Identification of *Monilinia fructigena*, *M. fructicola, M. laxa,* and *Monilia polystroma* on Inoculated and Naturally Infected Fruit Using Multiplex PCR

Marie-José Côté, Canadian Food Inspection Agency, Ottawa Laboratory (Fallowfield), Centre for Plant Quarantine Pests, 3851 Fallowfield Road, Ottawa, Ontario, Canada, K2H 8P9; **Marie-Claude Tardif,** Health Canada, Food Directorate, Building #7, Tunney's Pasture, P.L. 0700E1, Ottawa, Ontario, Canada K1A 0L2; and **Allison J. Meldrum,** Canadian Food Inspection Agency, Ottawa Laboratory (Fallowfield), Centre for Plant Quarantine Pests, 3851 Fallowfield Road, Ottawa, Ontario, Canada, K2H 8P9

ABSTRACT

Côté, M.-J., Tardif, M.-C., and Meldrum, A. J. 2004. Identification of *Monilinia fructigena*, *M. fructicola*, *M. laxa,* and *Monilia polystroma* on inoculated and naturally infected fruit using multiplex PCR. Plant Dis. 88:1219-1225.

Monilinia fructigena, M. fructicola, *M. laxa,* and *Monilia polystroma* each have a different regulatory status. To monitor imported and exported fruit for the presence of quarantined *Monilinia* or *Monilia* species, a timely identification method is required. Random amplified polymorphic DNA analysis was used to generate an *M. fructigena*–specific band that was characterized by sequencing. Using the sequence obtained, primers were designed to amplify bands in the same genomic region of *M. fructicola* and *M. laxa*. These bands were also characterized by sequencing. From all three sequences, a multiplex polymerase chain reaction (PCR) method based on a common reverse primer (MO368-5) and three species-specific forward primers (MO368-8R, MO368-10R, and Laxa-R2) was established for the differentiation of the three *Monilinia* species. The multiplex PCR was tested with additional isolates and consistently produced a 402-bp PCR product for *M. fructigena*, a 535-bp product for *M. fructicola*, and a 351-bp product for *M. laxa*. The method was also used with isolates of the recently characterized *Monilia polystroma,* and all isolates amplified a 425-bp PCR product. The identification method was shown to amplify a PCR product directly from inoculated apples, and the PCR band produced was specific to the inoculated *Monilinia* or *Monilia* species. Furthermore, the multiplex PCR was used to identify *Monilinia* species on naturally infected stone fruits. The method correctly identified infections by both *M. laxa* and *M. fructicola* by successful amplification of corresponding PCR products for each species.

Additional keywords: brown rot, sequence characterized amplified region

Three *Monilinia* species cause brown rot of fruit. *M. fructicola* (G. Wint.) Honey and *M. laxa* (Aderhold & Ruhland) Honey attack mainly stone fruits. The third species, *M. fructigena* Honey in Whetzel, also attacks stone fruits but is found more frequently in pome fruits (1). Recently, based on morphological, biological, and genetic differences between European and Japanese isolates of *M. fructigena,* van Leeuwen et al. proposed that the Japanese isolates be considered a distinct species named *Monilia polystroma* van Leeuwen (17). *M. fructigena* and *M. laxa* have traditionally been considered Old World species. However, recent isolations of *M. laxa* suggest it has a more global distribution (1). *M. fructicola* is considered a New World species, whereas *Monilia polystroma* has to date been found only in Japan (17). In North America, brown rot of fruit is mainly caused by *M. fructicola* and to a lesser extent by *M. laxa*. In Europe, the main causal agents of the disease are *M. fructigena* and *M. laxa* (3). The identification of the three *Monilinia* species and *Monilia polystroma* relies on few morphological characteristics, and identification in culture is difficult, since their appearances vary from isolate to isolate within species (15). An identification protocol based on quantitative cultural and morphological features was established and requires up to 5 days (17,18). To prevent the entry of *M. fructigena* or *Monilia polystroma* into North America and entry of *M. fructicola* into Europe, a more timely method is needed to differentiate the four species without having to culture the fungi.

Among many efforts to establish a molecular method for the differentiation of *Monilinia* species, Fulton and Brown (6) located a group I intron in the ribosomal small subunit (SSU) gene of *M. fructicola*. Polymerase chain reaction (PCR) primers for the *M. fructicola* SSU rDNA intron and some of the SSU sequence were developed for species identification (6). Recently, there have been reports showing that the intron-containing PCR product is not amplified in some isolates of *M. fructicola*, suggesting that some isolates lack the intron (4,5,7,9). This observation means the species identification method is no longer reliable. The sequences of the ribosomal internal transcribed spacer (ITS) regions of the three *Monilinia* species and of *Monilia polystroma* have been reported, and sequence comparison reveals a high level of similarity (7,8). Several molecular methods for the identification of *Monilinia* species are either based solely on ITS sequence variations or combined with other genetic markers (5,10,16). Other molecular identification methods based on unique species-specific repetitive sequences and on a microsatellite region were also developed (2,11). Some of the methods described have been developed and tested only with two of the three *Monilinia* species, whereas other methods present a result for only one species and do not identify the other species (2,5,11,16). Yet another method presents a result for all three *Monilinia* species but produces PCR amplicons of the same size so that three PCR reactions have to be performed in order to identify the species (10). No method has been developed for the differentiation of all three *Monilinia* species and *Monilia polystroma*.

