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Nested PCR Assays for Detection of *Monilinia fructicola* in Stone Fruit Orchards and *Botryosphaeria dothidea* from Pistachios in California

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With 5 figures

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Abstract

Nested polymerase chain reaction (PCR) assays were developed based on microsatellite regions for detection of *Monilinia fructicola*, the causal agent of brown rot of stone fruits, and *Botryosphaeria dothidea*, the causal agent of panicle and shoot blight of pistachio. The nested PCR primers specific to *M. fructicola* were developed based upon the sequence of a species-specific DNA fragment amplified by microsatellite primer M13. The external and internal primer pairs EMfF + EMfR and IMfF + IMfR amplified a 571- and a 468-bp fragment, respectively, from *M. fructicola*, but not from any other fungal species present in stone fruit orchards. The nested PCR primer pairs specific to *B. dothidea* were developed based upon the sequence of a species-specific 1330-bp DNA fragment amplified by microsatellite primer T3B. The external and internal primer pairs EBdF + EBdR and IBdF + IBdR amplified a 701- and a 627-bp fragment, respectively, from *B. dothidea,* but not from any other fungal species associated with pistachio. The nested PCR assays were sensitive enough to detect the specific fragments in 1 fg of *M. fructicola* or *B. dothidea* DNA or in the DNA from only two conidia of *M. fructicola* or *B. dothidea*. The nested PCR assays could detect small numbers of *M. fructicola* conidia caught on spore-trap tapes and detect visible infections of *B. dothidea* in pistachio tissues. Microsatellite regions with high numbers of copies are widely dispersed in eukaryotic genomes. The results of this study indicate that microsatellite regions could be useful in developing highly sensitive PCR detection systems for phytopathogenic fungi.

Introduction

Brown rot, caused by *Monilinia fructicola* (G. Wint.) Honey, is a destructive disease of stone fruit (*Prunus* spp.) in California. Ascospores and conidia of *M. fructicola* produced on infected mummies disperse in the air and infect blossoms causing blossom blight under favourable microclimatic conditions in spring (Holtz et al., 1998). Subsequently, conidia produced from blighted blossoms can cause secondary infections of young fruits. When the microclimatic conditions in the orchards are unfavourable, infected blossoms develop into young fruit bearing latent infections (Luo and Michailides, 2001). These fruits may drop naturally or be removed during thinning and remain on the orchard floor where they may produce numerous conidia when the humidity is high (Hong et al., 1997; Luo et al., 2001a). These conidia can cause fruit infections in mid-season. Later, when favourable conditions occur, the latent infections may develop into fruit rot that can cause significant pre- and post-harvest losses. Our previous studies with prunes (*Prunus domestica* L.) demonstrated that inoculum potential in the orchards is an important factor affecting both blossom blight and fruit infections. Inoculum potential was used to estimate the possible risk of blossom blight (Luo and Michailides, 2001), of latent infection under various climatic conditions (Luo et al., 2001b), and possible threshold conditions that cause latent infections to develop into fruit rot (Luo and Michailides, 2001, 2003). Thus, determination of inoculum potential, particularly the amount of spores in the orchard air in early- and mid-season is critical for predicting and managing brown rot.

Spore traps are conventionally used to determine the spore density for air-borne disease agents (Calderon et al., 2002) including *M. fructicola*. As samples from traps require microscopic examination, it is a very time-consuming method that requires special training. Additionally, spore count may be an unreliable indicator of inoculum potential because of the abundance of dust and other fungal species having spores with

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similar morphology to *M. fructicola*. Culturing airborne spores collected on spore-trap tapes or slides is also tedious and subject to frequent contamination problems and is suitable for detection of airborne inoculum in small-scale experiments, but is impractical for disease management in large areas.

Molecular techniques based on DNA analysis, although expensive, are very useful because they are highly specific and sensitive. Polymerase chain reaction (PCR) methods have been developed to detect, identify, and classify plant pathogens (Martin et al., 2000). The potential of these techniques to detect airborne fungal spores in indoor air has been recognized and reported (Alvarez et al., 1995; MacNeil et al., 1995). PCR assays have been developed to detect fungal spores of *Penicillium roqueforti* (Williams et al., 2001), *Pneumocystis carinii* (Wakefield, 1996), and *Stachybotrys chartarum* (Vesper et al., 2000) in air samples. Previous studies have shown that fungal DNA could be extracted from samples taken by a Burkard spore-trap and detected by PCR (Wakefield, 1996). More recently, fungal DNA of the plant pathogens *Leptosphaeria maculans* and *Pyrenopeziza brassicae* have been extracted successfully from small numbers of spores deposited on pieces of Burkard spore-trap tapes and detected by PCR assays (Calderon et al., 2002). These results indicate that the use of PCR-based assays in conjunction with conventional spore-trapping methods has a potential to routinely monitor the airborne inoculum rates of phytopathogenic fungi.

Species-specific primers for *M. fructicola* have been developed based on the small-subunit rDNA gene (Fulton and Brown, 1997), sequences of the rDNA internal transcribed spacer (ITS) (Ioos and Frey, 2000), species-specific repetitive sequences (Boehm et al., 2001), and the sequence of a random-amplified polymorphic DNA (RAPD) region (Förster and Adaskaveg, 2000). These primers have been used to differentiate *M. fructicola* from other *Monilinia* species and to detect *M. fructicola* in plant material with visible symptoms or latent infections. These methods, to date, have not been used to detect airborne inoculum of *M. fructicola*. More importantly, PCR inhibitors are commonly encountered in spore-trap samples and present a drawback for conventional one-step PCR techniques (Alvarez et al., 1995; Williams et al., 2001; Calderon et al., 2002). Thus, a more sensitive PCR assay (e.g. nested PCR) is needed to detect spores of *M. fructicola* in the orchard environment.

Panicle and shoot blight of pistachio (*Pistacia vera* L.), caused by *Botryosphaeria dothidea* (Moug.:Fr.) Ces. & de Not., has been a major threat to California pistachio industry since the late 1980s (Michailides, 1991; Ma et al., 2001). Species identification in *Botryosphaeria* is complicated and difficult because the teleomorphs of these fungi are rarely encountered in nature and teleomorphic characters vary little between species. Furthermore, morphological characteristics of the anamorphs are also similar among some *Botryosphaeria* species and can be strongly influenced by the substrate on which they are produced (Jacobs and Rehner, 1998). Moreover, these fungi are not easily differentiated by host range because host range may be extensive, and fruiting structures of two to three *Botryosphaeria* spp. have been found together on a single host. Recently, we found both *B. dothidea* and *B. rhodina* together on pistachio shoots. More importantly, *B. rhodina* also causes pistachio shoot blight (Michailides et al., 2002).

In a previous study, we developed PCR primers based on ITS sequence for identifying *B. dothidea* in culture (Ma and Michailides, 2002). However, an attempt to use the PCR technique to detect latent infections of *B. dothidea* on pistachio was not successful. Thus, it is necessary to develop a new technique for the detection of *B. dothidea in planta*. In this study, highly sensitive nested PCR primers were developed based upon the sequences of microsatellite regions to detect *M. fructicola* on spore-trap tapes and *B. dothidea* from pistachio tissues in California.

