Species-Specific Detection of *Monilinia fructicola* from California Stone Fruits and Flowers

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ABSTRACT

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A set of molecular diagnostics was developed for *Monilinia fructicola*, causal agent of brown rot of stone fruits, capable of sensitive detection of the pathogen in planta. Species-specific repetitive sequences were identified from a partial library of 312 recombinant clones hybridized with total DNA, followed by subsequent screening for specificity. One hundred isolates, comprising 12 fungal species common to California stone fruits, were surveyed for specificity. Three clones hybridized to 60 geographically diverse *M. fructicola* isolates (California, Michigan, Georgia, Oregon, and Australia) to the exclusion of all other fungi surveyed, including the closely related *M. laxa* (n = 12). Two clones were identical and of extrachromosomal origin (pMF73 and pMF150), whereas the third (pMF210) migrated with uncut DNA. The sensitivity of all three was comparable and capable of detecting 50 pg of fungal DNA in dot blot hybridizations. Six species-specific primer pair sets were designed. They maintained the same specificity patterns observed in the initial hybridization surveys and were sensitive enough to detect 50 fg of fungal DNA template, approximately equivalent to 10 spores. The species-specific clones were capable of detecting the pathogen in planta, specifically from infected plum flowers and nectarine fruit tissue, using both hybridization- and polymerase chain reaction-based methodologies.

Additional keywords: diagnostic probes, fruit diseases.

Brown rot of stone fruits (*Prunus* spp.), caused by the ascomycete *Monilinia fructicola* (Wint.) Honey, and to a lesser extent *M. laxa* (Aderhold & Ruhland) Honey, is a serious disease in the California Central Valley and often results in severe postharvest yield losses, in some cases in excess of 30% (16,27). Currently, an estimated 100,000 ha are under cultivation in the Sacramento and San Joaquin Valleys, producing annually some 800,000 t of stone fruit (peaches, nectarines, apricots, plums, and prunes), valued at over 700 million dollars (24,27).

In California, brown rot caused by *M. fructicola* is usually initiated in the spring as blossom blight from inoculum derived from overwintered mummified fruit that produce apothecia (15). Under favorable environmental conditions, blossom blight can progress to twig blight and branch canker, which can serve as additional sources of secondary inoculum, and may eventually lead to latent infection of immature green fruit, and pre- and postharvest brown rot of mature stone fruit (15,16). Postharvest losses are typically more severe than preharvest losses in California and routinely occur during storage and transport, in some cases even affecting fruit at the processing stage (27).

The development of a species-specific molecular diagnostic capable of detecting *M. fructicola* from infected host tissues could help the stone fruit industry to (i) predict the incidence of disease prior to the onset of visual symptoms; (ii) determine the efficacy of various chemical and cultural control strategies; (iii) evaluate proper postharvest fruit storage conditions; and (iv) establish appropriate quarantine measures based on a species-specific detection method. Currently, *M. fructicola* is a quarantine-listed organism in Europe (8). Detection of *M. fructicola* from asymptomatic preharvest stone fruits is presently based on an overnight freeze-thaw incubation assay (25). However, this technique requires time, is subject to frequent contamination problems, and does not directly quantify the pathogen.

Species-specific molecular diagnostics are being used increasingly to detect fungal plant pathogens with a high degree of specificity and sensitivity (13). One approach used to identify species-specific DNA sequences centers on the hybridization of genomic libraries with total DNA to detect repetitive DNA sequences that are then screened for specificity and sensitivity (10,12,18,28,35). Alternatively, conserved primers to amplify and sequence the variable internal transcribed spacers or nontranscribed intergenic regions of fungal ribosomal (rDNA) genes can provide the basis for species-specific primer development (2,7, 8,19,20,26,32,33). Sequence-characterized, randomly amplified regions have also provided the basis for species-specific primer development among fungal plant pathogens (7,30,34), as have mitochondrial (6,14) and microsatellite sequences (11). A few of these studies have also been successful in directly quantifying fungal pathogens from infected host tissues (11,17,18,21,26).

Recently, species-specific primers have been developed to an intron within the small-subunit rDNA gene capable of differentiating worldwide isolates of *M. fructicola* (n = 16) from both *M. laxa* (n = 20) and *M. fructigena* (n = 20) (8,9). The same small-subunit rDNA intron was also present in Michigan isolates of *M. fructicola* (n = 32) but not those of *M. laxa* (n = 8) (31). However, in another study (7), these same primers amplified rDNA from only 8 out of 21 California *M. fructicola* isolates surveyed and led these authors to develop two additional sets of primers. The first primer pair was capable of differentiating California *M. fructicola* isolates (n = 22) from those of *M. laxa* (n = 15) and other stone fruit fungal pathogens, based on the presence of an additional diagnostic band. The second primer pair was genus-specific to *Monilinia* (7).

The present study was initiated to develop a series of molecular diagnostics, based on both full-length probes and derived primers, capable of detection of *M. fructicola* from stone fruit and flower

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tissues. The strategy was based on the cloning and characterization of repetitive regions of the genome unique to *M. fructicola* to the exclusion of other stone fruit-associated fungi, including the closely related *M. laxa*. Here, we report on the cloning and characterization of three highly repetitive, nonribosomal sequences that are species-specific to a worldwide assemblage of *M. fructicola* isolates. We also present hybridization and polymerase chain reaction (PCR) data on sensitivity limits, genomic organization, and copy number estimates. We further describe their use as a sensitive diagnostic to detect *M. fructicola* from both stone fruit and flower tissues.

MATERIALS AND METHODS

Cloning highly repetitive sequences. The hybridization strategy used to identify species-specific repetitive clones in this study was threefold: (i) identification of highly repeated plasmid clones using total DNA as the probe source; (ii) exclusion of ribosomal clones using the entire 9-kb ribosomal repeat from *Nectria haematococca* as a heterologous probe (H. C. Kistler, USDA-Cereal Disease Laboratory, University of Minnesota, St. Paul); and (iii) determination of specificity and sensitivity of the identified putative repeat clones. Under nonsaturating probe con-