Random amplified polymorphic DNA (RAPD) analysis has been used to identify DNA polymorphisms between genomes (19,21). Generating information from a RAPD fragment for the design of specific primers is an alternative technique used when sequences, such as ribosomal ITS regions, are very conserved and restrict the design of a species-specific PCR method. The objective of this work was to use the RAPD technique to identify polymorphisms among *M. fructigena*, *M. fructicola,* and *M. laxa* and to use the sequence information obtained from the RAPD polymorphic band to design species-specific primers for a multiplex PCR assay that would, in a single tube reaction, discriminate among the three *Monilinia* pathogens on inoculated apples and naturally infected stone fruits. The assay was further enhanced to enable the discrimination of *Monilia polystroma* from all three *Monilinia* species, including the closely related *M. fructigena*.

Corresponding author: M.-J. Côté
E-mail: cotemj@inspection.gc.ca

Accepted for publication 18 June 2004.

Publication no. D-2004-0825-01R

MATERIALS AND METHODS

Fungal isolates. All isolates used and their sources are listed in Table 1. Cultures were received lyophilized and therefore were rehydrated before transferring to potato dextrose agar plates (39 g/liter). In order to limit the presence of contaminating potato DNA from the culture media, DNA extractions were done from mycelia that had grown over glass coverslips which had been deposited on the culture media surface.

Table 1. *Monilinia* and *Monilia* species, their origins, and multiplex polymerase chain reaction (PCR) results

Species	Isolate no.[a]	Origin (host, country)	PCR product (bp)
Monilinia fructigena	ATCC 11790	Unknown	402
	ATCC 24976	Apple, U.K.	402
	ATCC 26106	Pome fruit, England	402
	ATCC 38358	Pear, unknown	402
	ATCC 48167	Unknown, U.K.	402
	CBS 231.57	Pear, Netherlands	402
	CBS 494.50	Cherry, Netherlands	402
	LMK 433	Apple, Norway	402
	LMK 434	Apple, Norway	402
	MUCL 570	Crab apple, Belgium	402
	NRRL 22703	Pear, Belgium	402
Monilia polystroma	CBS 102686 (Jap 1815)	Apple, Japan	425
	CBS 102687 (Jap 2314)	Apple, Japan	425
	CBS 102688 (Jap 2316)	Apple, Japan	425
Monilinia fructicola	ATCC 42248	Peach, New Zealand	535
	ATCC 46606	Peach, U.S.	535
	ATCC 62879	Prune, U.S.	535
	CD 6	Sweet cherry, Canada	535
	CD 7	Sweet cherry, Canada	535
	CPQP 1	Peach, Canada	535
	CPQP 3	Nectarine, Canada	535
	DAOM 110195	Cherry, Canada	535
	DAOM 144446	Plum Queen Anne, Canada	535
	DAOM 144721	Plum, Canada	535
	DAOM 186890	Unknown, Canada	535
	JN Mer3-4 (DAOM 208461)	Peach, Canada	535
	JN TC3-13 (DAOM 208464)	Peach, Canada	535
	JN AN3-6 (DAOM 208467)	Peach, Canada	535
	NRRL A-28151	Peach, Canada	535
	URY 2	Unknown, Uruguay	535
	URY 8	Unknown, Uruguay	535
Monilinia laxa	ATCC 9961	Apricot, unknown	351
	ATCC 32671	Nectarine, U.S.	351
	ATCC 62881	Plum, U.S.	351
	ATCC 66106	Apricot, Spain	351
	CBS 165.24	Quince, unknown	535[b]
	CBS 202.25	Pear, U.S.	351
	CBS 298.31	Apple, Ireland	351
	CBS 488.50	Peach, Netherlands	351
	DAOM 209913	Peach, unknown	535[b]
	LMK 656	Unknown, Norway	351
	LMK 723	Nectarine, Italy	351
	MUCL 18236	Peach, Belgium	351
	MUCL 30841	Peach, Belgium	351
	UAMH 3523	Plum, Canada	535[b]
	UAMH 4801	Cherry, Canada	351
Monilinia demissa	CBS 151.22	Chokecherry, unknown	---[c]
Monilinia mespili	CBS 139.23	Medlar, Netherlands	---
Monilinia padi	ATCC 58545	Chokecherry, U.S.	---
Monilinia seaveri	DAOM 43511	*Prunus* spp., Canada	---
	DAOM 91807	Wild cherry, Canada	---
Botrytis cinerea	DAOM 189076	Potato, Canada	---
Sclerotinia sclerotiorum	DAOM 138182	Apple, Canada	---

[a] ATCC strains are maintained by the American Type Culture Collection, CBS isolates by the Centraalbureau voor Schimmelcultures, CPQP and CD isolates of *Monilinia* are from our laboratory and were identified by George P. White. DAOM isolates are maintained by the Canadian Collection of Fungal Cultures (Agriculture and Agri-Food Canada), JN isolates were obtained from John Northover (Agriculture and Agri-Food Canada, Research Farm, Vineland, Ontario, Canada), LMK isolates from Linda M. Kohn (University of Toronto, Toronto, Ontario, Canada), MUCL isolates by the Mycotèque de l'Université Catholique de Louvain, NRRL isolates are maintained by the USDA Agricultural Research Service Culture Collection, UAMH isolates by the University of Alberta Devonian Botanic Garden, Canada, and URY isolates were provided by Pedro Mondino (Catedra de Fitopatologia, Montevideo, Uruguay).

[b] Isolates originally identified as *M. laxa*, correctly identified as *M. fructicola* by PCR.

[c] No PCR product.

Naturally infected fruits. Cherries, nectarines, and peaches showing symptoms of brown rot were used for the multiplex PCR. Half of each localized rotting area on the fruit was removed and put into a microtube. The sample was then either stored at –80°C or processed for DNA extraction.