Materials and Methods

DNA amplification with microsatellite primers and design of nested PCR primers for *M. fructicola* and *B. dothidea*

Isolates of *M. fructicola, M. laxa,* and other fungi from stone fruit and isolates of *B. dothidea, B. rhodina,* and other fungi associated with pistachio used in this study are listed in Tables 1 and 2, respectively. To extract fungal genomic DNA, each isolate was grown in Petri dishes with potato dextrose broth (DIFCO Laboratories, Detroit, MI, USA) at 25°C for 3 days in darkness. Mycelia were harvested and washed in sterile water, snap-frozen in liquid nitrogen, and lyophilized. Fungal genomic DNA was extracted using FastDNA® Kit (BIO 101, Vista, CA, USA) in a FP120 FastPrep™ Cell Disruptor (Savant Instrument, Inc., Holbrook, NY, USA). DNA concentrations were determined using the Hoefer® DyNA Quant® 200 Fluorometer (Hoefer Pharmacia Biotech. Inc., San Francisco, CA, USA).

In microsatellite primed- (MP-) PCR amplifications, the microsatellite primer M13 (GAG GGT GGC GGT TCT) (Meyer et al., 1993) and T3B (AGG TCG CGG GTT CGA ATC C) (Thanos et al., 1996) were used for *M. fructicola* and *B. dothidea,* respectively. PCR was performed using an Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany) in 50-μl volumes containing 50 ηg fungal DNA template, 1.0 μM of the primer, 0.2 mM of each dNTP, 2.0 mM $MgCl_2$, 1 × Promega Taq Polymerase Buffer (10 mM Tris–HCl, pH 9.0, 50 mM KCl, 0.1% Triton® X-100, Promega, WI, USA), and 1.5 U of Promega Taq Polymerase. Amplification was performed using the following parameters: an initial pre-heat for 3 min at 95°C, 40 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 1.5 min, and a final extension at 72°C for 10 min. PCR products were separated in 1.5% agarose gels in Tris–acetate (TAE) buffer and photographed after staining with ethidium bromide.

Table 1
List of fungal species and isolates from stone fruit trees used in this study

Species	Isolate	Host	Origin	Date of isolation	PCR assay[a]
Monilinia fructicola	1656	Peach	Parlier, Fresno Co.	1994	+ +
M. fructicola	1660	Peach	Parlier, Fresno Co.	1994	+ +
M. fructicola	1679	Peach	Sanger, Fresno Co.	1994	+ +
M. fructicola	1716	Peach	Parlier, Fresno Co.	1995	+ +
M. fructicola	1915	Peach	Bakersfield, Kern Co.	1996	+ +
M. fructicola	527	Nectarine	Dinuba, Tulare Co.	1992	+ +
M. fructicola	806	Nectarine	Parlier, Fresno Co.	1993	+ +
M. fructicola	1704	Nectarine	Parlier, Fresno Co.	1995	+ +
M. fructicola	519	Prune	Colusa Co.	1992	+ +
M. fructicola	1688	Prune	Parlier, Fresno Co.	1995	+ +
M. fructicola	1055	Prune	Redbluff, Tahama Co.	1993	+ +
M. fructicola	1050	Prune	Marysville, Yuba Co.	1993	+ +
M. fructicola	2449	Prune	Hamilton, Glenn Co.	1998	+ +
M. fructicola	1048	Prune	Yuba city, Sutter Co.	1993	+ +
M. fructicola	2377	Prune	Gridley, Butte Co.	1998	+ +
M. fructicola	2378	Prune	Gridley Butte Co.	1998	+ +
M. fructicola	2381	Plum	Dinuba, Tulare Co.	1998	+ +
M. fructicola	1696	Plum	Parlier, Fresno Co.	1995	+ +
M. fructicola	1697	Plum	Parlier Fresno Co.	1995	+ +
M. fructicola	DH10	Prune	Butte Co.	2001	+ +
M. fructicola	DH12	Prune	Butte Co.	2001	+ +
M. fructicola	TH2	Prune	Tehama Co.	2001	+ +
M. fructicola	TH24	Prune	Tehama Co.	2001	+ +
M. fructicola	E1	Plum	Reedley, Fresno Co.	2001	+ +
M. fructicola	E6	Plum	Reedley Fresno Co.	2001	+ +
M. fructicola	F2	Plum	Reedley, Fresno Co.	2001	+ +
M. fructicola	F6	Plum	Reedley, Fresno CO.	2001	+ +
M. fructicola	C4	Plum	Reedley, Fresno Co.	2001	+ +
M. fructicola	Gf82	Prune	Fresno Co.	2001	+ +
M. fructicola	Gf104	Prune	Fresno Co.	2001	+ +
M. fructicola	2537	Cherry	Michigan	1998	+ +
M. fructicola	2538	Cherry	Michigan	1998	+ +
M. fructicola	532	Cherry	Oregon	1990	+ +
M. fructicola	533	Cherry	Oregon	1990	+ +
M. fructicola	12A	Peach	Bolivia	2001	+ +
M. fructicola	12B	Peach	Bolivia	2001	+ +
M. fructicola	A6	Peach	Australia	1997	+ +
M. fructicola	BR6	Nectarine	Australia	1997	+ +
M. laxa	515	Prune	Colusa Co.	1992	−−
M. laxa	516	Prune	Colusa Co.	1992	−−
M. laxa	568	Prune	Davis, Yolo Co.	1993	−−
M. laxa	572	Prune	Davis, Yolo Co.	1992	−−
M. laxa	993	Prune	Glenn Co.	1993	−−
M. laxa	994	Prune	Glenn Co.	1993	−−
Botrytis cinerea	1333	Prune	Fresno Co.	1994	−−
B. cinerea	1334	Prune	Fresno Co.	1994	−−
B. cinerea	2386	Plum	Dinuba Co.	1998	−−
B. cinerea	2367	Nectarine	Fresno Co.	1998	−−
Alternaria alternata	AA04	Stone fruit	Parlier, Fresno Co.	1999	−−
Aureobasidium pullulans	1606	Peach	Parlier, Fresno Co.	1995	−−
A. pullulans	1604	Nectarine	Parlier, Fresno Co.	1995	−−
Cladosporium herbarum	AA03	Prune	Parlier, Fresno Co.	1999	−−
Gilbertella persicaria	701	Peach	Clovis, Fresno Co.	1992	−−
G. persicaria	1321	Nectarine	Reedley, Fresno Co.	1995	−−
Mucor piriformis	2292	Plum	Fresno Co.	1997	−−
M. piriformis	1172	Peach	Fresno Co.	1993	−−
M. piriformis	1382	Prune	Parlier, Fresno Co.	1993	−−
Penicillium digitatum	1816	Plum	Parlier, Fresno Co.	1996	−−
P. digitatum	1874	Nectarine	Parlier, Fresno Co.	1996	−−
Phomopsis sp.	AA05	Nectarine	Parlier, Fresno Co.	1999	−−
Rhizopus stolonifer	453	Nectarine	Tulare Co.	1991	−−
R. stolonifer	299	Peach	Hanford, Kings Co.	1993	−−
Sclerotinia sclerotiorum	AA02	Plum	Parlier, Fresno Co.	1999	−−

[a] + + and −− indicate the presence and absence of the expected 571- and 468-bp DNA fragment amplified by the external primer pair EMfF + EMfR and the internal primer pair IMfF + IMfR, respectively.

