF357538	...
Fp	99-16	<i>P. vera</i>	Butte or Glenn Co., CA	T. Michailides	...	AF357539	...
Fp	99-17	<i>P. vera</i>	Butte or Glenn Co., CA	T. Michailides	...	...	...
Fp	99-18	<i>P. vera</i>	Butte or Glenn Co., CA	T. Michailides	...	...	...
Fp	99-19	<i>P. vera</i>	Butte or Glenn Co., CA	T. Michailides	...	...	...
Fp	99-20	<i>P. vera</i>	Butte or Glenn Co., CA	T. Michailides	...	...	...
Bd	94-104	<i>Liquidambar styraciflua</i>	South Carolina	R. Roncadori	B12	...	21
Bd	96-6	<i>Prunus persica</i>	Georgia	P. L. Pusey	Bd80, 94.27	AF027748	9,21,32
Bd	96-15	<i>Prunus persica</i>	Japan	P. L. Pusey	Bd73, 94.26	AF027749	9,21
Bd	97-5	<i>Malus</i> sp.	Wisconsin	P. McManus	...	AF241173	4,21
Bd	97-94	<i>Populus nigra</i>	New Zealand	G. Samuels	GJS 83-545, ATCC 58188	AF241175	14,21,32
Br	94-128	<i>Melaleuca quinquenervia</i>	Florida	M. Rayachhetry	M4, 94.09, 96-7, DAOM 229436	AF027743	9,16,21,32
Br	94-131	<i>Rhizophora mangle</i>	Florida	M. Rayachhetry	R7, 94.11, 96-8	...	9,16,21,32
Br	96-12	<i>Malus</i> sp.	Washington, D.C.	K. Jacobs	93.42	AF027741	9,21,32
Br	96-187	<i>Ribes rubrum</i>	Not known	N.E. Stevens	CBS 121.26	AF241177	21,32
Br	97-113	<i>Rhus typhina</i>	Wisconsin	D. R. Smith	...	AF241176	21,32
Fl	97-38	<i>Banksia caleyi</i>	Australia	H. White	WA 6731	...	21
Fl	97-40	<i>B. caleyi</i>	Australia	H. White	WA 6733	AF241178	21,32
Fl	97-45	<i>Jacksonia horrida</i>	Australia	H. White	WA 6738	...	21
Fl	97-78	<i>Pistacia</i> sp.	Italy	L. Sparapano	...	AF293479	21
Fl	97-88	<i>Actinidia chinensis</i>	New Zealand	G. Samuels	ATCC 58194, 93.52	AF027745	9,14,21,32
SsA	239	<i>Pinus resinosa</i>	Wisconsin	M. Palmer	...	...	24
SsA	411	<i>P. resinosa</i>	Minnesota	M. Palmer	DAOM 229440	AF243409	24
SsA	92-19	<i>P. resinosa</i>	Wisconsin	G. R. Stanosz	...	...	24
SsA	92-66	<i>P. sylvestris</i>	Michigan	G. R. Stanosz	...	...	24

^w Random amplified polymorphic DNA (RAPD) marker group as characterized in this study or previously determined (21); Bd = *Botryosphaeria dothidea*, Br = *B. ribis*, Fp = pistachio *Fusicoccum*, Fl = *Fusicoccum luteum*, and SsA = *Sphaeropsis sapinea* RAPD marker group A.

^x Isolate numbers assigned by collectors or culture collections.

^y GenBank accession numbers for isolates from which an internal transcribed spacer region sequence was obtained.

^z *P. vera* cv. Kerman.

CA), labeled with the ABI Prism BigDye Terminator Cycle Sequencing Kit (PE Biosystems, Foster City, CA), then sequenced by the University of Wisconsin-Madison Biotechnology Center on a PE Biosystems 377XL automated sequencer.