Apple inoculations. Asymptomatic apples were chosen for inoculation experiments for ease of use and year-round availability (J. Northover, *personal communication*). Apples were surface-sterilized with a 0.5% (vol/vol) sodium hypochlorite solution. Three apples were inoculated at one or two sites per experiment. At each inoculation site, three incisions of 5 mm and at 90 degrees to each other forming a U shape were made on the apple surface, leaving an uncut side acting as a hinge allowing the piece of tissue to be attached to the fruit. Inoculation sites for mixed infections were 30 to 40 mm apart. A piece of the apple skin was lifted, and a 4 × 4 mm piece of agar plug with mycelia from a 1- to 6-month-old potato dextrose agar culture was inserted. Isolates used for inoculation were *M. fructicola* ATCC 42248, *M. fructigena* ATCC 26106, *M. laxa* ATCC 9961, and *Monilia polystroma* CBS 102686. Replicate apples for each inoculation experiment were incubated together in a sealed container. One sealed container was used per species or mixed species experiment to prevent contamination from other fungi. Cubic pieces of apples (skin and flesh) approximately 5 × 5 mm square were harvested at 7 to 10 days after inoculation.

DNA extractions. DNA was extracted following the method of Möller et al. (14) with modifications. Mycelia were rinsed off the glass coverslip with 550 µl of extraction buffer (100 mM Tris-HCl, pH 8.0, 10 mM EDTA, 2% sodium dodecyl sulfate [SDS]) into a 1.5-ml microcentrifuge tube and crushed with a disposable pestle in a microtube. Apple samples or 100 µl of flesh taken from the infected area of stone fruit were placed in a 1.5-ml microcentrifuge tube with 550 µl of extraction buffer and crushed with a disposable pestle in a microtube. Proteinase K (100 µg) (Roche Diagnostics, Laval, QC, Canada) was then added to the mixture and incubated at 55 to 60°C for 60 min. The mixture was vortexed twice during the incubation period. After incubation, 140 µl of 5 N NaCl was added, followed by 65 µl of 10% CTAB (hexadecyltrimethylammonium bromide). After 10 min at 65°C, 700 µl of chloroform:isoamyl alcohol (24:1) was added and the mixture was placed on ice for 30 min. After 10 min centrifugation at 14,000 × *g* at 4°C, 600 µl of the supernatant was transferred to fresh microcentrifuge tubes containing 225 µl of 5 M ammonium acetate and placed on ice for 30 min. Follow-

ing a 10-min centrifugation at 14,000 × *g* at 4°C, the supernatant was added to 0.55 volume of isopropanol, mixed well, and incubated on ice for at least 10 min. The mixture was centrifuged for 10 min at 14,000 × *g* at 4°C and the supernatant was discarded. The DNA pellets were washed with 70% ethanol, dried, and resuspended in 100 µl of sterile water. DNase-free RNase (Roche Diagnostic) was added (1 µg), and the DNA extracts were incubated at 37°C for 20 min. Some of the DNA extractions from infected cherries were done using the DNeasy Plant Mini Kit (Qiagen, Mississauga, ON, Canada). Extraction was performed on 0.1 g of infected fruit flesh following the manufacturer's protocol, and the DNA was eluted in a total volume of 100 µl of AE buffer. DNA extracted from pure mycelia was quantified by running an aliquot along with known quantities of a DNA mass ladder (Invitrogen Life Technologies, Burlington, ON, Canada). DNA concentration was estimated by comparing the intensity relative to the DNA mass ladder band of the most comparable size.

DNA amplifications. RAPD reactions were performed using the GeneAmp kit (Applied Biosystems, Streetsville, ON, Canada). Approximately 20 to 25 ng of DNA was amplified in 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 250 µM of each dNTP, 1.5 mM $MgCl_2$, 1.2 µM 10-mer random primer, and 0.5 units of *Taq* DNA polymerase (Invitrogen Life Technologies) for a total volume of 20 µl. The reactions were carried out in a Perkin-Elmer 9600 thermocycler (Applied Biosystems) with an initial denaturation at 94°C for 3 min followed by 35 cycles of 94°C for 5 s, 35°C for 30 s, a 2-min ramping to 72°C (0.3°C/s), and an extension at 72°C for 2.5 min. After 35 cycles, there was a final extension at 72°C for 5 min. The random primers RAPD screening were purchased from John Hobbs (Nucleic Acid Protein Services (NAPS) Unit, Biotechnology Laboratory, University of British Columbia, Vancouver, BC, Canada). Primers 308, 316, 322, 336, 346, 351, 353, 354, 356, 358, 360, 361, 364, 366, 368, 369, 370, 372, 374, and 375 were tested (Sequence available online from University of British Columbia, Biotechnology Laboratory).

The PCR reactions to recover the fragment corresponding to the 491-bp *M. fructigena* RAPD fragment in the other species were done as follows. Primers were designed from the *M. fructigena* sequence obtained from the cloned RAPD fragment. MO368-6R (5′-AGT TAT CGG CTT GGG AGC GG-3′) and MO368-1 (5′-ACT TGT GCG GCA AAA GAG TA-3′) (synthesized by Invitrogen Life Technologies) were used with the same conditions as for the species-specific multiplex PCR with the exception of an annealing temperature of 35°C for *M. fructicola* and *M. laxa* and 55°C for *Monilia polystroma.*