TABLE 1. List of fungal species and isolates used in this study

Species	ID	Host	Origin and collector[a]	Date	Grid[b]	Species	ID	Host	Origin and collector[a]	Date	Grid[b]
Monilinia						*M. fructicola*	D1B3	Peach	Georgia, HS	1998	E8
M. fructicola	1656	Peach	Parlier, Fresno Co., TM	1994	A1	*M. fructicola*	W519	Peach	Georgia, HS	1998	E9
M. fructicola	1654	Peach	Parlier, Fresno Co., TM	1994	A2	*M. fructicola*	W619	Peach	Georgia, HS	1998	E10
M. fructicola	1660	Peach	Parlier, Fresno Co., TM	1994	A3	*M. fructicola*	BR5	Cherry	South Australia, SM	1997	E11
M. fructicola	1671	Peach	Parlier, Fresno Co., TM	1994	A4	*M. fructicola*	BR6	Nectarine	Donnybrook, Western Australia, SM	1997	E12
M. fructicola	1673	Peach	Parlier, Fresno Co., TM	1994	A5						
M. fructicola	1675	Peach	Parlier, Fresno Co., TM	1994	A6	*M. laxa*	515	Prune	Colusa Co., TM	1992	F1
M. fructicola	1716	Peach	Parlier, Fresno Co., TM	1995	A7	*M. laxa*	516	Prune	Colusa Co., TM	1992	F2
M. fructicola	1679	Peach	Sanger, Fresno Co., TM	1994	A8	*M. laxa*	517	Prune	Colusa Co., TM	1992	F3
M. fructicola	1658	Peach	Sanger, Fresno Co., TM	1994	A9	*M. laxa*	537	Prune	Davis, Yolo Co., TM	1992	F4
M. fructicola	1681	Peach	Sanger, Fresno Co., TM	1994	A10	*M. laxa*	567	Prune	Davis, Yolo Co., TM	1992	F5
M. fructicola	1683	Peach	Sanger, Fresno Co., TM	1994	A11	*M. laxa*	568	Prune	Davis, Yolo Co., TM	1992	F6
M. fructicola	1915	Peach	Bakersfield, Kern Co., TM	1996	A12	*M. laxa*	569	Prune	Davis, Yolo Co., TM	1992	F7
M. fructicola	527	Nectarine	Dinuba, Tulare Co., TM	1992	B1	*M. laxa*	571	Prune	Davis, Yolo Co., TM	1992	F8
M. fructicola	528	Nectarine	Dinuba, Tulare Co., TM	1992	B2	*M. laxa*	572	Prune	Davis, Yolo Co., TM	1992	F9
M. fructicola	529	Nectarine	Dinuba, Tulare Co., TM	1992	B3	*M. laxa*	992	Prune flws.	Glenn Co., TM	1993	F10
M. fructicola	794	Nectarine	Parlier, Fresno Co., TM	1993	B4	*M. laxa*	993	Prune flws.	Glenn Co., TM	1993	F11
M. fructicola	806	Nectarine	Parlier, Fresno Co., TM	1993	B5	*M. laxa*	994	Prune flws.	Glenn Co., TM	1993	F12
M. fructicola	808	Nectarine	Parlier, Fresno Co., TM	1993	B6	*Botrytis*					
M. fructicola	810	Nectarine	Parlier, Fresno Co., TM	1993	B7	*B. cinerea*	1333	Prune	Fresno Co., TM	1994	G1
M. fructicola	1703	Nectarine	Parlier, Fresno Co., TM	1993	B8	*B. cinerea*	1334	Prune	Fresno Co., TM	1994	G2
M. fructicola	1704	Nectarine	Parlier, Fresno Co., TM	1995	B9	*B. cinerea*	2386	Plum	Dinuba, Tulare Co., TM	1998	G3
M. fructicola	1717	Nectarine	Parlier, Fresno Co., TM	1995	B10	*B. cinerea*	2367	Nectarine	Fresno Co., TM	1998	G4
M. fructicola	1721	Nectarine	Parlier, Fresno Co., TM	1995	B11	*Penicillium*					
M. fructicola	1722	Nectarine	Parlier, Fresno Co., TM	1995	B12	*P. digitatum*	1816	Plum	Parlier, Fresno Co., TM	1996	G5
M. fructicola	519	Prune	Colusa Co., TM	1992	C1	*P. digitatum*	1872	Plum	Parlier, Fresno Co., TM	1995	G6
M. fructicola	520	Prune	Colusa Co., TM	1992	C2	*P. digitatum*	1874	Nectarine	Parlier, Fresno Co., TM	1996	G7
M. fructicola	523	Prune	Colusa Co., TM	1992	C3	*P. digitatum*	1875	Nectarine	Parlier, Fresno Co., TM	1996	G8
M. fructicola	524	Prune	Colusa Co., TM	1992	C4	*Aureobasidium*					
M. fructicola	1688	Prune	Parlier, Fresno Co., TM	1995	C5	*A. pullulans*	1606	Peach	Parlier, Fresno Co., TM	1995	G9
M. fructicola	1689	Prune	Parlier, Fresno Co., TM	1995	C6	*A. pullulans*	1612	Peach	Parlier, Fresno Co., TM	1995	G10
M. fructicola	1055	Prune	Redbluff, Tehama Co., TM	1993	C7	*A. pullulans*	1613	Peach	Parlier, Fresno Co., TM	1995	G11
M. fructicola	1050	Prune	Marysville, Yuba Co., TM	1993	C8	*A. pullulans*	1604	Nectarine	Parlier, Fresno Co., TM	1995	G12
M. fructicola	2449	Prune	Hamilton, Glenn Co., TM	1998	C9	*Gilbertella*					
M. fructicola	1048	Prune	Yuba City, Sutter Co., TM	1993	C10	*G. persicaria*	701	Peach	Clovis, Fresno Co., TM	1992	H1
M. fructicola	2377	Prune	Gridley, Butte Co., TM	1998	C11	*G. persicaria*	1321	Nectarine	Reedley, Fresno Co., TM	1995	H2
M. fructicola	2378	Prune	Gridley, Butte Co., TM	1998	C12	*G. persicaria*	697	Peach	Parlier, Fresno Co., TM	1994	H3
M. fructicola	2381	Plum	Dinuba, Tulare Co., TM	1998	D1	*G. persicaria*	2489	Peach	Clovis, Fresno Co., TM	1994	H4
M. fructicola	2388	Plum	Dinuba, Tulare Co., TM	1998	D2	*Rhizopus*					
M. fructicola	2392	Plum	Dinuba, Tulare Co., TM	1998	D3	*R. stolonifer*	453	Nectarine	Tulare Co., TM	1991	H5
M. fructicola	2394	Plum	Dinuba, Tulare Co., TM	1998	D4	*R. stolonifer*	299	Peach	Hanford, Kings Co., TM	1993	H6
M. fructicola	2435	Plum	Dinuba, Tulare Co., TM	1998	D5	*R. stolonifer*	492	Peach	Parlier, Fresno Co., TM	1992	H7
M. fructicola	2438	Plum	Dinuba, Tulare Co., TM	1998	D6	*R. stolonifer*	476	Peach	San Joaquin Co., TM	1992	H8
M. fructicola	1696	Plum	Parlier, Fresno Co., TM	1995	D7	*Mucor*					
M. fructicola	1697	Plum	Parlier, Fresno Co., TM	1995	D8	*M. piriformis*	2292	Plum	Fresno Co., TM	1997	H9
M. fructicola	1698	Plum	Parlier, Fresno Co., TM	1995	D9	*M. piriformis*	1172	Peach	Fresno Co., TM	1993	H10
M. fructicola	1699	Plum	Parlier, Fresno Co., TM	1995	D10	*M. piriformis*	1382	Prune	Parlier, Fresno Co., TM	1993	H11
M. fructicola	1701	Plum	Parlier, Fresno Co., TM	1995	D11	*M. piriformis*	2212	Plum	Parlier, Fresno Co., TM	1990	H12
M. fructicola	1702	Plum	Parlier, Fresno Co., TM	1995	D12	*Monilinia*					
M. fructicola	2537	Cherry	Michigan, AJ	1998	E1	*M. camelliae*	AA01	Camellia	Parlier, Fresno Co., TM	1999	…
M. fructicola	2538	Cherry	Michigan, AJ	1998	E2	*Sclerotinia*					
M. fructicola	2539	Cherry	Michigan, AJ	1998	E3	*S. sclerotiorum*	AA02	Plum	Parlier, Fresno Co., TM	1999	…
M. fructicola	532	Cherry	Oregon, HW	1990	E4	*Cladosporium*					
M. fructicola	533	Cherry	Oregon, HW	1990	E5	*C. herbarum*	AA03	Prune	Parlier, Fresno Co., TM	1999	…
M. fructicola	536	Cherry	Oregon, HW	1990	E6	*Alternaria*					
M. fructicola	D1B2	Peach	Georgia, HS	1998	E7	*A. alternata*	AA04	Stone fruit	Parlier, Fresno Co., TM	1999	…
						Phomopsis spp.	AA05	Nectarine	Parlier, Fresno Co., TM	1999	…

[a] Collectors: TM, T. Michailides (Kearney Agricultural Center) and C. Hong (Virginia Polytechnical Institute and State University); AJ, A. L. Jones (Michigan State University); HW, H. Wittig (Oregon State University); HS, H. Scherm (University of Georgia); SM, S. McKirby (Agriculture Western Australia Quarantine Office).

[b] Grid reference refers to the dot blot specificity survey shown in Figure 1.

ditions and high-stringency hybridization and wash temperatures, plasmid clones containing highly repeated sequences generated stronger signals than clones containing moderate- to low- or single-copy sequences. This difference was the basis for the initial screen (12,18).

DNA extractions. All fungal species and isolates (Table 1) were grown in stationary potato dextrose broth cultures at 27°C, frozen in liquid nitrogen, and lyophilized. Total genomic DNA was isolated by a modification of a previously published protocol (22). Briefly, lyophilized mycelia were ground with a motor-driven pestle and sterile white quartz sand in hot (68°C) cetyltrimethylammnium bromide (CTAB) extraction buffer (1% hexadecyltrimethylammonium bromide, 700 mM NaCl, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 1% β-mercaptoethanol). An equal volume of chloroform/isoamyl alcohol (24:1) was added to the supernatant to form an emulsion, tubes were centrifuged and an equal volume of isopropanol was added to the aqueous phase to precipitate nucleic acids. Recovered nucleic acid pellets were washed two times with 70% ethanol and suspended in Tris-EDTA (50 mM Tris-Cl, pH8.0, and 10 mM EDTA) + RNase (20 µg/ml) and transferred to microcentrifuge tubes. DNA was subjected to two rounds of phenol/chloroform/isoamyl alcohol (25:24:1) extraction, followed by chloroform/isoamyl alcohol extraction. DNA in the final supernatant was precipitated with ethanol and quantified with a fluorometer (DyNAQuant; Hoefer Pharmacia Biotech Inc., San Francisco, CA). DNA extractions from aliquots of quantified fungal spore suspensions were performed in microcentrifuge tubes in a scaled-down version of the protocol outlined above and suspended in 20 µl of water.

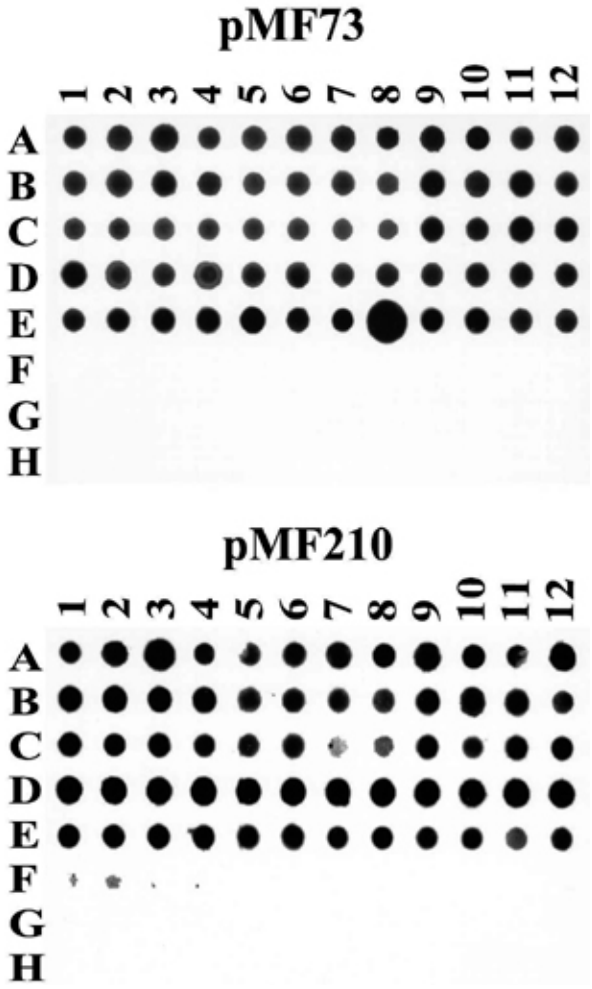

Fig. 1. Dot blot specificity survey of 96 fungal isolates, comprising eight species, commonly associated with California stone fruits (Table 1). Details for grid references are as follows: **A,** (lanes 1 to 12) California isolates of *Monilinia fructicola* from peaches; **B,** (1 to 12) nectarines; **C,** (1 to 12**)** prunes; and **D,** (1 to 12) plums. For geographically disparate *M. fructicola* isolates **E,** (1 to 3) from Michigan; **E,** (4 to 6) Oregon; **E,** (7 to 10) Georgia; and **E,** (11 to 12) Australia. For California isolates from stone fruits of **F,** (1 to 12) *M. laxa*; **G,** (1 to 4) *Botrytis cinerea*; **G,** (5 to 8) *Penicillium digitatum*; **G,** (9 to 12) *Aureobasidium pullulans*; **H,** (1 to 4) *Gilbertella persicaria*; **H,** (5 to 8) *Rhizopus stolonifer*; and **H,** (9 to 12) *Mucor piriformis*. Dot blots were hybridized, under high stringencies, to the gel-purified inserts from clones pMF73 and pMF210.