In previous studies, we observed that the primer M13 amplified a DNA fragment of approximately 740 bp in size from each of more than 500 isolates of *M. fructicola* collected worldwide (Y. Luo, Z. Ma and T. J. Michailides, unpublished data), and the primer T3B amplified a DNA fragment of approximately 1.3 kb in size from more than 300 isolates of *B. dothidea* collected from pistachios in California (Z. Ma

Table 2
List of *Botryosphaeria dothidea* isolates and other fungal species isolated from shoots of pistachio in California used in this study

Species	Isolate	Origin	Isolation date	PCR assay[a]
B. dothidea	MP1	Montgomery, Butte	04/04/00	+ +
B. dothidea	MP2	Montgomery, Butte	04/04/00	+ +
B. dothidea	CP2	Chico	04/04/00	+ +
B. dothidea	CP3	Chico	04/04/00	+ +
B. dothidea	HP7	Hansen, Glenn	09/01/94	+ +
B. dothidea	HP8	Hansen, Glenn	09/01/94	+ +
B. dothidea	HP10	Hansen, Glenn	09/01/94	+ +
B. dothidea	HP13	Hansen, Glenn	04/04/00	+ +
B. dothidea	SP81	San Joaquin	09/07/97	+ +
B. dothidea	SP132	San Joaquin	10/27/97	+ +
B. dothidea	SP133	San Joaquin	10/27/97	+ +
B. dothidea	SP200	San Joaquin	01/28/99	+ +
B. dothidea	KP95	Kings	10/23/97	+ +
B. dothidea	KP97	Kings	10/23/97	+ +
B. dothidea	MaP106	Madera	10/23/97	+ +
B. dothidea	MaP108	Madera	10/23/97	+ +
B. dothidea	MaP111	Madera	10/23/97	+ +
B. dothidea	BP169	Butte	10/21/98	+ +
B. dothidea	BP170	Butte	10/21/98	+ +
B. dothidea	BP171	Butte	11/5/98	+ +
B. dothidea	BP172	Butte	11/5/98	+ +
B. dothidea	FP497	Fresno	05/03/00	+ +
B. dothidea	FP499	Fresno	05/03/00	+ +
B. dothidea	FP579	Fresno	05/24/99	+ +
B. dothidea	FP624	Fresno	03/06/00	+ +
B. dothidea	FP719	Fresno	05/03/00	+ +
B. dothidea	TP565	Tulare	05/12/00	+ +
B. dothidea	TP566	Tulare	05/12/00	+ +
B. dothidea	TP764	Tulare	05/24/00	+ +
B. dothidea	TP766	Tulare	05/24/00	+ +
Alternaria alternata	CH1	Fresno	08/12/00	− −
Aspergillus ochraceus	2568	Colusa	09/02/99	− −
A. parasiticus	2535	San Joaquin	01/15/99	− −
A. terreus	2570	Colusa	09/02/99	− −
Bipolaris bicolor	CH2	Glenn	08/12/00	− −
B. spicifera	CH3	Glenn	08/12/00	− −
Botryosphaeria rhodina	315	Stanislaus	05/24/97	− −
B. rhodina	Br30	Reedley, Fresno	11/12/2001	− −
B. rhodina	Br32	Reedley, Fresno	11/12/2001	− −
Botrytis cinerea	549	Fresno	1992	− −
Cladosporium cladosporioides	1887	Fresno	1996	− −
Curvularia inaequalis	CH3	Glenn	09/20/00	− −
C. lunata	CH4	San Joaquin	09/20/00	− −
Chaetomium sp.	CH5	Yolo	08/12/00	− −
Drechslera biseptata	CH6	San Joaquin	0920//00	− −
Emericella sp.	2536	Glenn	01/15/99	− −
Epicoccum sp.	CH7	Glenn	0920//00	− −
Fusarium acuminatum	CH8	San Joaquin	08/12/00	− −
F. culmorum	CH9	Yolo	08/12/00	− −
F. moniliforme	CH10	Glenn	09/20/00	− −
F. dimerum	CH11	Glenn	09/20/00	− −
Humicola sp.	CH12	San Joaquin	08/12/00	− −
Monilinia sp.	608	…[b]	1992	− −
Mucor sp.	2541	Tulare	03/03/99	− −
Nigrospora sp.	CH13	Glenn	09/20/00	− −
Paecilomyces lilacinus	CH14	Yolo	08/12/00	− −
Penicillium expansum	CH15	San Joaquin	09/20/00	− −
P. chrysogenum	CH16	Glenn	09/20/00	− −
Phomopsis sp.	CH17	Yolo	08/12/00	− −
Rhizomucor sp.	1886	Kings	1996	− −
Stemphyllium botryosum	CH18	Glenn	09/20/00	− −
Torula sp.	CH19	San Joaquin	09/20/00	− −
Trichoderma sp.	CH20	Glenn	09/20/00	− −
Ulocladium atrum	CH21	San Joaquin	09/20/00	− −

[a] + + and − − indicate the presence and absence of the expected 701- and 627-bp DNA fragment amplified by the external primer pair EBdF + EBdR and the internal primer pair IBdF + IBdR, respectively.
[b] Unknown.

and T. J. Michailides, unpublished data). In the present study, we cloned and sequenced these amplification regions to develop nested PCR assays for detection of *M. fructicola* and *B. dothidea*. The characterized amplification regions from *M. fructicola*, *M. laxa*, and *B. dothidea* were purified, respectively,

using the QIAquick gel extraction kit (QIAGEN Inc., Valencia, CA, USA). The purified fragments were ligated into the pGEM-T Easy vector (Promega, Madison, WI, USA), and transformed into *Escherichia coli* (strain JM109) cells. Plasmids pMF02, pML04, and pBD01, which contained the fragments from *M. fructicola*, *M. laxa*, and *B. dothidea*, respectively, were selected for further characterization. PCR and restriction endonuclease analysis of the plasmids confirmed that the characterized fragments from *M. fructicola*, *M. laxa*, and *B. dothidea* had been cloned into the plasmids pMF02, pML04, and pBD01, respectively. The microsatellite primer M13 was used in PCR analysis for the plasmids pMF02 and pML04, and the primer T3B was used for analysis of the plasmid pBD01. *Eco*RI (Promega, Madison, WI, USA) was used for restriction endonuclease analysis of the plasmids. The cloned fragments were sequenced by DBS Sequencing Inc. (Division of Biological Sciences, University of California at Davis, CA, USA).

The sequences of the fragments from *M. fructicola* and *M. laxa* were aligned using the computer program CLUSTAL W 1.82 (European Bioinformatics Institute, Cambridge, UK). The nested primer pairs EMfF + EMfR (external primers) and IMfF + IMfR (internal primers), which are specific to *M. fructicola,* and the primer pairs EBdF + EBdR (external primers) and IBdF + IBdR (internal primers), which are specific to *B. dothidea,* were designed using the computer program XPRIMER (http://alces.med.umn.edu/VGC.html). The primers were synthesized by Invitrogen (Life Technologies, Grand Island, NY, USA).