Multiplex PCR reactions using species-specific primers were performed using Invitrogen Life Technologies reagents. Approximately 10 to 50 ng of DNA extracted from pure mycelia or 1:10, 1:30, or 1:100 dilutions of the DNA extracted from inoculated or infected fruits were amplified in 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 200 µM of each dNTP, 2.5 mM $MgCl_2$, 0.2 µM of each of the specific primers, and 0.25 units of *Taq* DNA polymerase (Invitrogen Life Technologies) in a total volume of 10 µl. The reactions were carried out in a PTC-200 DNA engine thermocycler (MJ Research, Watertown, MA) or a Perkin-Elmer 9600 thermocycler (Applied Biosystems), with an initial denaturation at 95°C for 2 min followed by 35 cycles of 95°C for 15 s, 60°C for 15 s, an extension at 72°C for 1 min, and at the end of the last cycle a final extension at 72°C for 3 min. The specific primers (synthesized by Invitrogen Life Technologies) are: MO368-5 (common reverse primer) 5′-GCA AGG TGT CAA AAC TTC CA-3′ and the three forward primers: MO368-8R (specific to *M. fructigena* and *Monilia polystroma*) 5′-AGA TCA AAC ATC GTC CAT CT-3′, MO368-10R (specific to *M. fructicola*) 5′-AAG ATT GTC ACC ATG GTT GA-3′, and Laxa-R2 (specific to *M. laxa*) 5′-TGC ACA TCA TAT CCC TCG AC-3′. Amplified products were electrophoresed through 1.5% agarose gels in 1× TBE buffer, stained with ethidium bromide, and visualized under ultraviolet light.

Cloning and sequencing RAPD or PCR products. Selected amplified DNA products of the *Monilinia* species were isolated from agarose gels using a GeneClean II kit (Bio101, Qbiogene, Carlsbad, CA) following the manufacturer's protocol and ligated into the vector pBluescriptII SK+ (Invitrogen Life Technologies). After transformation of *Escherichia coli* DH5α, white colonies were selected and plasmid DNA was prepared. Restriction digests were undertaken on plasmids to confirm the presence of an insert of the expected size, and these plasmids were then sequenced using the T7 Sequenase v2.0 Quick-Denature plasmid sequencing kit (Amersham Biosciences, Baie D'Urfé, QC, Canada) following the manufacturer's protocol. The PCR fragments obtained for *Monilia polystroma* were sent to Mobix Lab (McMaster University, Hamilton, ON, Canada) for direct sequencing. Sequence comparisons were done using DNASIS Ver 2.6 for Windows (Hitachi Software Engineering Co. Ltd., San Francisco, CA).

RESULTS

Screening for polymorphic RAPD patterns. Twenty random primers were used in RAPD reactions with *M. fructigena*, *M. fructicola,* and *M. laxa* DNA to find a polymorphic DNA band among the three species. RAPD patterns were characteristic for each pathogen. Using primer 368, a 491-bp fragment was amplified strongly from *M. fructigena* and not from *M. fructicola* and *M. laxa* (Fig. 1). This 491-bp fragment was chosen for its strong intensity and its size, which would facilitate characterization.

Characterization of the 491-bp *M. fructigena* RAPD fragment. The 491-bp RAPD fragment amplified from *M. fructigena* isolate ATCC 11790 (GenBank accession AF506701) was cloned for sequence analysis. From the sequence obtained, specific primers MO368-6R and MO368-1 were designed. MO368-6R contains the sequence just downstream of the primer 368 site at the 5′ end of the RAPD fragment, and MO368-1 contains all of the RAPD primer 368 sequence at the other end plus 10 bases upstream of the RAPD primer (Fig. 2). These primers designed from the *M. fructigena* sequence were used with *M. fructicola, M. laxa,* and *Monilia polystroma* DNA to produce PCR fragments from the same area of the genome for each of these species. A 620-bp PCR fragment from *M. fructicola* isolate DAOM 144721 and a 414-bp PCR fragment from *M. laxa* isolate LMK 723 were cloned and sequenced (GenBank accessions AF506700 and AF506702, respectively). A 504-bp PCR fragment from *Monilia polystroma* isolate CBS 102686 was directly sequenced (GenBank accession AY456197). A comparison of the sequences obtained from the four species

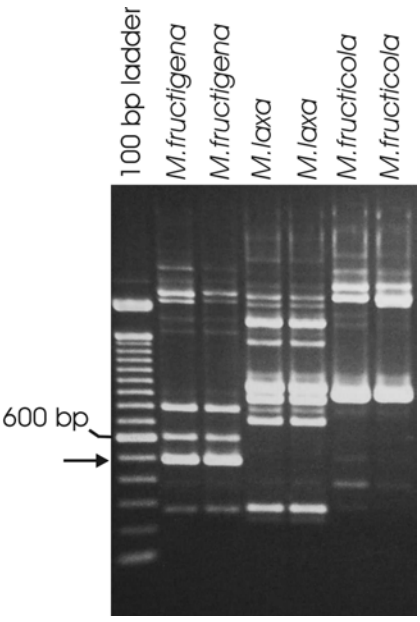

Fig. 1. Random amplified polymorphic DNA (RAPD) assay on selected isolates of *Monilinia* spp. using primer 368. Arrow on left indicates position of 491-bp RAPD fragment obtained for the *M. fructigena* isolates. Isolates used for *M. fructigena* are LMK 433 in lane 2 and LMK 434 in lane 3; for *M. laxa*, isolate LMK 656 in lane 4 and LMK 723 in lane 5; and for *M. fructicola,* isolate JN Mer3-4 in lane 6 and JN TC3-13 in lane 7. Sizes (base pairs) of selected fragments of the 100-bp ladder are indicated on the left.

is presented in Figure 2. All three other species' sequences are very similar to the *M. fructigena* sequence for the last 33 bp (Fig. 2, bases 583 to 615). Apart from two sections of approximately 40 bp each (Fig. 2, bases 449 to 490 and 527 to 568), there are no other significant similarities among the four sequences. Analysis of the sequences shows the *Monilia polystroma* sequence to be the closest to *M. fructigena* with 93% similarity. *M. laxa* is 72%, similar to *M. fructigena*, whereas *M. fructicola* is the most distant with 41% similarity due to several insertions (Fig. 2). At the 3′ end of the sequence, all four species share a common ATG codon (Fig. 2, bases 583 to 585) that initiates a stretch of 11 amino acids that is identical for all sequences. Using the BLAST search program (National Centre for Biotechnology Information), there were no obvious similarities with any of the sequences available in GenBank other than those in this study.