Partial library and hybridizations. A partial genomic library was constructed for *M. fructicola* isolate 1656 (Table 1). Briefly, *Sau*3AI partially digested genomic DNA was size-fractionated in a 0.6% agarose gel, and fragments ranging from approximately 0.2 to 5.0 kb were gel-purified. These were ligated to *Bam*HI-digested, dephosphorylated pGEM®-4Z (Promega Corp., Madison, WI) vector DNA and used to transform competent JM109 cells. A total of 312 random white recombinant clones were selected by blue-white screening according to the manufacturer's protocol (Promega Corp., Madison, WI). Plasmid extractions were done for these 312 recombinant clones with a miniprep DNA purification system (Wizard® Plus SV; Promega Corp.). Denatured plasmid DNA (1.0 µg) was applied to a series of nylon filters with a 96-well dot blot vacuum manifold apparatus (Gibco BRL Life Technologies, Inc., Rockville, MD). Additionally, *Eco*RI/*Pst*I digests were done for all 312 clones to characterize insert size and also transferred to a series of nylon filters. Both the dot blot filters and the *Eco*RI/*Pst*I digests of the 312 bacterial clones were hybridized to total labeled genomic DNA of *M. fructicola* isolate 1656 (Table 1) to identify repetitive clones.

All probes in this study were constructed from gel-purified inserts using GeneClean® (Q-BIOgene, Carlsbad, CA) and were labeled by random primers using either the Prime-It® Fluor (Stratagene, La Jolla, CA) or the DIG-High-Prime® (Roche Molecular Biochemicals, Indianapolis) fluorescence labeling kits. All hybridizations were carried out at high stringency temperatures either according to the manufacturer's instructions or at 68°C in 7% sodium dodecyl sulfate (SDS), 10 mM EDTA, and 0.5 M $NaPO_4$, pH 7.5. High-stringency washes were done at 68°C, first in 1× SSC (0.15M sodium chloride, 0.015M sodium citrate, pH 7.7), followed by 0.1×/0.1% SDS (2 × 15 min). Detection followed the manufacturer's instructions for either the Illuminator™ Chemiluminescent Detection System (Stratagene) or the DIG Luminescent Detection System (Roche Molecular).

Dot blot specificity screen. Inserts identified from repetitive, nonribosomal clones were sequentially tested for species specificity by hybridizing to a dot blot of 96 isolates, comprising eight fungal species, commonly encountered from California stone fruits (27). In addition to the 60 *M. fructicola* isolates surveyed (California, Michigan, Oregon, Georgia, and Australia; Fig. 1, rows A to E, and Table 1), the survey also included 12 California isolates of *M. laxa* (row F) and 4 California isolates each of *Botrytis cinerea*, *Penicillium digitatum*, *Aureobasidium pullulans*, *Gilbertella persicaria*, *Rhizopus stolonifer,* and *Mucor piriformis* (Fig. 1, rows G to H, and Table 1). Dot blot specificity surveys used 1.0 µg of denatured fungal genomic DNA.

In planta dot blot detection. Plum flowers (cv. Royal Diamond) were collected in March-April from a local plum orchard in Reedley, CA. Flowers were divided into three groups based on visual symptoms: (+) flowers were heavily infected with *M. fructicola* and showed obvious signs of fungal sporulation on the stem and calyx surface; (+/–) flowers displayed patches of brown discoloration on the same organs, but showed no external signs of fungal sporulation; and (–) flowers were asymptomatic, without any evidence of fungal infection. DNA extractions from individual plum flowers were obtained using the Fast-Prep System FP-120 biohomogenizer instrument, following the manufacturer's instructions for plant DNA extraction (Q-BIOgene Inc.), and routinely yielded 3 to 8 µg total DNA per flower. For the flower

dot blot hybridization assay, known amounts of fungal DNA (*M. fructicola* isolate 1654; Table 1) were applied as a calibration standard, ranging from 10 pg to 1,000 ng. Equal amounts of flower DNA (1,000 ng) were also loaded and the filter hybridized to pMF73 under high-stringency conditions.

Nectarine fruit (cv. Fantasia) was surface-sterilized in a 0.05% chlorine solution, prepared from 5.25% sodium hypochlorite (commercial bleach) and 0.05% Tween 20, rinsed in sterile water and surface-wounded with a sterile pin. Fungal spores of *M. fructicola* were quantified with a hemocytometer, and 20-µl drops containing either 2,000 or 20,000 spores per ml were placed on the wound (i.e., 40 or 400 spores per wound). Inoculated fruits were incubated at 25°C at high humidity for 0, 12, 24, 36, 48, 60, and 72 h. Following each time point, pieces of fruit tissue (≈0.5 × 0.5 cm^2) were excised and DNA was extracted as previously described, then quantified, and 500 ng was applied for each sample for dot blot hybridizations. The calibration standard was obtained from *M. fructicola* isolate 1656 (Table 1) and applied in a concentration series from 10 pg to 500 ng.

PCR amplification. Specific 22-bp primers were designed from the species-specific repetitive inserts pMF73 (204 bp) and pMF210 (1,526 bp). For pMF73, two forward (F) and two reverse (R) primer pairs were designed: 73F1, AGA ACC TTC TTT CTT TCC ACC C; 73F2, CCT GAT GTC TCC ATG TAG AAC C; 73R1, TTG TCT GAC GCT AAT GAA GGA G; and 73R2, TGT AAG GGT TAT TGT CTG ACG C. For pMF210, three forward (F) and three reverse (R) primers were designed: 210F1, CAA GAG AAA TCG GTC AAG TTC A; 210F2, GTG GAA AGG TGA GGA TTC ATT C; 210F3, AGA AGG AAT CGG CTT TAT CGT C; 210R1, GAT TCC ACC CCC TTT ATT TTT C; 210R2, TAC GAG CTA CTT GTA CAC TTT C; and 210R3, GGT ACT AGC CCC TAT AAT GAG T.

All PCR reactions were performed with a Perkin-Elmer Model 480 thermal cycler (Applied Biosystems, Foster City, CA) in 50-µl volumes containing 25 ng of template (either fungal or plant-fungal DNA), 0.5 µM of each forward and reverse primer, 0.2 mM each of dNTP, 1.5 mM $MgCl_2$, 1× Promega *Taq* polymerase buffer (10 mM Tris-Cl, pH 9.0, 50 mM KCl, 0.1% Triton X-100), and 1 unit of Promega *Taq* polymerase. For PCR specificity experiments, 15 µl of the 50-µl PCR reaction volume was typically run on the gel for each sample, whereas PCR sensitivity experiments used 25-µl half-volume reactions, loading the full volume for each sample. For the PCR detection experiments from known quantities of fungal spores, 25-µl PCR reaction volumes were run, and a 5-µl aliquot of template DNA was used for each spore concentration series.

A range of annealing temperatures was assayed for retention of species specificity. Primer pair combinations, amplicon size and empirically derived, species-specific annealing temperatures were as follows: for pMF73, 73F1 + 73R1 (134 bp at 66°C) and 73F2 + 73R2 (161 bp at 66°C); for pMF210, 210F1 + 210R1 (633 bp at 64°C), 210F2 + 210R1 (321 bp at 64°C), 210F3 + 210R2 (520 bp at 64°C), and 210F3 + 210R3 (619 bp at 64°C). The same conditions were used for testing primer-pair PCR sensitivities. Other primer-pair combinations (e.g., 73F1 + 73R2, 73F2 + 73R1, 210F1 + 210R2 or 210R3, and 210F2 + 210R2 or 210R3) were not tested in this study. Cycling profiles consisted of a 95°C premelt for 3 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealment for 1 min at the temperature required for retention of species specificity, extension at 72°C for 1.5 min, followed by a 10-min final extension at 72°C. All amplicons were run out on 2.0% agarose gels. Molecular weight standards were obtained commercially (Cat. no. G4521 and G5711, Promega).

PCR specificity survey. In the PCR specificity survey, 38 representative isolates were surveyed, including 19 *M. fructicola* isolates from central, southern, and northern California, as well as isolates originating from Michigan, Oregon, Georgia, and Australia. Also surveyed for specificity were seven California isolates of *M. laxa* and one isolate each of *M. camelliae*, *Sclerotinia sclerotiorum*, *B. cinerea* (*n* = 2), *Cladosporium herbarum*, *Alternaria alternata*, *Phomopsis* sp., *Penicillium digitatum*, *Aureobasidium pullulans*, *Gilbertella persicaria*, *R. stolonifer*, and *Mucor piriformis* (Table 1). Tested primer pairs included 73F2 + 73R2, 210F1 + 210R1, 210F2 + 210R1, and 210F3 + 210R2 at high annealing temperatures required for retention of species specificity.