Specificity and sensitivity of primer pairs

Thirty-eight isolates of *M. fructicola* collected from three states of US and from two other countries and 25 isolates of other fungal species associated with stone fruit (Table 1) were used for specificity tests for the primer pairs EMfF + EMfR and IMfF + IMfR. Thirty isolates of *B. dothidea* collected from pistachios throughout California in different years, as well as 29 other fungal species associated with pistachio (Table 2), were used for specificity tests for the primer pairs EBdF + EBdR and IBdF + IBdR. PCR reactions were performed as described above using 0.2 μM of each primer. The PCR amplification parameters were an initial pre-heat for 3 min at 95°C, 35 cycles of denaturation at 94°C for 40 s, annealing at 64°C for the primer pairs EMfF + EMfR and IMfF + IMfR or 68°C for the primer pairs EBdF + EBdR and IBdF + IBdR for 40 s, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. PCR products (10 μl per sample) were analysed in 1.5% agarose gels in TAE buffer.

In sensitivity tests, serial dilutions of DNA (0.01 fg to 1 ng) and DNA extracted from known numbers of conidia of *M. fructicola* or *B. dothidea* were used as template for PCR amplification. The genomic DNA from conidia was extracted using FastDNA® Kit. To rupture spores, each sample of 200–20 000 conidia of *M. fructicola* or *B. dothidea* together with 200 μl Cell Lysis/DNA Solubilizing Solution for Yeast, Algae, and Fungi (CLS-Y) were added to a FastDNA tube containing garnet matrix and a 1/4-inch ceramic sphere (as shipped). The mixture was shaken in the FastPrep Cell Disruptor twice for 40 s at 4.5 m/s with 2 min cooling on ice between the shaking periods. Previous tests showed that under these conditions, more than 90% of the spores were disrupted. The final DNA was dissolved in 50 μl of H_2O and a 5-μl aliquot each of 1 : 10 DNA dilutions was used for PCR amplification with either the external or internal primer pair. For the nested PCR, 1 μl of the products from the PCR using the external primer pair was added as template DNA for the second-round PCR using the internal primer pair. The PCR amplifications were performed using the parameters described above. PCR products (10 μl/reaction) were separated in 1.5% agarose gels in TAE buffer.

Detection of *M. fructicola* on spore-trap tapes by nested-PCR

Naturally released spores of *M. fructicola* were collected from a commercial prune orchard in Glenn County, CA, using a Burkard 7-day recording spore-trap (Burkard Manufacturing Co. Ltd, Rickmansworth, UK). The trap collected airborne particles on the wax-coated Melinex tape attached to a slowly rotating drum. The spore-trap tapes were replaced every 7 days. After exposure, each tape was cut into seven 48-mm long sections, representing 1-day exposure periods. Each piece of tape was put on a slide, and the slides were examined under a light microscope to determine the number of spores of *M. fructicola*. Tape sections without *M. fructicola* conidia were selected and inoculated with known numbers of *M. fructicola* conidia to generate spiked air samples.

To remove spores from the tapes for DNA extraction, each 48-mm tape section was cut into six pieces and put into a 2-ml FastDNA tube containing 1.5 ml of 0.1% Nonidet® (Sigma, MO, USA). The tubes were incubated at 55°C for 10 min, and then shaken for 2 min in an Eppendorf mixer. The tubes were spun at 10,600 g for 4 min and the supernatants were decanted. Extraction of DNA from the spores was performed using the FastDNA® Kit according to the protocol described above. The final DNA was dissolved in 50 μl of H_2O and 5-μl aliquots of DNA dilutions (1 : 0, 1 : 10, 1 : 100, or 1 : 1000) were used for the nested PCR amplifications. The nested PCR amplifications using primer pairs EMfF + EMfR and IMfF + IMfR were performed using the parameters as described above. The PCR products (10 μl/reaction) were separated in 1.5% agarose gels in TAE buffer.

Detection of *B. dothidea* on infected pistachio shoots by nested PCR

During the 2002 growing season, 20 pistachio blighted shoots showing wilting, which believed to be caused by *B. dothidea,* were collected from an orchard at the

Kearney Agricultural Center, University of California in Parlier. Both culturing and PCR methods were used to detect *B. dothidea* on these samples. For the PCR method, 100 mg of bark was cut using a sterile surgical blade from each shoot and used for DNA extraction by using the FastDNA® Kit with the buffer cell lysis/DNA solubilizing for vegetation (CLS-VF) and protein precipitation solution (PPS). The final DNA was dissolved in 50 μl of H_2O and 5-μl aliquots of 1 : 10 DNA dilutions were used for the nested PCR amplifications. The nested PCR amplifications using primer pairs EBdF + EBdR and IBdF + IBdR were conducted using the parameters as described above. For the culturing method, a small piece of bark (0.2×0.2 cm) was taken from each sample, surface-disinfested with 0.5% sodium hypochlorite (10% commercial bleach, Western Family Foods, Inc., Portland, OR, USA) for 3 min, and rinsed with sterile water. Each surface-sterilized tissue was placed on an acidified [2.5 ml of 25% (vol/vol) lactic acid per litre] Difco potato dextrose agar (APDA) plate. After incubation at 30°C for 7 days, *B. dothidea* mycelia growing from these samples were examined and identified as *B. dothidea* based on morphological characters. Colonies of the anamorph of *B. dothidea* on APDA grew rapidly, covering a 9-cm petri plate within 4–5 days. Initial colonies were white and cottony, became mouse grey, and turned dark grey and floccose after 3–5 days.

Results

Design of primers for *M. fructicola* and *B. dothidea*

The microsatellite primer M13 amplified a 735-bp DNA fragment from *M. fructicola* and a 732-bp fragment from *M. laxa* that could not be differentiated in a 1.5% agarose gel (Fig. 1A). However, the nucleotide sequences of these two fragments showed significant differences with an overall 58% dissimilarity. The GenBank accession numbers of the microsatellite regions from *M. fructicola* and *M. laxa* are AY237426 and AY237427, respectively. Based on the sequences of the fragments, we designed *M. fructicola* specific nested primer pairs [the external primer pair EMfF (5′-ACA ACG AGA GCT TTC TGT AAG AAT TCC ATC A-3′) + EMfR (5′-ACG TAT ATG ATC CCT CCA ACA TCG TTG A-3′) and the internal primer pair IMfF (5′-ATG CAG AAG TGT GAA TAG GGC CT-3′) + IMfR (5′-CGA AGG ATG AGA GGA AGA TTA GGG-3′)].