PCR analysis of *Monilinia* and *Monilia* species. Multiplex PCR analysis of *Monilinia* and *Monilia* species was carried out using primers MO368-8R, MO368-10R, and LaxaR-2 in combination with primer MO368-5. Primers MO368-8R, MO368-10R, and LaxaR-2 were designed to bind specifically to the highly variable 5′ end region of the sequence for each *Monilinia* species. Primer MO368-5 corresponds to base pairs 584 to 603 at the 3′ end of the RAPD 368 fragment, located

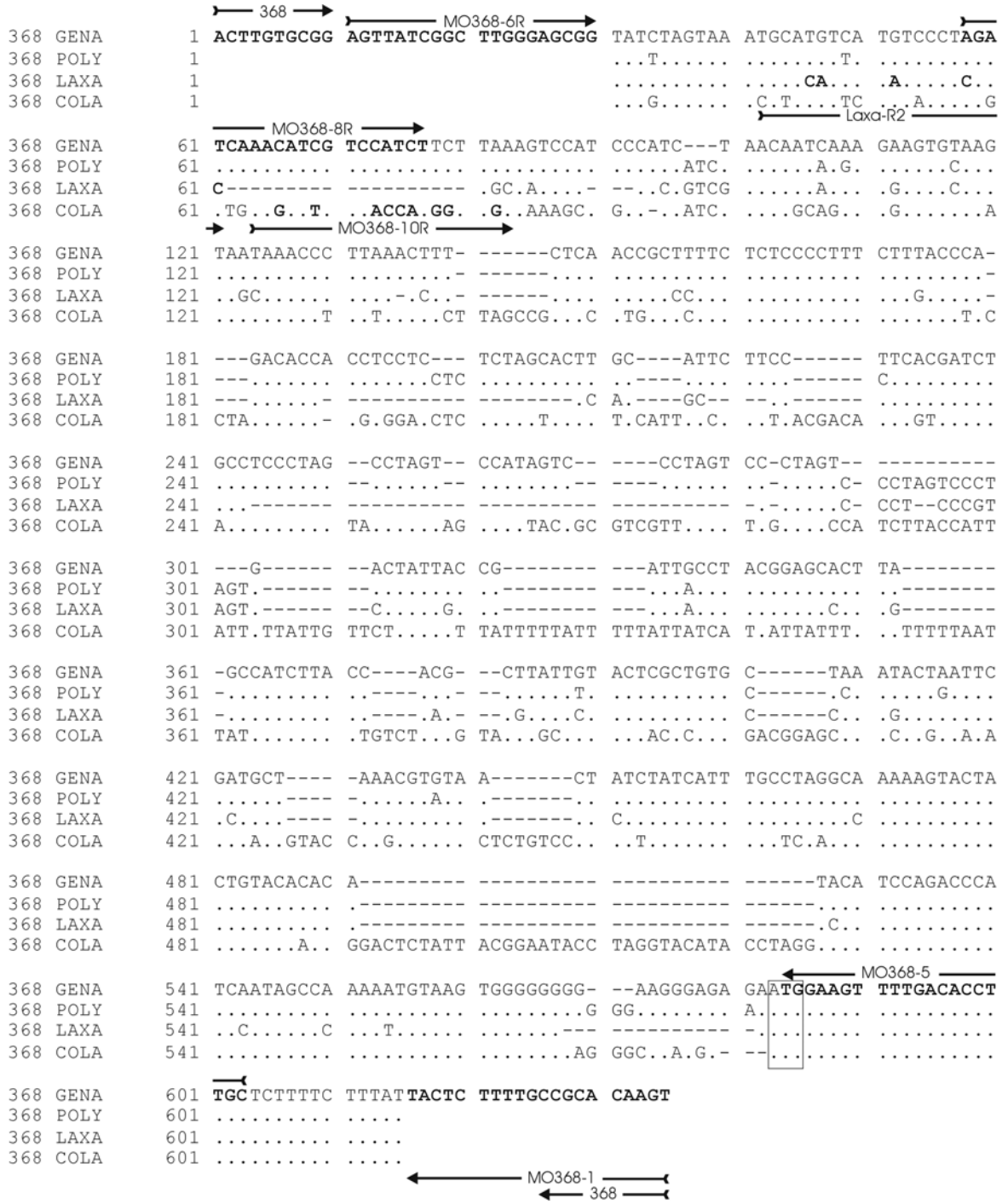

Fig. 2. Sequence alignment of the genome area corresponding to the *Monilinia fructigena* 491-bp random amplified polymorphic DNA (RAPD) product for the three *Monilinia* spp. and *Monilia polystroma*. Identical nucleotides are represented by dots, and absent nucleotides are shown by hyphens. Spaces at beginning and end of alignment are undetermined sequences. Primers used for polymerase chain reaction (PCR) assays and sequence analysis in this study are indicated in the sequence by bold letters and arrows indicating direction of amplication reaction. Putative translation initiation codon is boxed.