RESULTS

Library screening. Out of 312 recombinant plasmid clones, with insert sizes ranging from 0.2 to 6.5 kb, only 12 (3.8%) consistently generated strong signals when hybridized with total genomic DNA of *M. fructicola* isolate 1656 in both the initial dot blot screen and the *Eco*RI/*Pst*I double digests of individual clones under high-stringency conditions. Among these 12 repetitive clones, four hybridized strongly and three hybridized weakly to the 9-kb ribosomal repeat from *N. haematococca* and were not considered further (data not shown). Thus, only five repetitive, nonribosomal clones were identified (pMF73, pMF134, pMF150, pMF210, and pMF228). Upon *Eco*RI/*Pst*I digestion, single fungal DNA inserts were found for clones pMF73 (204 bp), pMF150 (204 bp), and pMF134 (260 bp), whereas clones pMF210 and pMF228 contained three (≈0.4, 1.5, and 3.5 kb) and two (≈1.2 and 5.0 kb) inserts, respectively. Hybridizing the *Eco*RI/*Pst*I digests of clones pMF210 and pMF228 with labeled total DNA from *M. fructicola* isolate 1656 identified which of these fragments carried repetitive fungal DNA: 1.5 kb for pMF210 and 5.0 kb for pMF228. To create a probe enriched for the repetitive sequence in clone pMF210, the 1.5-kb *Eco*RI/*Pst*I fragment was further subcloned into pGEM®-4Z. Cross-hybridization experiments for each of the individual clones indicated that clones pMF73 and pMF150 shared extensive regions of homology, whereas the other three clones were distinct (data not shown).

Dot blot specificity screen. Inserts from clones pMF73 (204 bp), pMF150 (204 bp), and pMF210 (1,526 bp) proved to be specific to *M. fructicola*, to the exclusion of all other fungi surveyed, including isolates of the closely related *M. laxa* (Fig. 1; Table 1). The specificity screen revealed that pMF73, pMF150, and pMF210 hybridized to *M. fructicola* isolates collected locally from Fresno and Tulare counties in the San Joaquin Valley (Fig. 1, rows A, B, and D), as expected, because the library originated

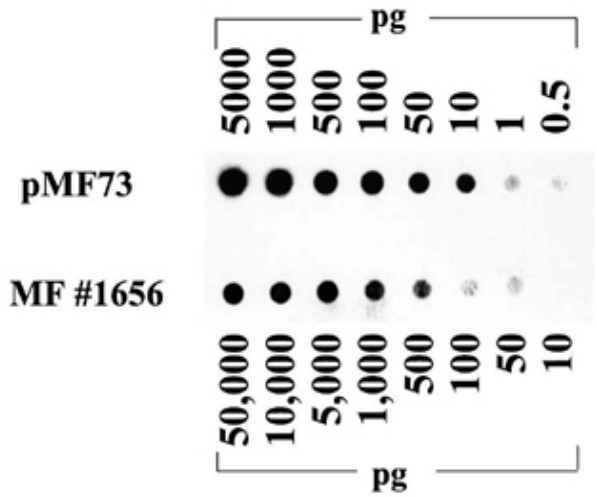

Fig. 2. Dot blot sensitivity assay and reconstruction experiment. A concentration series, in picograms of DNA, was prepared from both the gel-purified pMF73 insert and genomic DNA from *Monilinia fructicola* isolate 1656 (Table 1) and hybridized to the insert. Copy number estimate was derived by comparing the signal intensities of fungal DNA to that of the insert.

from a Fresno County isolate. In addition, the three clones also hybridized to recognized isolates collected from outlying northerly counties in the Sacramento Valley, such as Colusa (C1 to C4), Tehama (C7), Yuba (C8), Glenn (C9), Sutter (C10), and Butte (C11 and C12) counties, as well as one isolate from Kern County (A12) in the south (Fig. 1). Furthermore, the resolving power of these three clones extended to include *M. fructicola* isolates collected from Michigan (E1 to E3), Oregon (E4 to E6), Georgia (E7 to E9), and Australia (E11 and E12) (Fig. 1; Table 1). Signal intensity differences are presumably due to copy number, because equal amounts of genomic DNA were loaded for each isolate (Fig. 1).

Copy number and sensitivity determinations. Estimation of copy number for pMF73 was determined by quantitative dot blot hybridization to membranes containing known amounts of genomic and insert DNA (Fig. 2). This reconstruction experiment was based on comparing relative signal intensities between the pMF73 insert and fungal genomic DNA, when both were simultaneously hybridized to the insert. The signal intensity obtained with 50 pg of genomic DNA from *M. fructicola* isolate 1656 corresponded to that with 1 pg of the 204-bp pMF73 insert. The sequence was calculated to comprise approximately 2.0% of the genome, assuming that the repeat was present once in the insert sequence. The haploid genome size of *M. fructicola* is unknown. However, if it is assumed to be roughly the same as that of other ascomycetes described in the literature (e.g., *Aspergillus*, *Neurospora*, or *Colletotrichum*; 29), $\approx 4 \times 10^7$ bp, then the pMF73 repeat comprises approximately 2,500 copies per haploid genome. Repeated reconstruction experiments yielded comparable values. A similar copy number was obtained with pMF150 (data not shown).

Dot blot sensitivity thresholds indicated that the pMF73 insert was capable of detecting as little as 50 pg of fungal genomic DNA (Fig. 2). This was confirmed over several separate determinations using varying wash stringencies and exposure times. The same level of sensitivity was also observed for pMF150 and pMF210 (data not shown). Different *M. fructicola* isolates contain different copy numbers or degrees of reiteration of the pMF73/150 and pMF210 repeats (Fig. 1, E8, *M. fructicola* isolate D1B3 from Georgia). Because detection sensitivity is based on copy number, the value of 50 pg as a detection limit appears to be isolate-dependent.

Genomic organization. Genomic southerns (*Eco*RI, *Pst*I, and *Bam*HI) were done for a number of *M. fructicola* isolates and several *M. laxa* isolates and hybridized to the pMF73 and pMF210 inserts. Surprisingly, pMF73 did not hybridize to uncut genomic DNA run as a control. Rather, pMF73 hybridized to three bands, ≈1.8, 3.7, and 9.0 kb, that ran ahead of the uncut genomic DNA (data not shown), indicating that this sequence was extrachromosomal in origin. A similar finding was observed for pMF150. Conversely, the probe derived from pMF210 did hybridize to uncut genomic DNA, indicating a chromosomal origin for this sequence. Few restriction sites were observed for pMF73 and none were observed for pMF210, indicating that recognition sites for these three enzymes were rare or absent in the reiterated sequence.

The genomic organization of the species-specific clones was more fully determined by following a time course of partially digested genomic DNA, using the restriction sites flanking the repetitive fungal inserts: *Sau*3AI for pMF73 or *Eco*RI/*Pst*I for pMF210 (data not shown). Autoradiograms for both partial restriction digests yielded discrete ladders of oligomers, which were rough integers of the basic monomer repeat, and distinctive of the tandem genomic arrangement of conventionally reiterated satellite sequences, as opposed to that of a randomly interspersed sequence.

Sequence characterization. Full-length sequences were obtained in both directions for all three clones, using the SP6 and T7 sequencing primers flanking the insert (Fig. 3). To obtain the full-length sequence for pMF210 (1,526 bp), two additional sequencing primers were designed (data not shown). The 204-bp inserts for clones pMF73 and pMF150 were identical in sequence, confirming earlier cross-hybridization data. The sequence was flanked on each end by a *Sau*3AI site, was 51% A+T, and contained several direct repeats: ACCCCCCTC (sites 38 and 86), CCCTTAC (81 and 157), and a single inverted repeat (i.e., palindrome): GGCTACGTGGGG (52 and 72). The 1,526-bp repeat from pMF210 was 63% A+T, and contained many homopolymeric runs of either A or T. In addition, pMF210 had a number of direct repeats greater than 9 bp in length: ATTCCCGGTGAAC (sites 1444 and 1471), ACCGGTGAGCC (118 and 673), CCCCCGGTGA (375 and 578), TACCGGTGAG (672 and 1412), GTTTACTATA (356 and 984), CCGGTGAGC (310 and 378), TTTCTATTA (712 and 1002), AAAAATTAA (830 and 1029), and ATCAATACT (1011 and 1190). GenBank accession numbers for pMF73/150 and pMF210 are AF288574 and AF288575, respectively. No open reading frames were detected for either clone. BLASTN searches revealed no significant sequence similarity to any other sequences in the GenBank database for both pMF73 and pMF210. The only exception was the first 44 bp of clone pMF210, which had partial homology to the intergenic spacer region of the 28S and 18S rDNA from *Sclerotinia sclerotiorum*, a taxon closely related to *M. fructicola* (GenBank Accession No. AF040080-85).

PCR specificity survey. At the high annealing temperatures required for retention of species specificity, the same specificity profiles observed earlier in the 96 isolate dot blot hybridization (Fig. 1), were again observed for a subset of the same isolates (Fig. 4). Nonspecific amplification products were sometimes observed at lower or more relaxed annealing temperatures for some of the *M. laxa*, *M. camelliae*, and *B. cinerea* isolates (data not shown). However, this was observed only for primer pairs designed to the pMF73 sequence (i.e., 73F1 + 73R1 and 73F2 + 73R2) when annealing temperatures were relaxed to 62°C and below. This primer set was species-specific at 66°C. Nonspecific amplification products were never observed for any of the pMF210 primer pair combinations (i.e., 210F1 + 210R1, 210F2 + 210R1, 210F3 + 210R2, and 210F3 + 210R3), even when the annealing temperatures were relaxed significantly.