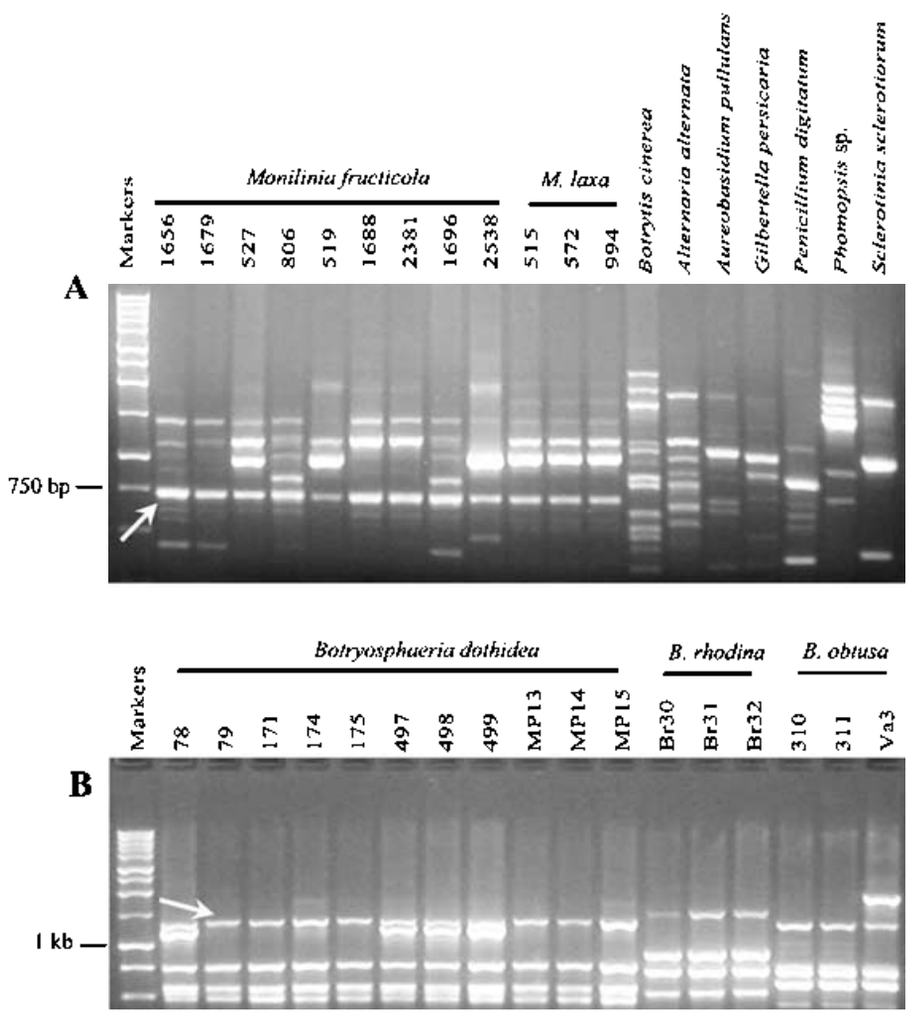

Fig. 1 DNA fingerprinting patterns generated from (A) genomic DNA of *Monilinia fructicola, M. laxa,* and other species from stone fruit with the microsatellite primer M13, and (B) genomic DNA of *Botryosphaeria dothidea* and other species with the microsatellite primer T3B. The arrows in (A) and (B) indicate the characteristic DNA fragment for *M. fructicola* and *M. laxa,* and for *B. dothidea,* respectively, that were cloned and sequenced for the development of nested PCR primers

The microsatellite primer T3B amplified a 1330-bp DNA fragment from *B. dothidea,* but not from other species (Fig. 1B). The GenBank accession number of this microsatellite region from *B. dothidea* is AY237428. Based on the sequences of this DNA fragment, we designed *B. dothidea* specific nested primer pairs [the external primer pair EBdF (5′-CCC CGG CAG TCA GTG CAA GGC-3′) + EBdR (5′-GTT TCG GGT ATC CCG CAC ACC ATG G-3′) and the internal primer pair IBdF (5′-CTG CAT GAA CCA ATG TCC GAC-3′) + IBdR (5′-AGG ATG GAG AGC ACA GTC CGT-3′)].

Specificity and sensitivity of primer pairs

In specificity tests, the external primer pair EMfF + EMfR and the internal primer pair IMfF + IMfR amplified a 571- and a 468-bp DNA fragment, respectively, from all 38 *M. fructicola* isolates tested that were collected from different stone fruit hosts at different locations in different years. No fragments were amplified from *M. laxa* and any other fungus associated with stone fruit (Table 1, Fig. 2A,B). The external primerpair EBdF + EBdR and internal primer pair IBdF + IBdR amplified a 701- and a 627-bp DNA fragment, respectively, from all 30 *B. dothidea* isolates tested that were collected from pistachios at different locations in different years. No fragments were amplified from other fungi associated with pistachios (Table 2, Fig. 3A,B).

Sensitivity assays showed that a single-round PCR with the primer pair EMfF + EMfR or IMfF + IMfR detected the specific fragment in 10 pg of DNA (Fig. 4A,B) or in the DNA from 200 conidia of *M. fructicola* (Fig. 4D). The nested PCR assays were 100–10 000 times more sensitive than the single-round PCR assays and could detect the specific fragment in as little as 1 fg of *M. fructicola* DNA (Fig. 4C) or in the DNA from only two spores of *M. fructicola* (Fig. 4E). The external primer pair EBdF + EBdR detected the specific fragment in 1 pg of *B. dothidea* DNA (Fig. 5A). The nested PCR using primer pairs EBdF + EBdR and IBdF + IBdR, however, could detect the specific fragment in 1 fg of DNA (Fig. 5B) or in the DNA from only two conidia of *B. dothidea* (Fig. 5C).

Detection of *M. fructicola* on spore-trap tapes by nested PCR

The results of the experiments for the nested PCR assay using primer pairs EMfF + EMfR and IMfF + IMfR in detecting *M. fructicola* from spiked spore-trap tapes are summarized in Table 3. Using 1 : 10 dilutions of DNA extracts, the nested PCR consistently detected two spores/PCR reaction (Table 3). However, the nested PCR could not detect the expected fragment efficiently in the undiluted DNA extraction from even 20 spores (Table 3). This was probably because of the presence of PCR inhibitors in the samples. Using 1 : 10 dilution of DNA extracts as templates, the nested PCR could also consistently detect as few as two conidia of *M. fructicola* per PCR reaction from the Burkard spore-trap tape samples collected from a commercial prune orchard in California.

Detection of *B. dothidea* on infected pistachio shoots by nested PCR

PCR assay using primer pairs EBdF + EBdR and IBdF + IBdR for detecting visible infections of *B. dothidea* on pistachio tissues was in agreement with the

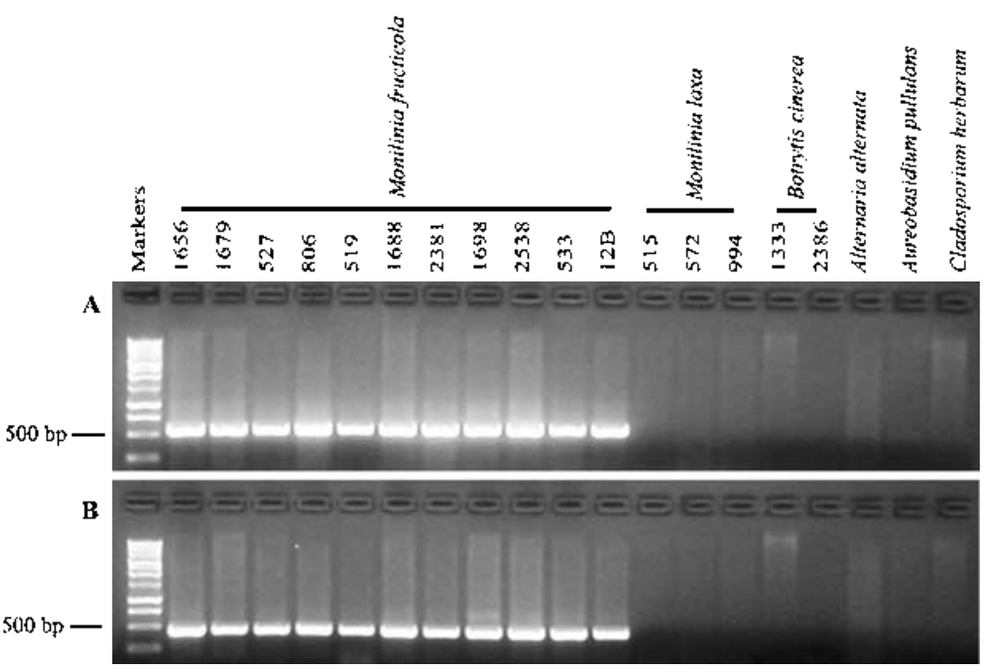

Fig. 2 Specificity of (A) external primer pair (EMfF + EMfR), and (B) internal primer pair (IMfF + IMfR) for detection of *Monilinia fructicola*

