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Genomic variation within *Monilinia laxa*, *M. fructigena* and *M. fructicola*, and application to species identification by PCR

Renaud Ioos[1] and Pascal Frey[2]
[1]*LNPV, Unité de Mycologie Agricole et Forestière, 38 rue Sainte Catherine, 54043 Nancy, France (Phone: +33 383 304 151; Fax: +33 383 320 045; E-mail: renaud.ioos@agriculture.gouv.fr);* [2]*INRA, Unité Ecosystèmes Forestiers, Equipe de Pathologie Forestière, 54280 Champenoux, France*

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Abstract

Brown rot and twig canker of fruit trees are caused by *Monilinia laxa*, *M. fructigena* and *M. fructicola*. The Internal Transcribed Spacer (ITS) between the 18S and the 28S rRNA genes of four *M. laxa* and four *M. fructigena* isolates collected in France was amplified by Polymerase Chain Reaction (PCR) using universal primers and sequenced. Multiple alignment of the ITS sequences and comparison with published sequences revealed very little intraspecific variation and a low interspecific polymorphism clustered in two regions. Species-specific PCR primers were designed to amplify a 356 bp fragment for each of the three species. The specificity of the three primer pairs was successfully tested with a collection of 17 *M. laxa*, 18 *M. fructigena* and 6 *M. fructicola* isolates collected from different hosts and different countries, unequivocally confirming the identification of each isolate based on morphological and cultural traits. Using stringent PCR conditions, no cross-reaction was observed with any of the isolates tested. The specificity of the PCR assays was also successfully confirmed with DNA extracted from different fungal species, either phylogenetically close to the genus *Monilinia* or commonly found on diseased fruits. Using this new reliable technique, doubtful isolates can be directly identified in a single PCR run. Moreover, detection and identification of the *Monilinia* species were successfully achieved directly on diseased fruits. This simple and rapid method can be particularly useful to detect *M. fructicola* which is a listed quarantine fungus in all European countries.

Introduction

Brown rot and twig canker of *Prunus* spp., *Malus pumila* and *Pyrus communis* are currently caused by three species of the genus *Monilinia*: *M. laxa* Aderhold & Ruhland, *M. fructigena* Honey and *M. fructicola* (Wint.) Honey. These pathogens cause significant losses on stone and pome fruits, both before and after harvest.

M. fructicola is listed as a quarantine pathogen in the European Union (Directive du conseil 77/93/CEE, 1976; OEPP, 1996) whereas *M. fructigena* is recorded as a quarantine pathogen in the USA (Code of Federal Regulation, 1996) and in Australia (Commonwealth Department of Health, 1984). The reliable identification of these pathogens is therefore important for these countries. Furthermore, *M. fructicola* is subjected to a particular phytosanitary survey as resistance to benzimidazole fungicides has been described in orchards in North America and in Australia (Penrose, 1990).

Monilinia laxa, *M. fructigena* and *M. fructicola* have been differentiated for many years on morphological and cultural traits (Hewitt and Leach, 1939; Willetts, 1969; Sonoda et al., 1982). However, some morphological criteria are overlapping and these classical diagnostic methods are not reliable enough to be used in routine (Penrose et al., 1976). Therefore,

alternative methods have been developed, such as isozyme analysis (Penrose et al., 1976), and the effect of long-wave UV light on colony growth (De Cal and Melgarejo, 1999). PCR-based diagnostic assays have been successfully developed for several phytopathogenic fungi (Nazar et al., 1991; Liew et al., 1998; Ristaino et al., 1998).

Recently, a molecular identification strategy was applied to distinguish *M. fructicola* from the two other species (Fulton and Brown, 1997) and *M. laxa* from *M. fructicola* (Snyder and Jones, 1999). Fulton and Brown (1997) proposed an assay based on the presence of a group-I intron within the 18S rRNA gene of *M. fructicola*. This method permitted the rapid and sensitive detection of *M. fructicola* from infected fruits, but could not distinguish *M. laxa* from *M. fructigena*. This method was clearly intended to detect the EU quarantine listed pathogen *M. fructicola* from infected fruits.