PCR sensitivity assay. To determine the detection limits of the species-specific primer pair sets developed for *M. fructicola*, PCR sensitivity assays were done for known concentrations of both fungal DNA template (Fig. 5A to C) and template derived from DNA extractions from known concentrations of fungal spore suspensions (Fig. 5D and E). It was assumed that the former test would be a more accurate means of assessing the true limits of sensitivity, whereas the latter probably more accurately reflected detection under field conditions. PCR sensitivity limits were also assessed in planta at various times after inoculation.

The sensitivity of a subset of the species-specific primer pair sets, derived from pMF73 and pMF210, was determined by incorporating progressively smaller amounts of *M. fructicola* template DNA in the PCR reaction, ranging from 50 ng to 0.5 ag (Fig. 5A to C). The sensitivity of all three primer pairs tested (73F2 + 73R2, 210F2 + 210R1, and 210F1 + 210R1) was comparable at the high annealing temperatures required for retention of species specificity, and capable of detecting 50 fg of fungal genomic DNA template (Fig. 5A to C). The same results were obtained over several separate determinations. PCR detection of *M. fructicola* was 1,000 times more sensitive than dot blot hybridizations to total fungal genomic DNA (50 fg versus 50 pg, respectively; Figs. 2 and 5A to C).

PCR detection from fungal template derived from known quantities of fungal spores was also used to evaluate the sensitivity of the primer pairs for both pMF210 (201F3 + 210R2) and pMF73 (73F2 + 73R2) (Fig. 5D and E, respectively). This was done to correlate the detection limits obtained from known quantities of DNA template with that obtained from known

quantities of fungal spores. Both primer pair sets from pMF210 and pMF73 were capable of amplifying consistently from template derived from as few as 10 spores (Fig. 5D and E), approximately equivalent to 50 fg of DNA (Fig. 5A to C).

In planta detection of *M. fructicola* by dot blot hybridization. Inserts from pMF73 and pMF210 were capable of detecting *M. fructicola* directly from infected plant material. None of the flower hybridization signals exceeded the intensity of the highest fungal DNA calibration standard (i.e., 1,000 ng). Thus, from a single hybridization experiment, both detection of the pathogen and an estimation of pathogen biomass (in relation to percentage of total flower DNA) could be determined. All 10 flowers classified as (+) gave intense hybridization signals when probed with the pMF73 insert. Eight out of ten of the (+/–) flowers and 1 out of 10 of the asymptomatic (–) flowers hybridized to the probe (Fig. 6). In general, (+) flowers had stronger

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pMF73
                          73F1> >>>>>>>>>> >>>>>>>>>> >
1       GATCCTGATG TCTCCATGTA GAACCTTCTT TCTTTCCACC CCCCTCCCAC CGGCTACGTG
      73F2>>>>>>> >>>>>>>>>> >>>>>

61      GGGCTAGCGC CCCCACGTAG CCCTTACCCC CCTCTATTTT TACAATCCTT AGTTTGCTCT

                                <<<<<<< <<<<<<<<<< <<<<<73R2
121     AGTAGCTTTC CCTCCTTCAT TAGCGTCAGA CAATAACCCT TACATCTTTA TGTGAGACAG
                   <<<<<<<<< <<<<<<<<<< <<<73R1

181     GTATTTTCTA ACACCTAGCA GATC


pMF210

1       GAATTCAACC GATTTCTTTC AAGAGAAATC GGTCAAGTTC ATGTTATGAT TATAAGCTCA
                        210F1> >>>>>>>>>> >>>>>>>>>> >
61      CCGGGAGACA TATAATTTAT AACGTAGGTC CATGTTAATC TTTTAAAACT TTCTACCACC
121     GGTGAGCCAC ACATAATATA TGCGGTCGGT TTATTAAGTG GTCGAAAGGT AAATTATTTG
181     AATAACAGAC TTTAAAACGC TTATACAATA GTACTGTACT TGTATGCTCC AGAAGTACAA
241     TACTTGTGGT GGGACCCTAA ACGAAAGCTT CAAGGCTTAT CTTTGTTATT ATAGAAAATG
301     TATACGTCTC CGGTGAGCAC CCTGTTAAAG AGTGGAAAGG TGAGGATTCA TTCTAGTTTA
                                     210F2>>>>>>>>> >>>>>>>>>> >>>
361     CTATAGAGAT AAAGCCCCCG GTGAGCCCAA AGTATACAGC AGATTATATA ACCGCTCAAG
421     CTTAACCTTA ACGAGTGCTG GAATGGGCCC TTAGTGCCTC CCGGTGACCC TTAAGGCTGC
481     AATTGTAAAA AATTAATCTT TCCTGAATTT AATAAGTAGA GTCCATCCCC GGTGAGCATA
541     TCGAAATATA ATTGATATAA GGTAGGTATT TTAAGAACCC CCGGTGAAGT TTAATAATAT
601     AGTAATTATA AGTATATATA TTCGCCTAAG TGAAAAATAA AGGGGGTGGA ATCGCGTAAG
                                       <<<<<<<<< <<<<<<<<<< <<<210R1
661     CGAAGTTTCG CTACCGGTGA GCCCTTATAT AATCACAATT TGAATCATAT ATTTCTATTA
721     CTGTGATTTC AATCTTAAAA TGGATTTATC GTTAAAAATA GGATGTCAAT GAGTATATAG
781     AAGCCAAGTC GTAGAGTAAA TAAGTAAGGT AATATAATTA TATTAAGTCA AAAATTAAAT
841     ATATAAAAGT TAAAGTATTA TAAATATAAG TACCGATCGT TATAGAAGGA ATCGGCTTTA
                                                 210F3>>>>>>> >>>>>>>>>>
901     TCGTCGCCGA TAGCCTTAAC GTATCGACAA GATCGGCATA GTTAATATTA AGTAAAGAGA
      >>>>>
961     TTAAGTAGTT TTAATAAAGT TTAGTTTACT ATAATCTACG TTTTCTATTA ATCAATACTA
1021    TTACAATTAA AAATTAAGCT TCTTAGACTT ATAAAGTAAA AGTATATATA TTTAAGAATA
1081    TAGTATATAA AAATAGAGAT ATATAAAGAA TGGTATAGAG TTATAATAAT TATTAAAATT
1141    AAATTCAAAC GGATTAACGA GTATAAGGTA TAAAATAATA GAAGAGGATA TCAATACTTT
1201    ATAAGTGGAT TGAAAGCTCG CCGGCGACGG ACCCCGGTGG CGCCCCGGTG ACCCCCCCTC
1261    GCGACAGGGG GCGATGGGGG ATGGGGTCGG GCGAATTTTT TAATTTTTTT TCACTTTAGA
1321    AGGGGTCTTC CACTGTACAA ACTCTCAAGT GATGGTCCCG GTGATGTGTT CTATAAGAGG
1381    GTGAAAGTGT ACAAGTAGCT CGTAAGTATA ATACCGGTGA GGTGTACCTA ATGTGGAAGC
          <<<<<<<< <<<<<<<<<< <<<<210R2
1441    ACAATTCCCG GTGAACACTA ATTTATTATG ATTCCCGGTG AACTCATTAT AGGGGCTAGT
                                                   <<<<<<<<< <<<<<<<<<<
1501    ACCCTGAGCT TACTCCCGGT CTGCAG
      <<<210R3
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Fig. 3. Nucleotide sequences of the two species-specific *Monilinia fructicola* clones. The extrachromosomal pMF73 sequence was 204 bp in length, flanked on either side by a *Sau*3AI site (underlined), 51% A+T and contained several direct repeats and a single inverted repeat. The chromosomal pMF210 sequence was 1,526 bp in length, flanked by *Eco*RI and *Pst*I sites (underlined), 63% A+T and contained many homopolymeric runs of A+T and a number of direct repeats. GenBank searches provided no significant homologies to any other sequence in the databank. Forward (>) and reverse (<) species-specific primer pair sets designed to each sequence are indicated.

hybridization signals than did either (+/–) or (–) flowers carrying *M. fructicola*. Visual estimations of fungal biomass from single flowers indicated that some flowers had more than 50% of their total DNA comprised of *M. fructicola* (e.g., flower no. 1 in the (+) series), whereas other flowers had less than 1% of their total DNA comprised of fungal DNA (e.g., flowers 8 and 10 in the (+/–) series) (Fig. 6).