within the portion of the alignment that is identical for all species (Fig. 2). Amplification using a mixture of all four primers produced a 402-bp PCR product for *M. fructigena*, a 535-bp product for *M. fructicola*, a 351-bp product for *M. laxa,* and a 425-bp PCR product for *Monilia polystroma* (Fig. 3). All *Monilinia* and *Monilia* isolates listed in Table 1 were tested with MO368-8R, MO368-10R, LaxaR-2, and MO368-5. *Botrytis cinerea* and *Sclerotinia sclerotiorum* were included in the study due to their close relationship with the three *Monilinia* species of this study (8). The results of the species-specific multiplex PCR are shown in Table 1. There were no PCR products for *B. cinerea, S. sclerotiorum,* or the four other *Monilinia* spp. listed in Table 1. A DNA fragment of the expected size was amplified for all isolates of *M. fructigena*, *M. fructicola*, and *Monilia polystroma* tested. All but three isolates of *M. laxa* amplified a PCR product of the expected size. Isolates CBS 165.24, DAOM 209913, and UAMH 3523 amplified a PCR product of a size corresponding to the size of the product for *M. fructicola*. Fungal primers ITS1 and ITS2 (20), which amplify across the internal transcribed spacer 1, the 5.8SrRNA, and the internal transcribed spacer 2, were used on these specific isolates to amplify a PCR fragment that was isolated and sequenced (data not shown). The sequences obtained for isolates CBS 165.24, DAOM 209913, and UAMH 3523 were compared with sequences published for *M. fructicola, M. fructigena,* and *M. laxa* (8,10) and were found to be identical to the sequence for *M. fructicola*.

The *Monilinia* species DNA that did not yield a PCR fragment, as well as *B. cinerea* and *S. sclerotiorum* DNA, amplified a band of appropriate size with the ribosomal DNA primers NS5 and NS6 (20), demonstrating that DNA quality and quantity were not the reason for the absence of the specific PCR band (data not shown).

Detection of *Monilinia* and *Monilia* on inoculated apples. DNA was extracted from brown apple skin, brown flesh, brown apple skin and flesh together, and mycelia. Every sample taken from infected apples yielded the PCR fragment corresponding to the inoculated *Monilinia* or *Monilia* species. An example of the signal obtained for each species from DNA extracted from inoculated apple is shown in Figure 3.

Detection of *Monilinia* species from mixed inoculations. Apples were inoculated at two separate sites with one *Monilinia* species per site in a total of three experiments combining two of the three brown rot species of *Monilinia* per experiment. Rotting tissues surrounding each inoculated site located as far as possible from the other species were taken for DNA extraction. Rotting tissues located between the two inoculation sites were taken as well. Every tissue sample tested amplified the PCR fragment corresponding to the *Monilinia* species inoculated closest to the sampling site. Samples taken between two inoculation sites amplified PCR fragments corresponding to both inoculated *Monilinia* species. Results are shown in Figure 4.

***Monilinia* species identification from naturally infected stone fruits.** Rotting tissues from stone fruits showing brown rot symptoms were tested with the multiplex PCR assay. In cases of fruits demonstrating more than one localized bruised area, a sample was taken from each bruised area on the same fruit. The samples taken from naturally infected fruits amplified a PCR fragment corresponding to either *M. fructicola* or *M. laxa* confirmed by culture isolation. Results are shown in Table 2.

DISCUSSION

With increasing importation and exportation of various fresh fruits worldwide, border surveillance becomes more and more challenging. Especially with fresh commodities that may be detained, timely methods for the identification of potential quarantine pests are an important tool for inspection agencies to deliver the rapid service so essential to the import and export industries. Molecular biology based methods are progressively providing the means for timely identification of quarantine plant pests (13). These methods can potentially be designed to directly and specifically identify the species infecting a commodity. Molecular methods based on the PCR technique will give results more rapidly than identification following isolation of the potential quarantine pests. The multiplex PCR method described herein does not necessitate isolation of the fungal agent and therefore significantly accelerates the identification process compared with methods based on quantitative characteristics.

When the fungal agent infecting a commodity is suspected to belong to the *Monilinia* or *Monilia* genera, the multiplex PCR described herein can be used as a qualitative method to further identify the species among the four most likely to occur on stone fruits. In a single tube reaction, a PCR band specific to one of the species will lead to the identification of the *Monilinia* species and *Monilia polystroma*. Absence of amplification is not considered to be a final identification result, and the use of universal primers binding to a conserved area of the fungi ribosomal gene can be used to assess DNA quality or quantity.

The sequence information obtained from the RAPD fragment of *M. fructigena* corresponding to sequences obtained from the other *Monilinia* and *Monilia* species seems to correspond to the junction between the 5′ end noncoding and coding sequences. This would explain the sequence similarity at the 3′ ends, allowing the use of a common reverse primer, and sequence dissimilarity at the 5′ ends, facilitating the amplification of a species-specific fragment. The high variability of the sequence at the 5′ end of the marker also allows the specific amplification of fungal DNA on fruit without interference from plant DNA. The