Snyder and Jones (1999) proposed a diagnostic method based on RFLP of PCR-amplified ITS1, digested with the endonuclease *Mse* I, which allowed differentiation between *M. laxa* and *M. fructicola*. This method should also differentiate *M. fructigena* from the two other species, according to the published ITS1 sequences. Snyder and Jones (1999) also assessed a microsatellite-primed PCR assay with primers $(GACA)_4$ and $(GTG)_5$ in order to differentiate *M. laxa* from *M. fructicola*. The DNA fingerprints obtained showed that there was little intraspecific variation with both primers, but the banding patterns clearly distinguished *M. laxa* from *M. fructicola*. Nevertheless, microsatellite-primed PCR (or any other arbitrary-primed PCR method) would be of no use for direct species diagnostic from infected fruits, since plant DNA may be simultaneously amplified.

The objective of our work was to study the genetic variability within the ITS region of the three species in order to design species-specific PCR primer pairs for each of the three species.

Materials and methods

The ITS region of four French isolates of *M. fructigena* and four french isolates of *M. laxa* were sequenced and compared by multiple alignment with ITS sequences retrieved from Genbank: *M. laxa* (6 strains), *M. fructigena* (4 strains), *M. fructicola* (6 strains) (Figure 1). Total DNA from axenic fungal culture was extracted with the hexadecyltrimethylammonium

	GenBank Accession No	86	96	106		416	426	436	Reference*
M. fructicola	Z73777	...TGTATGCTCG	CCAGAGGATA	ATTAAACTCT...	.....	...GTTCTCAGTG	TGCTTCTGCC	AAAACCCAAA...	(1)
M. fructicola	Z73778	...TGTATGCTCG	CCAGAGGATA	ATTAAACTCT...	.....	...GTTCTCAGTG	TGCTTCTGCC	AAAACCCAAA...	(1)
M. fructicola	U21815	...TGTATGCTCG	CCAGAGGATA	ATTAAACTCT...					(2)
M. fructicola	AF010500	...TGTATGCTCG	CCAGAGGATA	ATTAAACTCT...					(3)
M. fructicola	AF010501	...TGTATGCTCG	CCAGAGGATA	ATTAAACTCT...					(3)
M. fructicola	AF010502	...TGTATGCTCG	CCAGAGGATA	ATTAAACTCT...					(3)