Detection of *M. fructicola* from artificially infected nectarine (cv. Fantasia) tissues was done using the pMF210 insert (Fig. 7). As in the flower dot blot (Fig. 6), none of the fruit hybridization signals exceeded the intensity of the highest fungal calibration standard (i.e., 500 ng). No hybridization to the control-fruit DNA was observed. The hybridization probe was capable of detecting *M. fructicola* from infected fruit tissues at 24 h postinoculation for the 400 spores-per-wound experiment and 36 h postinoculation for the 40 spores-per-wound experiment (Fig. 7). The earliest time points of detection for both experiments corresponded to 50 to 100 pg of fungal DNA template as deduced from the calibration

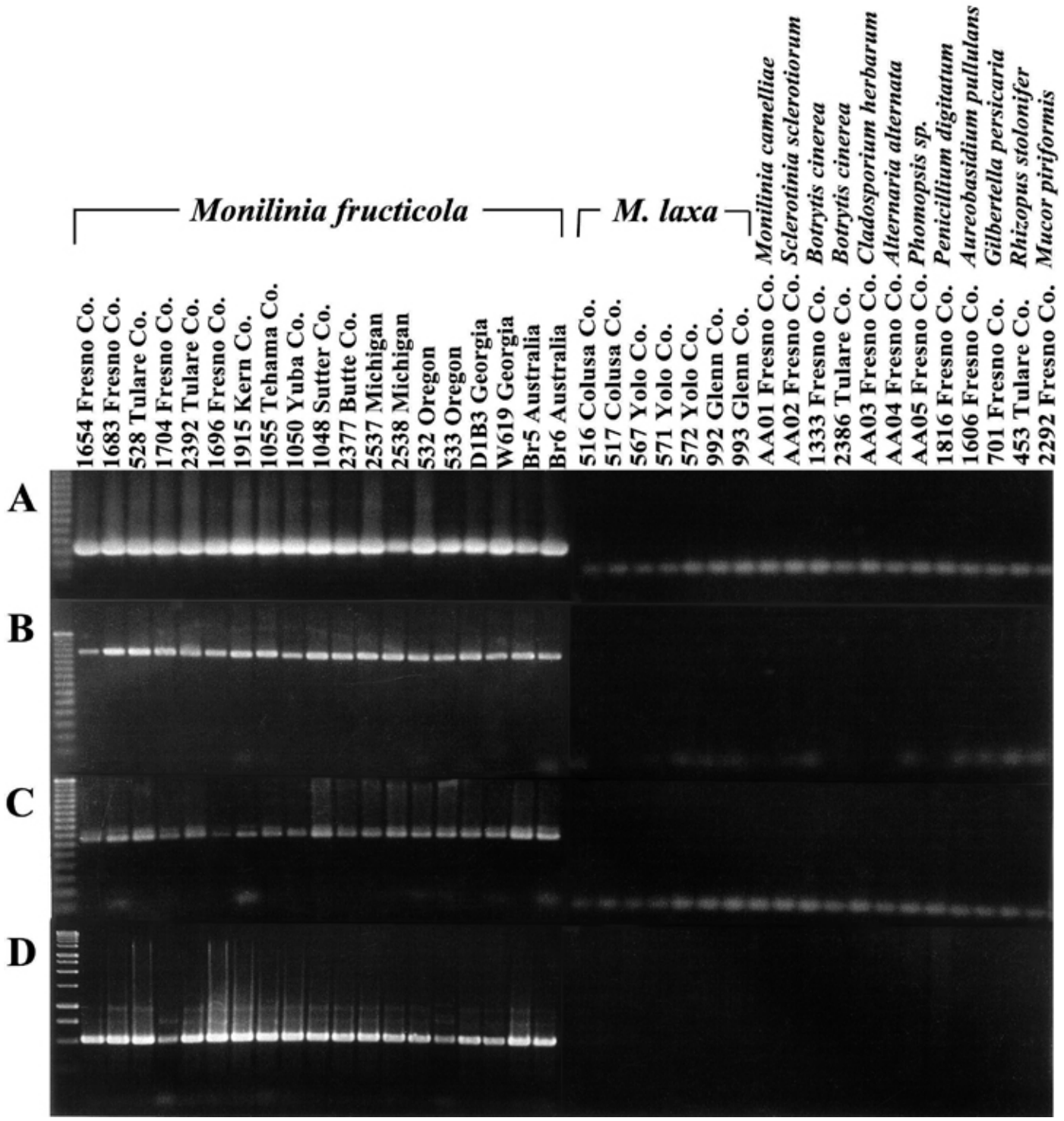

Fig. 4. Polymerase chain reaction specificity survey. Primers designed to the *Monilinia fructicola*-specific pMF73 and pMF210 sequences were used to assess the limits of specificity for a subset of the same isolates screened by dot blot hybridization in Figure 1. Primer pair combinations and final annealing temperatures included **A,** 73F2 + 73R2 (66°C; 161 bp); **B,** 210F1 + 210R1 (64°C; 633 bp); **C,** 210F2 + 210R1 (64°C; 321 bp); and **D,** 210F3 + 210R2 (64°C; 520 bp). **A to C,**The molecular weight standard ranges in size from 50 to 800 bp in successive increments of 50 bp**,** whereas the standard in **D** is from Promega Corp. (G5711; Madison, WI) and begins at 0.25 kb. The nonspecific lower band visible in these gels is excess or unincorporated primer.

standard. This was comparable to the sensitivity limits obtained from hybridization to purified fungal genomic DNA (Fig. 2).

In planta detection of *M. fructicola* by PCR. PCR detection of *M. fructicola* from naturally infected flower tissues was possible using the primer pair sets derived from pMF210 (210F1 + 210R1) at the high annealing temperatures required for retention of species specificity (Fig. 8A). Amplicons of the correct size were observed in PCR reactions using template derived from the same flower DNA samples as were used in the preparation of the dot blot hybridization (Fig. 6) and yielded the same specificity profiles (Fig. 8A). As was seen in the flower dot blot (Fig. 6), the (+) series of flowers had much stronger amplification signals than did either the (+/–) or the (–) flowers carrying *M. fructicola* (Fig. 8A). This was expected, because equal amounts of template (i.e., 25 ng of total fungal-plant DNA) were incorporated into each PCR reaction, and the proportion of fungal versus plant template would be expected to differ between flowers, reflecting varying pathogen loads. The primer pair designed to pMF73 (73F2 + 73R2) yielded similar results (data not shown).

The species-specific primer pairs were also capable of detecting *M. fructicola* directly from artificially infected nectarine fruit tissues in PCR detection assays after varying time points following inoculation (Fig. 8B). No amplification occurred from the control-fruit DNA template alone. There was sufficient fungal DNA template present 12 h postinoculation in the 400-spores-per-wound experiment to yield amplification product, whereas this time point was 24 h in the 40-spores-per-wound experiment (Fig. 8B). Thus, PCR detection limits were more sensitive and capable of detection 12 h earlier than dot blot hybridization to the same DNA template (Fig. 7). However, these detection thresholds were less sensitive than PCR with fungal DNA template alone (Fig. 5A to E).

DISCUSSION

The cloning and characterization of DNA sequences specific to *M. fructicola* provide an important new tool for unambiguous detection of a major plant pathogen. The two identified species-specific probes (pMF73 and pMF210) and derived primer sets should permit the identification of most isolates of *M. fructicola*, since they detected a diverse assemblage of isolates collected from all commonly encountered hosts, over different time periods and, most important, from highly divergent geographic sources. The clones were initially selected for maximum intensity of hybridization under high-stringency conditions, which resulted in the preferential selection of high-copy sequences (4,10,12,18). This was later reflected in hybridization- and PCR-based sensitivity assays. The advantage of such highly repetitive, species-specific probes is that the detection signal is multiplied, because multiple copies of the target sequence exist. This should provide much greater sensitivity than probes or primers designed to low- or single-copy target sequences (12). Furthermore, detection of a repetitive sequence should prove to be more stable over evolutionary time, because mutations in one copy of a highly reiterated sequence should not, in theory, affect the overall sensitivity of either hybridization- or PCR-based detection methods (10).

The specificity surveys included a wide range of isolates and co-occurring fungal species on stone fruits. Thus, 100 fungal isolates, representing 12 species of fungi commonly associated with California stone fruits, were surveyed for specificity. To verify retention of intraspecific fidelity, a geographically diverse assemblage of *M. fructicola* isolates was included in the initial dot blot hybridization specificity survey. These included not only isolates from all regions of California where stone fruits are grown, and from which the original partial plasmid library originated, but also included geographically disparate isolates from Oregon, Michigan, Georgia, and Australia. All *M. fructicola* isolates hybridized to the two probes (pMF73 and pMF210) to the