The complete sequences for the ITS region (partial 18S, ITS1, 5.8S gene, ITS2, and partial 28S sequences) were aligned using ClustalX 1.64b (30) with gap penalty = 25 and gap extension penalty = 5. The alignment was manually adjusted in the data editor of PAUP* (29). Phylogenetic relationships were determined using the same program. A heuristic search with tree bisection-reconnection and random addition of sequences was performed. Nucleotide substitutions were unordered, but transversions were more heavily penalized (weight = 5) than transitions (weight = 1). Alignment gaps were treated as missing data. The sequence from *S. sapinea* isolate 411 was used as an outgroup. To determine the support for each clade, bootstrap analysis was performed with 1,000 replicates.

Conidium morphology. Sizes and shapes of conidia were compared using methods that previously permitted differentiation of the conidia of *B. dothidea* from those of some other fungi with *Fusicoccum* anamorphs (21). Isolates of the pistachio panicle and shoot blight pathogen (99-7, 99-12, and 99-13), *B. dothidea* (96-6, 96-14, 97-5, and 97-114), *B. ribis* (94-131), and *F. luteum* (96-130 and 97-45) (Table 1) were grown on Difco potato dextrose agar at 12°C with 12 h of light (fluorescent and black lights) per day. After 42 days of incubation, conidia from squash mounts of pycnidia were observed by light and differential interference contrast microscopy and photographed. Three mature pycnidia (based on the predominance of apparently fully developed spores and scarcity of smaller, possibly less developed spores) were selected for each isolate after 52 or 53 days of incubation. Lengths and widths of 20 conidia from each pycnidium were measured in water with a light microscope with an ocular micrometer. Analyses of variance (ANOVA) and calculations of Fisher's protected least significant difference (LSD) for length, width, and length/width were performed using Minitab for Windows (ver. 12; Minitab Inc., State College, PA).

RESULTS

RAPD markers. Eight RAPD primers produced 90 scorable fragments. Most fragments were between 300 and 1,000 bp long. Seven fragments were unique to isolates of the pistachio panicle and shoot blight pathogen (subsequently referred to as the pistachio *Fusicoccum*). Only one fragment was shared between the pistachio *Fusicoccum* isolates and the *B. dothidea* isolates, one other fragment was shared between the pistachio *Fusicoccum* isolates and the *B. ribis* isolates, and yet another

fragment was shared between the pistachio *Fusicoccum* isolates and the *F. luteum* isolates. Three fragments were shared among the pistachio *Fusicoccum* isolates, the *B. ribis* isolates, and the *F. luteum* isolates. Two fragments were shared among the pistachio *Fusicoccum* isolates, the *B. dothidea* isolates, and the *B. ribis* isolates.

The phenogram generated by cluster analysis placed the pistachio *Fusicoccum*

isolates in a group separate from the other species (Fig. 1). The Jaccard's coefficients calculated for RAPD marker data indicated at least 68% similarity among the isolates in this pistachio *Fusicoccum* group. Similarity between this group and the group composed of *B. dothidea* isolates was only 12%. The pistachio *Fusicoccum* group was only 7% similar to the *F. luteum* group and 5% similar to the *B. ribis* group.

Fig. 1. Phenogram produced by unweighted pair-group method using arithmetic averages (UPGMA) analysis of similarity coefficients from random amplified polymorphic DNA (RAPD) marker data. Asterisk (*) denotes isolates for which internal transcribed spacer region DNA sequence was analyzed. Scale represents percent similarity (1.00 = 100%).

ITS sequence comparisons. The ITS sequence alignment indicated that the pistachio *Fusicoccum* isolates differed from their closest relative, *F. luteum*, by only five nucleotides in ITS1 and three nucleotides in ITS2. In contrast, the pistachio *Fusicoccum* isolates differed from the *B. dothidea* isolates by 14 nucleotides in ITS1 and 17 nucleotides in ITS2. Maximum parsimony analysis produced a single most parsimonious tree of 102 steps (consistency index = 0.8824 and retention index = 0.9344). The *B. dothidea* isolates

were distinctly separate from the pistachio *Fusicoccum* isolates and the other groups with 100% bootstrap support (Fig. 2). Bootstrap analysis also very strongly supported the grouping of the pistachio *Fusicoccum* isolates with *F. luteum* isolates and *B. ribis* (99%) but did not strongly support the pistachio *Fusicoccum* isolates as a group distinct from *B. ribis* and *F. luteum*.