M. laxa	Z73784	...TGTATGCTCG	CCAGAG*A*ATA	ATC*A*AACTCT...	.....	...GTTCTC*G*GTG	TGCTTCTGCC	AAAACCCAAA...	(1)
M. laxa	Z73785	...TGTATGCTCG	CCAGAG*A*ATA	ATC*A*AACTCT...	.....	...GTTCTC*G*GTG	TGCTTCTGCC	AAAACCCAAA...	(1)
M. laxa	Z73786	...TGTATGCTCG	CCAGAG*A*ATA	ATC*A*AACTCT...	.....	...GTTCTC*G*GTG	TGCTTCTGCC	AAAACCCAAA...	(1)
M. laxa	Z73787	...TGTATGCTCG	CCAGAG*A*ATA	ATC*A*AACTCT...	.....	...GTTCTC*G*GTG	TGCTTCTGCC	AAAACCCAAA...	(1)
M. laxa	AF150673	...TGTATGCTCG	CCAGAG*A*ATA	ATC*A*AACTCT...	.....	...GTTCTC*G*GTG	TGCTTCTGCC	AAAACCCAAA...	This study
M. laxa	AF150674	...TGTATGCTCG	CCAGAG*A*ATA	ATC*A*AACTCT...	.....	...GTTCTC*G*GTG	TGCTTCTGCC	AAAACCCAAA...	This study
M. laxa	AF150675	...TGTATGCTCG	CCAGAG*A*ATA	ATC*A*AACTCT...	.....	...GTTCTC*G*GTG	TGCTTCTGCC	AAAACCCAAA...	This study
M. laxa	AF150676	...TGTATGCTCG	CCAGAG*A*ATA	ATC*A*AACTCT...	.....	...GTTCTC*G*GTG	TGCTTCTGCC	AAAACCCAAA...	This study
M. laxa	AF010503	...TGTATGCTCG	CCAGAG*A*ATA	ATC*A*AACTCT...					(3)
M. laxa	AF010504	...TGTATGCTCG	CCAGGG*A*ATA	ATC*A*AACTCT...					(3)
M. fructigena	Z73779	...TG*CA*CGCTCG	CCAGAG*A*ATA	A*CC*AAACTCT...	.....	...GTTCTCAGTG	TGCTTCTG*G*C	AAAAC*A*C*C*AA...	(1)
M. fructigena	Z73780	...TG*CA*CGCTCG	CCAGAG*A*ATA	A*CC*AAACTCT...	.....	...GTTCTCAGTG	TGCTTCTG*G*C	AAAAC*A*C*C*AA...	(1)
M. fructigena	Z73781	...TG*CA*CGCTCG	CCAGAG*A*ATA	A*CC*AAACTCT...	.....	...GTTCTCAGTG	TGCTTCTG*G*C	AAAAC*A*C*C*AA...	(1)
M. fructigena	AF150677	...TG*CA*CGCTCG	CCAGAG*A*ATA	A*CC*AAACTCT...	.....	...GTTCTCAGTG	TGCTTCTG*G*C	AAAAC*A*C*C*AA...	This study
M. fructigena	AF150678	...TG*CA*CGCTCG	CCAGAG*A*ATA	A*CC*AAACTCT...	.....	...GTTCTCAGTG	TGCTTCTG*G*C	AAAAC*A*C*C*AA...	This study
M. fructigena	AF150679	...TG*CA*CGCTCG	CCAGAG*A*ATA	A*CC*AAACTCT...	.....	...GTTCTCAGTG	TGCTTCTG*G*C	AAAAC*A*C*C*AA...	This study
M. fructigena	AF150680	...TG*CA*CGCTCG	CCAGAG*A*ATA	A*CC*AAACTCT...	.....	...GTTCTCAGTG	TGCTTCTG*G*C	AAAAC*A*C*C*AA...	This study
M. fructigena	U21825	...TG*CA*CGCTCG	CCAGAG*A*ATA	A*CC*AAACTCT...					(2)