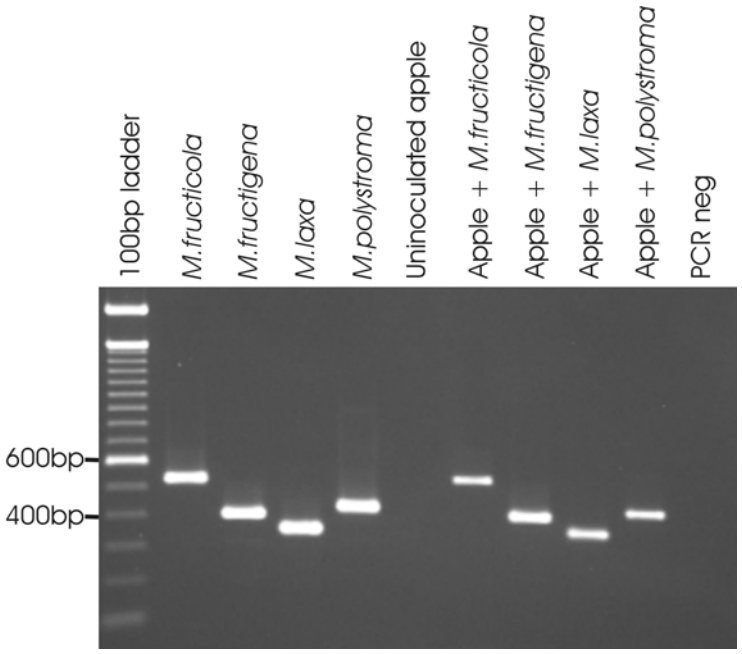

Fig. 3. Multiplex polymerase chain reaction (PCR) assay on selected isolates of the three *Monilinia* spp. and *Monilia polystroma*, from pure culture or from the rotting areas of inoculated apples. The isolate from pure culture used for *M. fructicola* is ATCC 42248, for *M. fructigena* ATCC 26106, for *M. laxa* ATCC 9961, and for *Monilia polystroma* CBS 102686. The isolate used to inoculate apples for *M. fructicola* is JN AN3-6, for *M. fructigena* ATCC 26106, for *M. laxa* ATCC 32671, and for *Monilia polystroma* CBS 102686. The last lane is a PCR negative control. Sizes (base pairs) of selected fragments of the 100-bp ladder are indicated on the left.

multiplex method has been used to identify isolates from *Monilinia*-infected fruits in Uruguay and was proven to be effective in identifying *M. fructicola* (12).

To verify the specificity of the multiplex method, several isolates originating from varied sources were tested. The three isolates identified as *M. laxa* that produced bands specific to *M. fructicola* were likely misidentified. One of the isolates, UAMH 3523, originated from the province of British Columbia, Canada, where *M. laxa* is known to occur. DAOM 209913 is suspected to originate from Uruguay, where *M. laxa* was believed to be the most likely species to occur until recently (12). As for isolate CBS 165.24, Fulton and Brown (6) also placed the isolate in the *M. fructicola* group based on the amplification of a DNA fragment indicative of the presence of an intron in the SSU rDNA.

Recently, *M. fructigena* isolates originating from Japan were assigned to a distinct species, *Monilia polystroma* (7,17). Three *Monilia polystroma* isolates were included in this study. Comparing the sequences of RAPD fragments from *M. fructigena* and *Monilia polystroma* confirmed that *Monilia polystroma* is indeed very closely related to *M. fructigena* but is sufficiently different to produce an amplicon of distinct size in the multiplex PCR method.

A procedure for the application of the multiplex method directly to infected fruit was established using inoculated apples. From these experiments, it was also shown that the multiplex PCR identified the two species present in mixed infection as long as tissue was collected from every infected area. This procedure is currently used for the identification of *Monilinia* species naturally infecting peaches, nectarines, and cherries submitted to our laboratory for testing. The *Monilinia* species identified so far on naturally infected fruits and presented in Table 2 are the species known to occur in Europe and Canada.

The multiplex PCR method described in this work has been demonstrated to be a relatively simple and rapid technique for identifying *Monilinia* and *Monilia* species known to infect stone fruits. The method can be universally used, because it identifies quarantined *Monilinia* and *Monilia* species occurring worldwide as well as *M. laxa*. Furthermore, the multiplex PCR method provides the basis for the future development of a quantitative and more sensitive PCR method using real-time PCR technology.

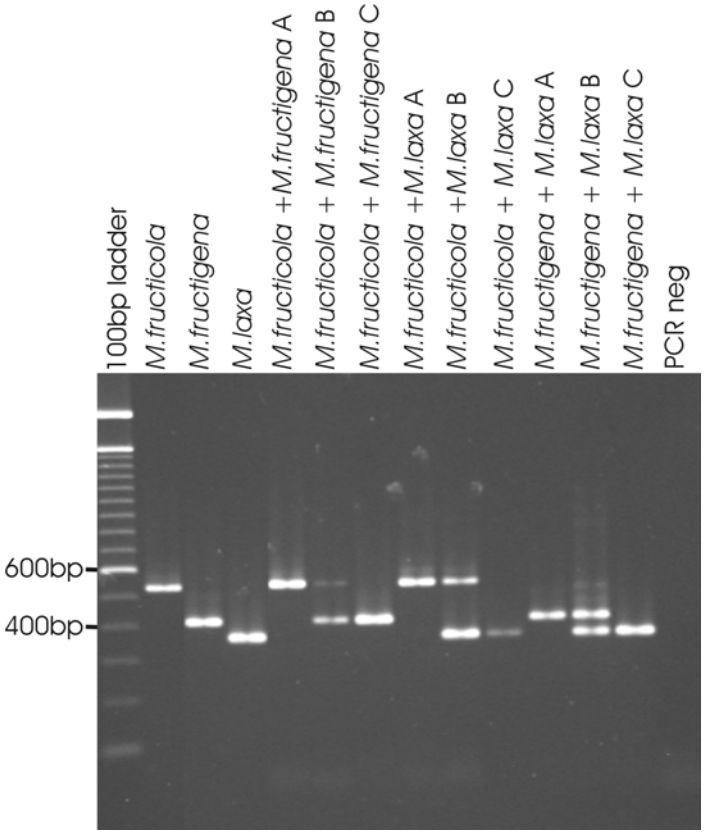

Fig. 4. Multiplex polymerase chain reaction (PCR) assay on selected isolates of the three *Monilinia* species from rotting areas of three inoculated apples. Apples were inoculated with *M. fructicola* JN AN3-6, *M. fructigena* ATCC 26106, and *M. laxa* ATCC 32671. Single inoculations for each species are shown in the first three lanes. The following nine lanes show mixed inoculations. The letters A, B, and C represent different samples taken from the same inoculated apple. A and C are samples taken from each side of the mixed inoculations and B are samples taken between the two inoculations. The last lane is a PCR negative control. Sizes (base pairs) of selected fragments of the 100-bp ladder are indicated on the left.