Conidium morphology. Lengths of conidia of the *B. dothidea* isolates tended to be greater (means of 26.7 to 30.0 μ m) than

those of the pistachio *Fusicoccum* isolates (means of 20.3 to 22.3 μ m), *B. ribis* (18.9 μ m), and *F. luteum* (22.7 to 25.5 μ m) (Table 2). Widths of the conidia of the *B. dothidea* isolates usually were less than those of the other fungi; therefore, ratios of length to width of the *B. dothidea* isolates (means of 4.2 to 5.0 μ m) were greater than those of the pistachio *Fusicoccum* isolates, *B. ribis*, and *F. luteum* (means of 2.6 to 3.3 μ m) ($P < 0.05$; Table 2). The shape of the conidia from the pistachio *Fusicoccum* isolates (Fig. 3C and D) also was more similar to the slightly ovoid to obovoid conidia of *B. ribis* (Fig. 3E) and *F. luteum* (Fig. 3F) than the fusiform conidia of *B. dothidea* (Fig. 3A and B).

DISCUSSION

Our results strongly support the conclusion that the pistachio *Fusicoccum* from California is not *B. dothidea*. RAPD marker analysis, ITS sequence analysis, and comparison of conidial morphology allow these fungi to be clearly differentiated. Recognition of this pistachio pathogen as distinct from *B. dothidea* has immediate implications for those involved in research concerning the serious disease it causes. For example, this distinction may explain the recently reported genetic dissimilarity between isolates of the pistachio *Fusicoccum* and putative *B. dothidea* isolates from other woody plant hosts in California (10). This finding also is of importance to further phylogenetic investigations of the genus *Botryosphaeria* and related anamorphic fungi.

Differences among RAPD markers and ITS sequences previously have been very useful for characterizing *Botryosphaeria* spp. and related anamorphic fungi. RAPD marker analyses allowed differentiation of discrete groups among fungi referred to as *Sphaeropsis sapinea* (23,24). In addition, analysis of RAPD markers of *Botryosphaeria* spp. has facilitated meaningful selections of isolates for subsequent sequence analyses (21). Previous analyses of ITS sequences of *Botryosphaeria* spp. and related anamorphic fungi have indicated that differences of only a very few nucleotides are found between well-defined, accepted species (7,9,32). Thus, the relatively large number of nucleotide differences in the ITS sequences of *B. dothidea* and the pistachio *Fusicoccum* very strongly support the distinction between them.

Confusion of the pistachio *Fusicoccum* with the anamorph of *B. dothidea* illustrates the problems with identification based on morphological and cultural comparison. Characteristics of fungi in this group are known to be influenced by substrates (5,9,28). Jacobs and Rehner (9) noted that colony morphology among isolates of *Botryosphaeria* spp. "was the most variable character evaluated" and observed 12 distinct colony types among the eight species examined. Similarly, Pennycook

Fig. 2. Phylogram produced by bootstrap analysis of internal transcribed spacer region DNA sequences. Numbers above lines indicate bootstrap support.

and Samuels (14) had difficulty distinguishing *F. luteum* from *B. dothidea* on each of three common media. Finally, the size ranges of conidia of *Botryosphaeria* spp. and related anamorphic fungi have long been reported to overlap, and conidial color and septation may change with age (5,14,18,19,25–27).