Primer sequence (5' to 3') for :

M. fructicola	**ITS1Mfcl**	**TAT GCT CGC CAG AGG ATA ATT**	**ITS4Mfcl**	**TGG GTT TTG GCA GAA GCA CAC T**
M. laxa	**ITS1Mlx**	**TAT GCT CGC CAG AGA ATA ATC**	**ITS4Mlx**	**TGG GTT TTG GCA GAA GCA CAC C**
M. fructigena	**ITS1Mfgn**	**CAC GCT CGC CAG AGA ATA ACC**	**ITS4Mfgn**	**GGT GTT TTG CCA GAA GCA CAC T**

Figure 1. Sequence alignment of the ITS1 and ITS2 regions showing species-specific bases substitutions (in bold italic). The regions chosen for the design of the species-specific primers are underlined. The respective forward and reverse species-specific primers are indicated in footnote. *References: (1): Holst-Jensen et al., 1997; (2): Carbone and Kohn, 1993; (3): Snyder and Jones, 1999.

bromide (CTAB)/proteinase K method (Henrion et al., 1994). Total DNA was diluted 20-fold in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8) and used as template for PCR amplification. The full-length ITS region (ITS1, ITS2 and the 5.8S rDNA) was amplified from total genomic DNA with the universal primers ITS1 and ITS4 (White et al., 1990). Amplifications were performed in 50 µl reactions containing Taq polymerase buffer (20 mM Tris–HCl pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 1.25 U Taq polymerase (Life Technologies, Cergy Pontoise, France), 200 µM of each dNTPs, 0.2 µM of each ITS1 and ITS4 primers, and 5 µl of template DNA. PCR amplifications were performed in a Hybaid thermal cycler model Omn-E with an initial denaturation (94 °C, 3 min) followed by 30 cycles of denaturation (94 °C, 30 s), annealing (55 °C, 30 s), and extension (72 °C, 1.5 min), with a final extension (72 °C, 10 min). PCR products were separated by electrophoresis on 1% agarose gel in 0.5 × TBE buffer. Gels were stained with ethidium bromide and photographed under UV. The amplified fragments (538 bp) were precipitated with two volumes ethanol and 0.25 volume 10 M ammonium acetate for 15 min at −20 °C, pelleted, rinsed twice with 70% ethanol, air dried, and resuspended in 50 µl of ultrapure sterile water.

The purified amplified fragments were sequenced for both strands by the dideoxy-chain termination method. The sequencing reactions were performed using the Big Dye Terminator Sequencing kit (Applied Biosystems, Foster City, CA, USA), *Taq* FS polymerase, and ITS1/ITS4 primers. The sequencing reaction products were analysed using the ABI Genotyper 310 DNA sequencer (Perkin Elmer Applied Biosystems). Raw sequence data were edited using Sequencher (Gene Codes Corporation, Ann Arbor, MI). The eight ITS sequences obtained have been deposited in GenBank (see accession numbers in Table 1).

Multiple sequence alignment with hierarchical clustering was performed using MultAlin program version 5.3.3 (Corpet, 1988).

Results and discussion

Analysis of the ITS sequences revealed almost no intraspecific polymorphism within *M. laxa* and *M. fructigena*. The sequences obtained for the four French *M. laxa* isolates shared 100% identity, and were also identical to three out of four full-length ITS sequences retrieved from GenBank, corresponding to isolates from Norway and Italy (Holst-Jensen et al., 1997). Similarly, the sequences obtained for the four French *M. fructigena* isolates shared 100% identity, and were identical to the three full-length ITS sequences retrieved from GenBank, corresponding to isolates from Norway and Denmark (Holst-Jensen et al., 1997). The nearly null intraspecific polymorphism of ITS sequences was recently confirmed on a worldwide collection of 71 isolates of *Monilinia* spp. (Fulton et al., 1999). In spite of the low interspecific polymorphism between the three *Monilinia* species, four to thirteen base substitutions were detected as specific to one of the three species. Most of these base substitutions were clustered in two slightly more polymorphic regions, one located in the ITS1 (bases 88–108; Figure 1) and one located in the ITS2 (bases 422–443; Figure 1). These two polymorphic regions were chosen to design primers specific for each of the three species. The sequences of the forward and the reverse primers specific for *M. laxa*, *M. fructigena* and *M. fructicola*, are indicated in Figure 1. The three species-specific primers pairs were custom synthesized by Life Technologies (Cergy Pontoise, France).

The cycling profile chosen to amplify species-specific fragments from total genomic DNA of *M. laxa*, *M. fructigena* and *M. fructicola* was exactly the same as described above except for the annealing temperature that was raised to 62.5 °C. Indeed, due to the low number of differences between the three primer pairs, cross-reactions were observed at low annealing temperatures (55 and 60 °C). Raising the annealing temperature to 62.5 °C resulted in an absence of cross-reaction, while giving a good amplification yield (Figure 2). The PCR products generated using each of the three pairs of primers were, as expected, about 350 bp long.

The specificity of the three primer pairs was tested with a large collection of *Monilinia* species, consisting of 17 isolates of *M. laxa*, 16 isolates of *M. fructigena*, and 6 isolates of *M. fructicola* (Table 1). No cross-reaction was observed with any isolate, and every isolate yielded one amplification product with only one of the three primer pairs. Thus, the method developed allowed the rapid identification of the pathogen in a single PCR run, testing its total DNA with each of the three PCR primer pairs.