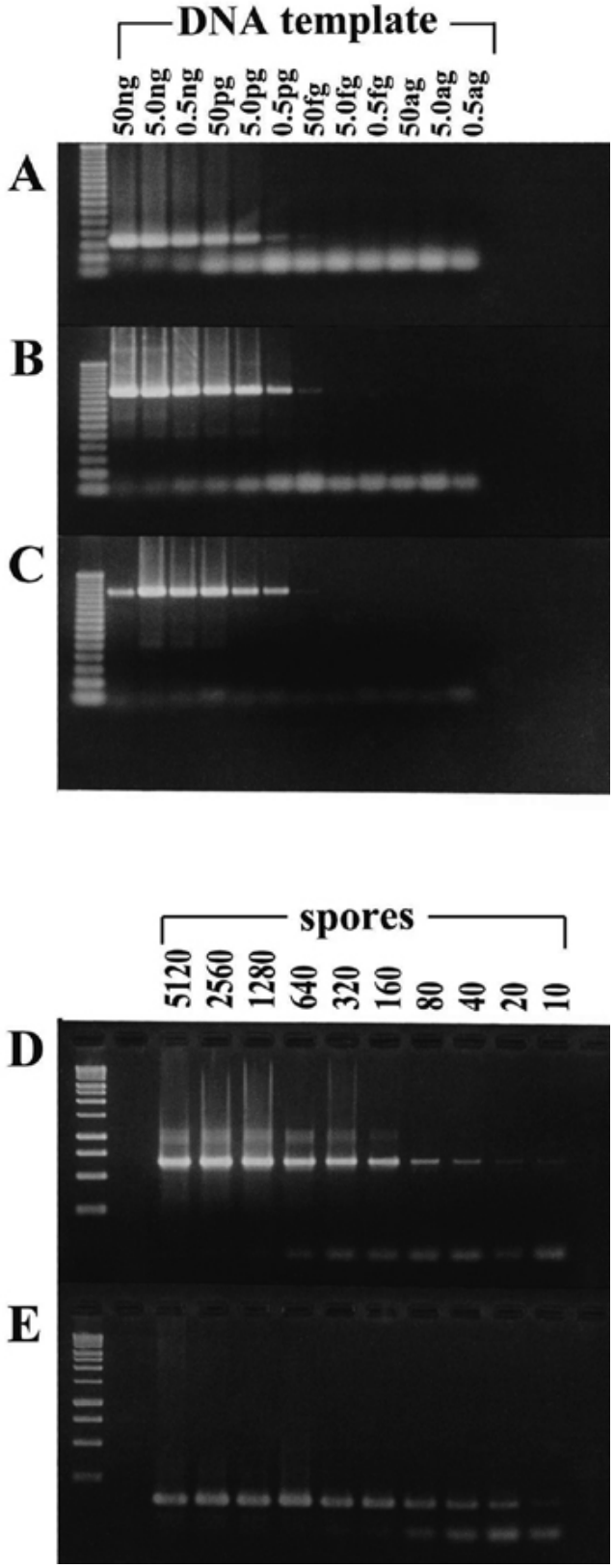

Fig. 5. A to C, PCR sensitivity assay from known concentrations of *Monilinia fructicola* DNA template and **D and E,** spore suspensions. Progressively smaller amounts of fungal DNA template, ranging from 50 ng to 0.5 ag, were tested to determine the sensitivity limits of the species-specific primer pairs **A,** 73F2 + 73R2; **B,** 210F2 + 210R1; and **C,** 210F1 + 210R1. The 50-bp molecular weight standard ranges in size from 50 to 800 bp. PCR sensitivity limits were also determined from DNA extractions from known amounts of spore suspensions using primer pairs **D,** 210F3 + 210R2, and **E,** 73F2 + 73R2 at high annealing temperatures. The molecular weight marker is the 1-kb ladder (G5711) from Promega Corp. (Madison, WI) and begins at 0.25 kb. The nonspecific lower molecular weight band is unincorporated primer.

exclusion of every other fungal species surveyed, including closely related species such as *M. laxa*, *B. cinerea*, *Sclerotinia sclerotiorum*, and one nonstone fruit pathogen, *M. camelliae*. This finding was repeated on a subset of these same fungal isolates, using the species-specific primer pair sets designed to pMF73 and pMF210 at the high annealing temperatures required for retention of species specificity. Repetitive sequences are often hypervariable between taxonomically related species (1,5) and, as such, represent a logical region of the genome to search for sensitive species-specific diagnostic sequences (10,12,18).

In this study, all three species-specific clones were assumed to be nonribosomal in origin, primarily because they failed to cross-hybridize with the full 9-kb ribosomal clone from *N. haematococca* in the initial partial library screen. However, upon sequencing, clone pMF210 revealed a distal 44-bp sequence homology to the ribosomal intergenic spacer region from *Sclerotinia sclerotiorum*, a species closely related to *M. fructicola*. Sequence alignments with GenBank *S. sclerotiorum* intergenic spacer regions failed to find significant homology other than the distal 44-bp tract. Because the heterologous ribosomal probe was derived from *N. haematococca*, a pyrenomycete, and because the brown rot pathogen *M. fructicola* is a discomycete, it is reasonable to expect that only conserved regions of the ribosomal gene cluster fully cross-hybridized under the stringent conditions used here in the initial screen. The internal transcribed spacer and intergenic nontranscribed regions of fungal ribosomal repeats are hypervariable (33) and have been used for species-specific molecular diagnostics among other plant pathogenic fungi (19,20, 26,32). Therefore, clone pMF210 may be ribosomal in origin, but it is of sufficient sequence dissimilarity that it can serve as the basis for the development of a species-specific molecular diagnostic.

The fraction of genomic clones hybridizing to a complex probe of total genomic DNA is a good indication of the frequency of reiteration of repeats in a given genome (5,12). In our partial library of 312 clones, only 3.8% generated a strong hybridization signal. This is somewhat comparable to what has been reported for other ascomycetes. For instance, in a survey of 34 random clones, 5.8% proved repetitive in *Stagonospora nodorum* (23) and in *B. cinerea*, a species closely related to *M. fructicola*, out of 870 clones only 23, or 2.6%, proved repetitive (4). The number of species-specific clones in our survey was 3 out of 312 or 0.96%. Similar values were found in *Phytophthora parasitica* (10) and *Fusarium oxysporum* f. sp. *canariensis* (28). However, in other studies much higher values have been reported for the repetitive fraction and for the fraction of species-specific clones (18,35).

A range of sensitivity values has been reported for molecular diagnostics developed to identify fungal plant pathogens, more or less consistent with the findings reported here for *M. fructicola*. However, few studies have compared sensitivity values derived from both hybridization- and PCR-based methodologies to fungal DNA alone and in planta, as was done in the present study. Sensitivity levels have been determined from full-length species-specific probes in hybridization studies (10,18,35) and from species-specific primers (2,6,7,26,30,32,34). Commonly reported sensitivity values include, for example, *Peronospora tabacina* (34), in which the minimum amount of fungal genomic DNA detected, using species-specific primers developed from a sequence-characterized randomly amplified region, was between 100 fg and 1 pg. Subsequent hybridization of the labeled amplicon to the PCR blot enhanced the sensitivity of detection to less than 10 fg of template. In another study using the same approach, species-specific primers amplified 50 pg of template in *F. culmorum*, and in *F. graminearum* 5 pg of template proved sufficient for amplification; sensitivity was again enhanced using hybridization to the PCR blot to the 500-fg level for both (30). Similarly, among *Phytophthora* spp. causing late blight and pink rot of potatoes (32), species-specific primers designed to the internal transcribed spacer region showed a sensitivity limit of 10 pg of genomic DNA template, with a 10-fold increase in sensitivity following hybridization. The enhancement of PCR-based

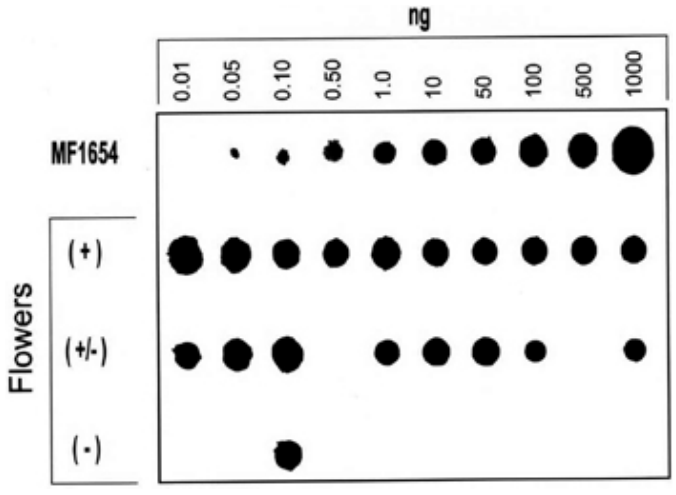

Fig. 6. In planta detection of *Monilinia fructicola* from plum flowers (cv. Royal Diamond) using the gel-purified pMF73 insert as the hybridization probe. Field-collected flowers were classified by visual symptoms: (+) flowers were heavily infected with *M. fructicola* and showed sporulation on stem and calyx; (+/–) flowers showed patches of brown discoloration on the same flower organs, but no evidence of fungal emergence; whereas (–) flowers appeared asymptomatic. Because none of the flower hybridization signals exceeded the highest fungal calibration standard, and equal amounts of flower DNA were loaded, an estimation of fungal biomass as a percentage of total DNA is based on comparisons to the fungal calibration standard.

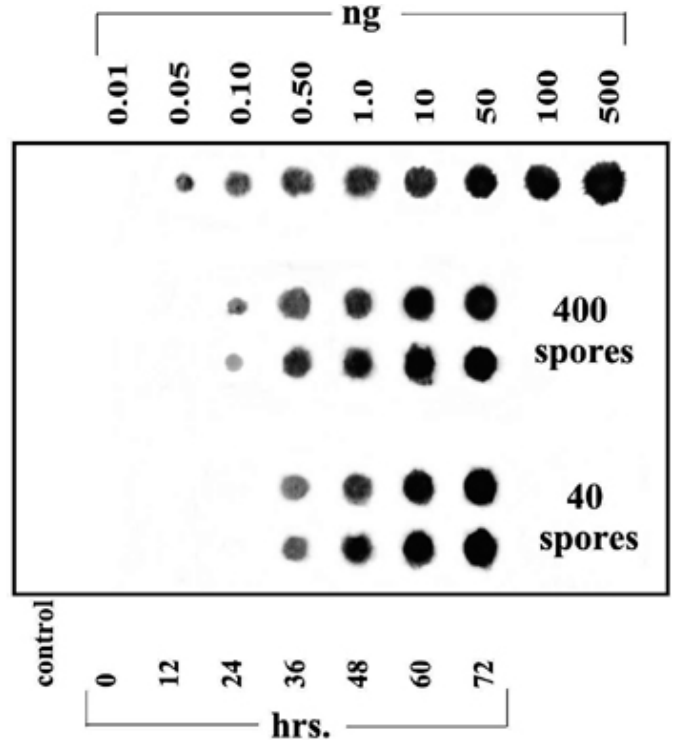

Fig. 7. In planta dot blot detection of *Monilinia fructicola* from inoculated nectarine fruit (cv. Fantasia) tissues, using the gel-purified pMF210 insert as the hybridization probe. The earliest detection times were 24 and 36 h postinoculation for the 400- and 40-spores-per-wound experiments, respectively. This corresponded to a detection threshold of 50 to 100 pg of fungal DNA. Similar levels of detection were observed in hybridizations to fungal DNA alone in Figures 2 and 6. None of the fruit hybridization signals exceeded the highest fungal calibration standard. No hybridization to the control fruit DNA was noted.

sensitivity thresholds based on the hybridization of the original PCR blot with labeled amplicon, however, should be distinguished from sensitivity limits derived from direct hybridizations to fungal genomic DNA, either alone or from infected host tissues. The latter more accurately reflects true sensitivity based on relative copy number in the genome.