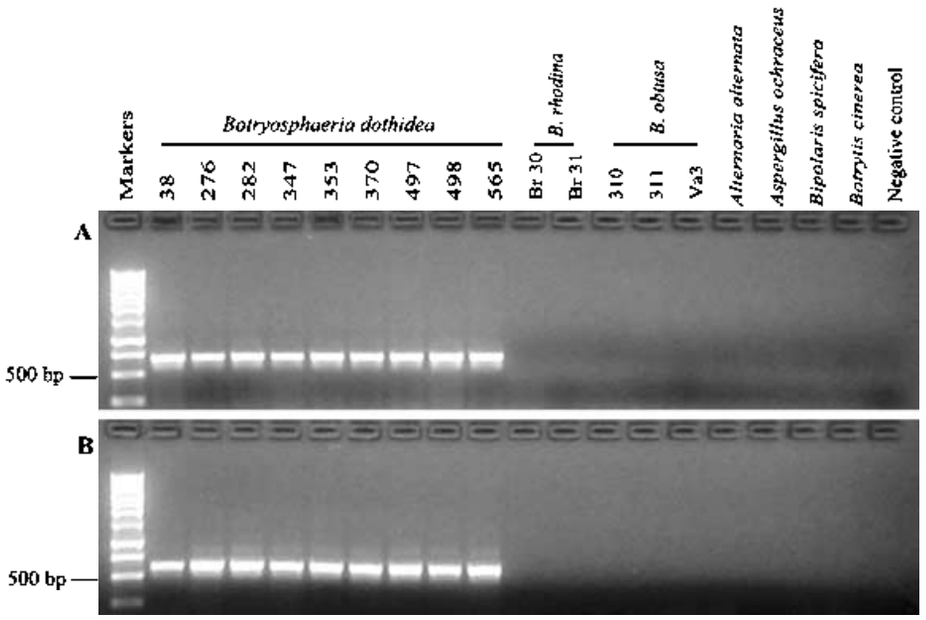

Fig. 3 Specificity of (A) external primer pair (EBdF + EBdR), and (B) internal primer pair (IBdF + IBdR) for detection of *Botryosphaeria dothidea* from California pistachios. **B. obtusa* isolates were collected from apples

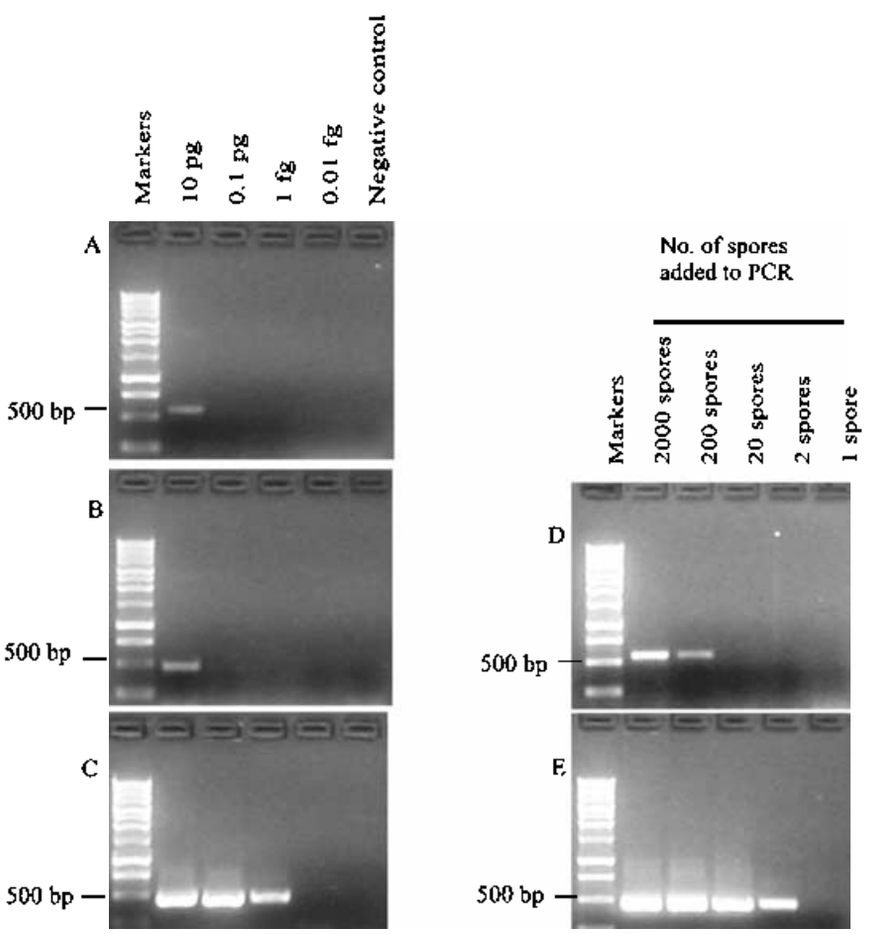

Fig. 4 Sensitivity of the *Monilinia fructicola* primers. Single-step PCR amplification with (A) external primer pair (EMfF + EMfR), (B) internal primer pair (IMfF + IMfR), (C) nested-PCR amplifications with external primer pair and internal primer pairs from known quantities of DNA template, (D) single-step PCR amplification with external primer pair, and (E) nested-PCR amplifications with external and internal primer pairs from genomic DNA extracted from known numbers of conidia of *M. fructicola*

culturing method. The PCR assay identified 18 of 20 shoot samples infected with *B. dothidea*. These 18 samples were also confirmed as having *B. dothidea* infections by culturing the infected tissue on APDA plates for 1 week. *B. dothidea* was not detected from the rest two samples by either PCR assay or culturing method.