Table 2. *Monilinia* species identification directly from naturally infected fruits using multiplex polymerase chain reaction (PCR)

Sample no.	Infected fruit	Origin	*Monilinia* species[a]
M98-128	Cherry	British Columbia, Canada	*M. laxa*
M98-129	Cherry	British Columbia, Canada	*M. fructicola*
M98-130	Cherry	British Columbia, Canada	*M. fructicola*
CPQP1	Peach	Ontario, Canada	*M. fructicola*
CPQP3	Nectarine	Canada	*M. fructicola*
MB00-06	Cherry	Romania	*M. laxa*
MB00-07	Cherry	Bulgaria	*M. laxa*
MB00-12	Peach	British Columbia, Canada	*M. laxa*
MB00-13	Cherry	British Columbia, Canada	*M. laxa*
MB00-14	Cherry	British Columbia, Canada	*M. laxa*

[a] *Monilinia* species determined from DNA fragment size amplified using the multiplex PCR.

ACKNOWLEDGMENTS

We thank G. P. White and S. J. Miller for providing morphological identification and technical help. A special thanks to J. Northover in guiding us in the apple inoculation. We also thank L. M. Kohn, P. Mondino, and J. Northover for supplying isolates. We are grateful to C. Dollard, S. Hambleton, and S. Nadin-Davis for reviewing the manuscript. We also thank A. Saparno for technical help.

LITERATURE CITED

1. Batra, L. R. 1991. World species of *Monilinia* (fungi): Their ecology, biosystematics and control. Mycologia Mem. 16:1-246.
2. Boehm, E. W. A., Ma, Z., and Michailides, T. J. 2001. Species-specific detection of *Monilinia fructicola* from California stone fruits and flowers. Phytopathology 91:428-439.
3. Byrde, R. J. W., and Willetts, H. J. 1977. The brown rot fungi of fruit. Their biology and control. Pergamon Press, Oxford.
4. Côté, M.-J., Prud'homme, M., Meldrum, A. J., and Tardif, M.-C. 2004. Variations in sequence and occurrence of SSU rDNA group I introns in *Monilinia fructicola* isolates. Mycologia 96(2):240-248.
5. Förster, H., and Adaskaveg, J. E. 2000. Early brown rot infections in sweet cherry fruit are detected by *Monilinia*-specific DNA primers. Phytopathology 90:171-178.
6. Fulton, C. F., and Brown, A. E. 1997. Use of SSU rDNA group-I intron to distinguish *Monilinia fructicola* from *M. laxa* and *M. fructigena*. FEMS Microbiol. Lett. 157:307-312.
7. Fulton, C. E., van Leeuwen, G. C. M., and Brown, A. E. 1999. Genetic variation among and within *Monilinia* species causing brown rot of stone and pome fruits. Eur. J. Plant Pathol. 105:495-500.
8. Holst-Jensen, A., Kohn, L. M., Jakobsen, K. S., and Schumacher, T. 1997. Molecular phylogeny and evolution of *Monilinia* (Sclerotiniaceae) based on coding and noncoding rDNA

sequences. Am. J. Bot. 84:686-701.
9. Hughes, K. J. D., Fulton, C. E., McReynold, D., and Lane, C. R. 2000. Development of new PCR primers for identification of *Monilinia* species. Page 54 in: EPPO conference on diagnostic techniques for plant pests. Wageningen, NL, 2000-02-01/04.
10. Ioos, R., and Frey, P. 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.
11. Ma, Z., Luo, Y., and Michailides, T. J. 2003. Nested PCR assays for detection of *Monilinia fructicola* in stone fruit orchards and *Botryosphaeria dothidea* from pistachios in California. J. Phytopathol. 151:312-322.
12. Malvárez, G., Rodríguez, A., Aguilar, C., Silvera, E., y Mondino, P. 2001. Identificación de especies de *Monilinia* spp., en aislamientos obtenidos de *Prunus* spp. por PCR con *primers* específicos. Agrociencia V(1):48-53.
13. Martin, R. R., Delano, J., and Lévesque, C. A. 2000. Impacts of molecular diagnostic technologies on plant disease management. Annu. Rev. Phytopathol. 38:207-239.
14. Möller, E. M., Bahnweg, G., Sandermann, H., and Geiger, H. H. 1992. A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected plant tissues. Nucleic Acids Res. 20:6115-6116.
15. Penrose, L. J., Tarran, J., and Wong, A.-L. 1976. First record of *Sclerotinia laxa* Aderh. & Ruhl. New South Wales: Differentiation from *S. fructicola* (Wint.) Rehm. by cultural characteristics and electrophoresis. Aust. J. Agric. Res. 27:547-556.
16. Snyder, C. L., and Jones, A. L. 1999. Genetic variation between strains of *Monilinia fructicola* and *Monilinia laxa* isolated from cherries in Michigan. Can. J. Plant Pathol. 21:70-77.
17. van Leeuwen, G. C. M., Baayen, R. P., Holb, I. J., and Jeger, M. J. 2002. Distinction of the asiatic brown rot fungus *Monilia polystroma* sp. nov. from *M. fructigena*. Mycol. Res. 106:444-451.
18. van Leeuwen, G. C. M., and van Kesteren, H. A. 1998. Delineation of the three brown rot fungi of fruit crops (*Monilinia* spp.) on the basis of quantitative characteristics. Can. J. Bot. 76:2042-2050.
19. Welsh, J., and McClelland, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res. 18:7213-7218.
20. White, T. J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: PCR Protocols. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds. Academic Press, San Diego, CA.
21. Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A., and Tingey, S. V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18:6531-6535.