In our study, the inserts from all three species-specific clones, when used as hybridization probes in dot blot sensitivity assays, were comparable and could readily detect as little as 50 pg of fungal genomic target DNA. This level of sensitivity was also observed for the fungal calibration standards in both flower and fruit dot blots. Comparable values were reported for *Phytophthora cinnamomi* (18), where 50 pg was also recorded as the detection threshold, with an enhancement to 5 pg after prolonged exposures. This sequence was calculated to comprise roughly 12,000 copies per haploid genome and to make up more than 5% of the genome

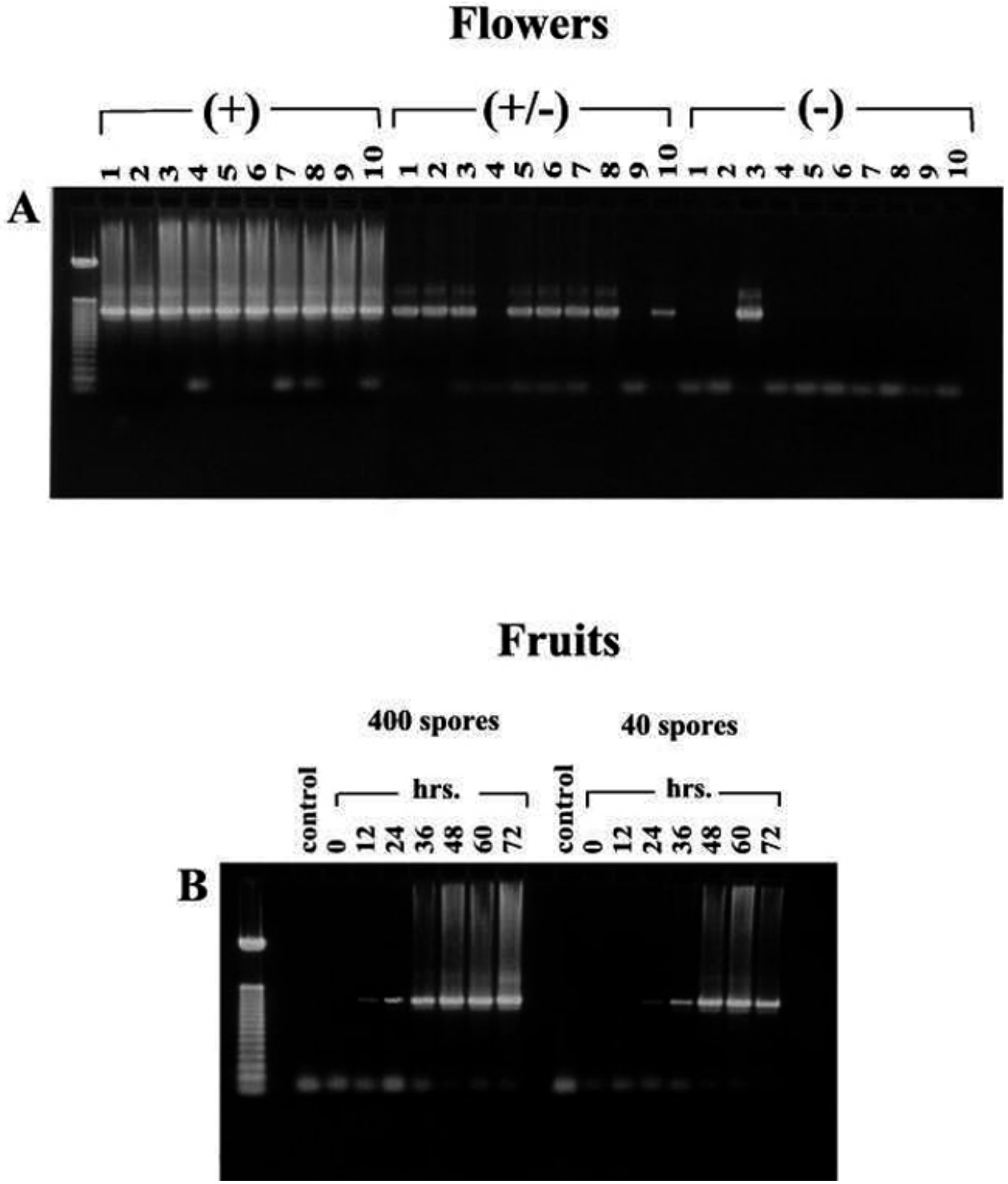

Fig. 8. In planta polymerase chain reaction (PCR) detection from **A,** flower, and **B,** fruit tissues. Detection of *Monilinia fructicola* from plum flowers (cv. Royal Diamond) used the species-specific primer pair 210F1 + 210R1 at the high annealing temperature required for retention of species specificity. **A,** DNA template was the same as that used for the flower dot blot hybridizations (Fig. 6). In planta PCR detection from inoculated nectarine (cv. Fantasia) tissues **B,** was done with the 210F3 + 210R2 primer pair at high annealing temperatures. Detection of *M. fructicola* from infected fruit tissues was possible 12 h postinoculation for the 400-spores-per-wound experiment and at 24 h postinoculation for the 40-spores-per-wound experiment. No amplification to the control fruit DNA was noted.

(18). Our calculations indicated that the copy number of pMF73 was about 2,500 copies per haploid genome, and comprised roughly 2% of the *M. fructicola* genome. The pMF210 copy number was not determined here, but we estimate it to be comparable to that of the pMF73 sequence, because pMF210 also had a detection threshold of 50 pg in dot blot hybridization, similar to that of fungal genomic DNA.

Although the sensitivity limits obtained from our hybridization data were comparable to previously published values, the species-specific primer pair sets designed to pMF73 and pMF210 were considerably more sensitive than many values previously reported in the literature. The primer pairs designed for both species-specific sequences were comparable, and capable of amplifying consistently from as little as 50 fg of purified fungal genomic DNA template, corresponding to a detection threshold of approximately 10 spores. This represents a 1,000-fold increase in sensitivity as compared to the dot blot hybridization. This also represents a higher level of sensitivity than species-specific fungal primers developed from sequence-characterized, randomly amplified regions (30,34), analysis of the rDNA internal transcribed spacer (2,32), and mitochondrial sequences (6). However, sensitivity values comparable to those in our study were reported by Forster and Adaskaveg (7) for species-specific primers to *M. fructicola*, where as little as 20 spores were detected from inoculated cherry tissues, and from single ascospores in *Gaeumannomyces graminis* (14).

The data in our study indicated that sensitivity limits were attenuated by the presence of plant DNA in PCR-based detection methods, but no such attenuation was noted for the dot blot hybridization. In planta dot blot hybridization indicated detection thresholds of 50 pg of fungal DNA 24 h postinoculation in the 400-spores-per-wound experiment, and approximately 100 pg in the 40-spores-per-wound experiment after 36 h. This was comparable to values obtained from hybridizations to fungal DNA alone. PCR enhanced this level of detection to 12 h earlier for both fruit-inoculation experiments. However, the sensitivities observed in PCR with infected fruit template were somewhat less than expected, given that PCR from fungal DNA template alone indicated detection limits equivalent to approximately 10 spores, or 50 fg of DNA. Lowered PCR sensitivities associated with in planta detection have been reported previously (14), and may be due to kinetic interference of plant DNA or plant-derived inhibitory compounds (3,14).

Nevertheless, in our study, both hybridization- and PCR-based methodologies were capable of detecting *M. fructicola* consistently from infected host tissues. Different flowers carried different levels of *M. fructicola* infection as deduced from comparisons of relative signal intensities among various flowers. We were able to detect *M. fructicola* not only from obviously infected (+) flowers, but also from both (+/–) and asymptomatic (–) flowers using both hybridization- and PCR-based methodologies. This allowed us to determine that most of the naturally infected (+/–) plum flowers were carrying *M. fructicola* and not some other pathogen. The species-specific flower detection method reported here could prove useful in early spring orchard surveys of random flower samplings, and thus could provide a tool for early prediction of blossom blight field incidence and impact on the timing of fungicide applications.

Both preharvest and postharvest losses have often been attributed to asymptomatic quiescent infections of *M. fructicola*, especially on bruised regions or fruit-to-fruit contact points (24). The development of a sensitive species-specific molecular diagnostic for *M. fructicola*, capable of detecting the pathogen in planta prior to the onset of visual symptoms, could provide a tool for determining disease risk and establishing guidelines on preharvest fungicide applications, and aid in marketing strategies of stored stone fruit. Regions of the fruit implicated as entry points for the pathogen (24) could be sampled during cold storage, for example, to monitor the incidence of latent infections. Furthermore, the tools developed here could be used to monitor latent spore load from stone fruit surface washes. Latent spore load has been implicated as a significant source of inoculum potential in stored fruits, and cannot be measured with the current freeze-thaw detection assays, which require surface sterilization (25).

In conclusion, we have developed a set of sensitive molecular diagnostics capable of detection of the brown rot pathogen *M. fructicola* from infected host tissues. We have also correlated sensitivity threshold values derived from both hybridization- and PCR-based methodologies to purified fungal DNA template alone, as well as to sensitivities obtained in planta. The retention of species-specificity patterns, observed among a diverse assemblage of *M. fructicola* isolates, suggests that both the extrachromosomal pMF73 and the chromosomal pMF210 are highly conserved within the taxon. The molecular tools developed here for *M. fructicola*, as well as those reported previously (7,8), should prove useful for the stone fruit industry in determining disease risk assessment and blossom blight incidence, and in developing preharvest and postharvest chemical control strategies.

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