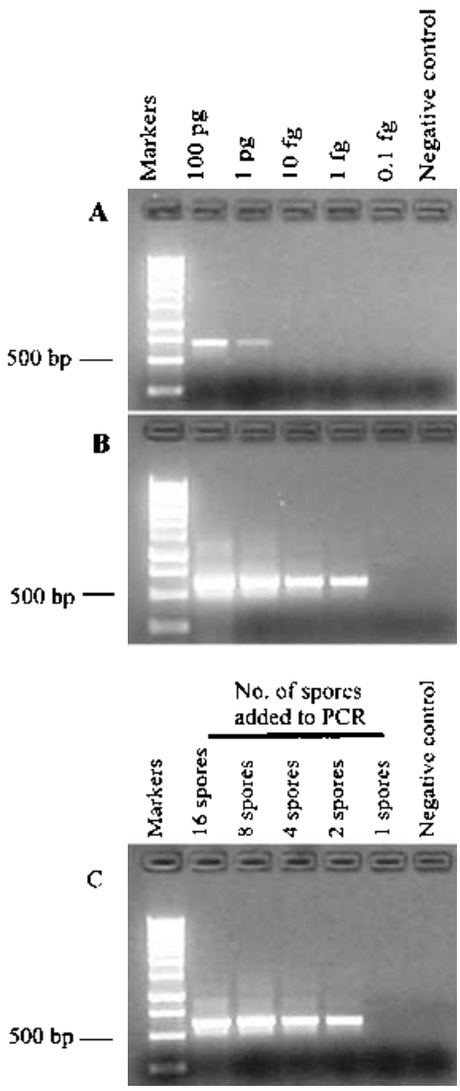

Fig. 5 Sensitivity of the *Botryosphaeria dothidea* primer pairs. (A) Direct PCR amplification with external primer pair (EBdF + EBdR), (B) nested-PCR amplification with external (EBdF + EBdR) and internal primer (IBdF + IBdR) pairs from known concentrations of DNA template, and (C) nested-PCR amplification with external and internal primer pairs from genomic DNA extracted from known numbers of conidia of *B. dothidea*

Discussion

In the present study, we developed nested PCR assays based on microsatellite regions for detection of two major plant pathogens, *M. fructicola* from stone fruit and *B. dothidea* from pistachio. These PCR assays had higher sensitivities (detected the specific fragments in 1 fg of DNA) than PCR methods described previously (Förster and Adaskaveg, 2000; Boehm et al., 2001; Ma and Michailides, 2002). To our knowledge, this is the first report of development of species-specific PCR primers based on microsatellite regions.

A reliable PCR assay depends upon having highly sensitive PCR primers that are specific to the target organism. There are several strategies that have been used recently to develop PCR primers for detection of plant pathogens. One strategy that has gained widespread usage, including *M. fructicola* (Fulton et al., 1999; Ioos and Frey, 2000) and *B. dothidea* (Ma and Michailides, 2002), is the use of PCR primers designed from the DNA sequences of internal transcribed spacer (ITS) regions of rDNA. A second strategy is designing PCR primers based on DNA sequences of protein genes that are unique to the target pathogen (Klein and Juneja, 1997). The third strategy used to detect phytopathogens is the design of primers using randomly amplified polymorphic DNA (RAPD) markers (Martin et al., 2000; Schaad and Frederick, 2002). After comparing RAPD markers of a target organism with the markers of non-target organisms, the unique bands specific to the target organism are removed from the gel, cloned and sequenced. Then, new specific PCR primers are designed based on the sequences of the unique DNA fragments. Recently, this method has been used successfully in developing *M. fructicola*-specific PCR primers (Förster and Adaskaveg, 2000). However, because short primers (10–12 base pairs in length) are used, RAPDs require low annealing temperature (35–37°C), which are not stringent and increase the chance of non-specific priming. RAPD markers are difficult to reproduce between laboratories and sometimes even within laboratories (McDonald, 1997). Microsatellite PCR (MP-PCR) is considered more robust than conventional RAPDs, because longer primers are used for MP-PCR as compared with RAPDs. This allows more stringent annealing temperatures and reaction conditions that enhance reproducibility (Weising et al., 1995; Ma et al., 2001). Microsatellite regions with high numbers of copies are widely dispersed in eukaryotic genomes (Bruford and Wayne, 1993; Balloux and Lugon-Moulin, 2002), including plant pathogenic fungi. In preliminary studies, we found microsatellite primers also generated unique bands for other than *M. fructicola* and *B. dothidea* phytopathogenic fungi, including *Botrytis cinerea* and *Alternaria alternata*. Thus, microsatellite regions have a potential for developing highly sensitive PCR detection systems for many phytopathogenic fungi.

Table 3
Detection of *Monilinia fructicola* from spiked air samples by the nested PCR assay using the external and internal primer pairs EMfF + EMfR and IMfF + IMfR

Number of spores processed for DNA extraction[a]	A 5-μl aliquot of DNA dilution added to PCR (the equivalent number of spores/PCR)	Positive results/total number of tests
0	0	0/6
1×10^2	1 : 0 dilution (10 spores)	0/6
1×10^2	1 : 10 dilution (1 spore)	0/6
1×10^2	1 : 100 dilution (0.1 spores)	0/6
2×10^2	1 : 0 dilution (20 spores)	1/6
2×10^2	1 : 10 dilution (2 spores)	6/6
2×10^2	1 : 100 dilution (0.2 spore)	0/6
2×10^3	1 : 0 dilution (200 spores)	6/6
2×10^3	1 : 10 dilution (20 spores)	6/6
2×10^3	1 : 100 dilution (2 spores)	6/6
2×10^3	1 : 1000 dilution (0.2 spore)	0/6

[a]Final DNA from each sample was dissolved in 50 μl sterile water.

Nested-PCR is a very sensitive technique and because of this, extreme care is required to avoid the risk of cross-contamination among samples. Negative controls are essential and were included in each of our tests to ensure that positive results were valid. Based on our experience, by careful separation of pipettes and areas of work for DNA handling extraction, PCR preparation and PCR product handing, problems with cross-contamination can be avoided.

Another major obstacle in using PCR for detection of pathogens from environmental samples and from plant tissues is the presence of PCR inhibitors. The nested-PCR assay developed in this study could detect small numbers of spores of *M. fructicola* in air samples, but it is still difficult to detect small number of spores resting on non-infected plant tissue (data not shown). This is probably because plant tissues might contain more PCR inhibitors than air samples did. Although a wide range of inhibitors has been reported, the identity and mode of action of most of them remain unclear (Wilson, 1997). Some of these inhibitors could inhibit PCR reactions by denaturing or binding to the thermostable DNA polymerase, by chelating the Mg^{2+} cofactor for *Taq* polymerase, or by binding to target DNA (Luisentti and Poussier, 2000). To attenuate the effect of inhibitors, Calderon et al. (2002) and Williams et al. (2001) purified DNA by phenol : chloroform extraction. However, in this study, PCR inhibitors were not removed efficiently by phenol : chloroform extraction (data not shown). Nor did the addition of 0.2% skim milk or Bovine Serum Albumin (BSA) (500 ng per reaction) improve the PCR amplification from undiluted DNA extracts of spore-trap tapes (data not shown). Both of these compounds have been previously described to overcome PCR inhibitors (Luisentti and Poussier, 2000; Pryor and Gilbertson, 2001). In this study, dilution of the DNA extracts (at 1 : 10) was efficient to prevent the effect of PCR inhibitors. Any reduction in sensitivity due to the dilution of DNA extraction was compensated by the highly sensitive nested PCR method.

In the present study, the PCR assays were not quantitative and only detected the presence of target pathogens in air samples or plant tissues. However, the recently developed real-time PCR technique can be used to directly determine the amount of target DNA in a sample (Schaad and Frederick, 2002). The highly sensitive nested PCR assays will be the basis for using real-time PCR to determine the population levels of *M. fructicola* or *B. dothidea* in orchards. Such information will enhance the accuracy of the disease models used for management of brown rot of stone fruit (Luo and Michailides, 2001; Luo et al., 2001b) and panicle and shoot blight of pistachio.