Despite these problems, conidial morphology recently was found to be useful in differentiating *B. dothidea* (*F. aesculi*) from a complex consisting of fungi with *Fusicoccum* anamorphs (21). As in the current study, conidia of isolates of *B. dothidea* were distinctly fusiform and had greater length:width ratios than the conidia of *B. ribis* and *F. luteum*. The validity of

this morphological distinction between conidia of *B. dothidea* and the pistachio *Fusicoccum* was strongly supported by the RAPD marker and ITS region sequence comparisons. Neither conidium morphology nor molecular markers, however, allowed clear differentiation of the pistachio *Fusicoccum* from isolates of *F. luteum*, a fungus reported from Australia and New Zealand, but also from pistachio in Italy (21). As suggested previously (21), more extensive research is necessary to clarify the status of taxa in this complex.

Differentiation of the pistachio *Fusicoccum* from *B. dothidea* should stimulate additional study. Different species of *Botryosphaeria* and related anamorphic fungi vary in many potentially important characteristics. For example, Pennycook and Samuels (14) found that *F. luteum* would not grow on benzimidazole-amended media, although *B. dothidea* was not inhibited, and Britton and Hendrix (3) found evidence that *B. dothidea* differs from *B. obtusa* and *B. rhodina* in its mode of infection of peach trees. Thus, further research should include well-characterized isolates of *B. dothidea* and other fungi with *Fusicoccum* anamorphs (21,32). Results of comparative studies should clarify potential differences in host range and disease biology that may lead to more effective and efficient control and decrease losses in commercial pistachio production.

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Fig. 3. Conidia of: *Botryosphaeria dothidea* isolates **A**, 96-6 and **B**, 97-5; pistachio *Fusicoccum* isolates **C**, 99-7 and **D**, 99-12; **E**, *B. ribis* isolate 94-131; and **F**, *F. luteum* isolate 97-45. Bar = 10 μ m.

Table 2. Conidial dimensions for isolates examined in an attempt to differentiate the pistachio *Fusicoccum* from other *Fusicoccum* and *Botryosphaeria* spp.^x

Species, isolate ^y	RAPD ^z	Conidial length (μ m)		Conidial width (μ m)		Length/width	
		Mean	Range	Mean	Range	Mean	Range
<i>Botryosphaeria dothidea</i>							
96-6	Bd	30.0 a	24–38	7.2 b	6–9	4.2 c	3.3–6.3
97-114	Bd	26.7 c	23–33	6.3 d	5–7.5	4.3 c	3.4–5.8
97-5	Bd	27.6 b	22–35	5.5 f	4.5–6.5	5.0 a	3.7–6.5
96-14	Bd	28.1 b	23–37	5.9 e	5–8.5	4.8 b	3.6–6.3
<i>B. ribis</i>							
94-131	Br	18.9 h	16–23	7.3 c	6–10	2.6 g	2.0–3.5
<i>Fusicoccum luteum</i>							
96-130	Fl	25.5 d	21–30	7.8 a	7–9	3.3 d	2.6–3.8
97-45	Fl	22.7 e	19–25	7.8 a	7–9	2.9 f	2.2–3.6
Pistachio <i>Fusicoccum</i>							
99-7	Fp	20.3 g	17–27	6.8 c	5.5–8	3.0 ef	2.2–4.2
99-12	Fp	21.3 f	17–27	6.8 c	5.5–9	3.1 de	2.5–4.4
99-13	Fp	22.3 e	17–27	7.4 b	6–9	3.0 ef	2.4–3.9

^x Sixty conidia per isolate were measured. Values (within columns) followed by the same letter were not significantly different at $P = 0.05$ using Fisher's protected least significant difference.

^y Species names are those used by collector/collection and are not necessarily indicative of identity.

^z Refers to group from results of random amplified polymorphic DNA (RAPD) marker analysis; Bd = *Botryosphaeria dothidea*, Br = *B. ribis*, Fl = *Fusicoccum luteum*, and Fp = pistachio *Fusicoccum*.

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