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References

Alvarez, A. J., M. P. Buttner, L. D. Stetzenbach (1995): PCR for bioaerosol monitoring: sensitivity and environmental interference. Appl. Environ. Microbiol. **61,** 3639–3644.

Balloux, F., N. Lugon-Moulin (2002): The estimate of population differentiation with microsatellite markers. Mol. Ecol. **11,** 155–165.

Boehm, E. W. A., Z. Ma, T. J. Michailides (2001): Species-specific detection of *Monilinia fructicola* from California stone fruits and flowers. Phytopathology **91,** 428–439.

Bruford, M. W., R. K. Wayne (1993): Microsatellites and their application to population genetic studies. Curr. Opin. Genetic. Dev. **3,** 939–943.

Calderon, C., E. Ward, J. Freeman, S. J. Foster, H. A. McCartney (2002): Detection of airborne inoculum of *Leptosphaeria maculans* and *Pyrenopeziza brassicae* in oilseed rape crops by polymerase chain reaction (PCR) assays. Plant Path. **51,** 303–310.

Förster, H., J. E. Adaskaveg (2000): Early brown rot infections in sweet cherry fruit are detected by *Monilinia*-specific DNA primers. Phytopathology **90,** 171–178.

Fulton, C. E., A. E. Brown (1997): Use of SSU rDNA group-I intron to distinguish *Monilinia fructicola* from *M. laxa* and *M. fructigena*. FEMS Microbiol. Lett. **157,** 307–312.

Fulton, C. E., G. C. M. van Leeuwen, A. E. Brown (1999): Genetic variation among and within *Monilinia* species causing brown rot of stone and pome fruits. Eur. J. Plant Pathol. **105,** 495–500.

Holtz, B. A., T. J. Michailides, C. Hong (1998): Development of apothecia from stone fruit infected and stromatized by *Monilinia fructicola* in California. Plant Dis. **82,** 1375–1380.

Hong, C. X., B. A. Holtz, D. P. Morgan, T. J. Michailides (1997): Significance of thinned fruit as a source of the secondary inoculum of *Monilinia fructicola* in California nectarine orchards. Plant Dis. **81,** 519–524.

Ioos, R., P. Frey (2000): Genomic variation within *Monilinia laxa, M. fructigena* and *M. fructicola* and application to species identification by PCR. Eur. J. Plant Pathol. **106,** 373–378.

Jacobs, K. A., S. A. Rehner (1998): Comparisons of cultural and morphological characters and ITS sequences in anamorphs of *Botryosphaeria* and related taxa. Mycologia **90,** 601–610.

Klein, P. G., V. K. Juneja (1997): Sensitive detection of viable *Listeria monocytogenes* by reverse transcription-PCR. Appl. Environ. Microbiol. **63,** 4441–4448.

Luisentti, J., S. Poussier (2000): Specific detection of biovars of *Ralstonia solanacearum* in plant tissue by nested-PCR-RFLP. Eur. J. Plant Pathol. **106,** 255–265.

Luo, Y., Z. Ma, T. J. Michailides (2001a): Analysis of factors affecting latent infection and sporulation of *Monilinia fructicola* on prune fruit. Plant Dis. **85,** 999–1003.

Luo, Y., T. J. Michailides (2001): Factors affecting latent infection of prune fruit by *Monilinia fructicola*. Phytopathology **91,** 864–872.

Luo, Y., T. J. Michailides (2003): Threshold conditions that lead latent infection to prune fruit rot caused by *Monilinia fructicola*. Phytopathology **93,** 102–111.

Luo, Y., D. P. Morgan, T. J. Michailides (2001b): Risk analysis of brown rot blossom blight of prune caused by *Monilinia fructicola*. Phytopathology **91,** 759–768.

Ma, Z., E. W. A. Boehm, Y. Luo, T. J. Michailides (2001): Population structure of *Botryosphaeria dothidea* from pistachio and other hosts in California. Phytopathology **91,** 665–672.

Ma, Z., T. J. Michailides (2002): A PCR-based technique for identification of *Fusicoccum* sp. from pistachio and various other hosts in California. Plant Dis. **86,** 515–520.

MacNeil, L., T. Kauri, W. Robertson (1995). Molecular techniques and their potential application in monitoring the microbiological quality of indoor air. Can. J. Microbiol. **41,** 657–665.

Martin, R. R., D. James, C. A. Levesque (2000): Impacts of molecular diagnostic technologies of plant disease management. Annu. Rev. Phytopathol. **38,** 207–239.

McDonald, B. A. (1997): The population genetics of fungi: tool and techniques. Phytopathology **87,** 448–453.

Meyer, W., T. G. Mitchell, E. Z. Freedman, R. Vilgalys (1993): Hybridization probes for conventional DNA fingerprinting used as

single primers in the polymerase chain reaction to distinguish strains of *Cryptococcus neoformans*. J. Clin. Microbiol. **31,** 2274–2280.

Michailides, T. J. (1991): Pathogenicity, distribution, sources of inoculum courts of *Botryosphaeria dothidea* on pistachio. Phytopathology **81,** 566–573.

Michailides, T. J., D. P. Morgan, D. Felts (2002): First report of *Botryosphaeria rhodina* causing shoot blight of pistachio in California. Plant Dis. **86,** 1273.

Pryor, B. M., R. L. Gilbertson (2001): A PCR-based assay for detection of *Alternaria radicina* on carrot seed. Plant Dis. **85,** 18–23.

Schaad, N. W., R. D. Frederick (2002): Real-time PCR and its application for rapid plant disease diagnostics. Can. J. Plant Pathol. **24,** 250–258.

Thanos, M., G. Schönian, W. Meyer, C. Schweynoch, Y. Gräser, T. G. Mitchell, W. Persber, H. J. Tietz (1996): Rapid identification of *Candida* species by DNA fingerprinting with PCR. J. Clin. Microbiol. **34,** 615–621.

Vesper, S., D. G. Dearborn, I. Yike, T. Allen, J. Sobolewski, S. F. Hinkley, B. B. Jarvis, R. A. Haugland (2000): Evaluation of *Stachybotrys chartarum* in the house of an infant with pulmonary haemorrhage: quantitative assessment before, during and after remediation. J. Urb. Health **77,** 68–85.

Wakefield, A. E. (1996): DNA sequences identical to *Pneumocystis carinii* f. sp. *carinii* and *Pneumocystis carinii* f. sp. *hominis* in samples of air spora. J. Clin. Microbiol. **34,** 1754–1759.

Weising, K., R. G. Atkinson, R. C. Gardner (1995): Genomic fingerprinting by microsatellite-primed PCR: a critical evaluation. PCR Meth. Appl. **4,** 249–255.

Williams, R. H., E. Ward, H. A. McCartney (2001): Methods for integrated air sampling and DNA analysis for detection of airborne fungal spores. Appl. Environ. Microbiol. **67,** 2453–2459.

Wilson, I. G. (1997): Inhibition and facilitation of nucleic acid amplification. Appl. Environ. Microbiol. **63,** 371–375.