The three primer pairs were also tested with DNA extracted from *Botrytis cinerea* and *Sclerotinia sclerotiorum*, two fungi genetically close to the genus *Monilinia* (Carbone and Kohn, 1993; Holst-Jensen et al., 1997), and with DNA extracted from other

Table 1. Origin of the *Monilinia* isolates studied and result of the species-specific PCR assays

Species	Isolate	Host	Year of isolation	Source	Locality	Amplification with the primer pair specific for			GenBank accession number
						M. laxa	*M. fructigena*	*M. fructicola*	
M. laxa	LUC1A	*Prunus insititia*	1997	LNPV Nancy*	N-E France	+	−	−	AF 150673
M. laxa	COY1B5	*Prunus insititia*	1997	LNPV Nancy	N-E France	+	−	−	AF 150674
M. laxa	LUCO2B1	*Prunus insititia*	1997	LNPV Nancy	N-E France	+	−	−	AF 150675
M. laxa	BUL1A1	*Prunus insititia*	1997	LNPV Nancy	N-E France	+	−	−	AF 150676
M. laxa	PECH3	*Prunus persica*	1997	SRPV Lyon**	S-E France	+	−	−	
M. laxa	CERI4	*Prunus cerasus*	1996	SRPV Lyon	S-E France	+	−	−	
M. laxa	ABRI1	*Prunus armeniaca*	1995	SRPV Lyon	S-E France	+	−	−	
M. laxa	CERI8	*Prunus cerasus*	1996	SRPV Lyon	S-E France	+	−	−	
M. laxa	ABRI8	*Prunus armeniaca*	1995	SRPV Lyon	S-E France	+	−	−	
M. laxa	CERI5	*Prunus cerasus*	1996	SRPV Lyon	S-E France	+	−	−	
M. laxa	CERI6	*Prunus cerasus*	1996	SRPV Lyon	S-E France	+	−	−	
M. laxa	PRUN4	*Prunus insititia*	1996	LNPV Nancy	N-E France	+	−	−	
M. laxa	POIR1	*Prunus communis*	1995	SRPV Lyon	S-E France	+	−	−	
M. laxa	NECT1	*Prunus persica* var *nectarina*	1995	SRPV Lyon	S-E France	+	−	−	
M. laxa	CERI15	*Prunus cerasus*	1996	SRPV Lyon	S-E France	+	−	−	
M. laxa	CERI1	*Prunus cerasus*	1995	SRPV Lyon	S-E France	+	−	−	
M. laxa	CERI11	*Prunus cerasus*	1997	SRPV Lyon	S-E France	+	−	−	
M. fructigena	LAI1B	*Malus pumila*	1997	LNPV Nancy	N-E France	−	+	−	AF 150677
M. fructigena	COY2N	*Prunus persica*	1997	LNPV Nancy	N-E France	−	+	−	AF 150678
M. fructigena	VIC3B	*Prunus insititia*	1997	LNPV Nancy	N-E France	−	+	−	AF 150679
M. fructigena	COY2M	*Prunus insititia*	1997	LNPV Nancy	N-E France	−	+	−	AF 150680
M. fructigena	POM1	*Malus pumila*	1995	SRPV Lyon	S-E France	−	+	−	
M. fructigena	POM10	*Malus pumila*	1996	SRPV Lyon	S-E France	−	+	−	
M. fructigena	PRUN2	*Prunus domestica*	1996	SRPV Lyon	S-E France	−	+	−	
M. fructigena	POIR7	*Prunus communis*	1996	SRPV Lyon	S-E France	−	+	−	
M. fructigena	POIR4	*Pyrus communis*	1996	SRPV Lyon	S-E France	−	+	−	
M. fructigena	PRUN1	*Pryus domestica*	1996	SRPV Lyon	S-E France	−	+	−	
M. fructigena	POM9	*Malus pumila*	1996	SRPV Lyon	S-E France	−	+	−	
M. fructigena	POM2	*Malus pumila*	1995	SRPV Lyon	S-E France	−	+	−	
M. fructigena	POM7	*Malus pumila*	1996	SRPV Lyon	S-E France	−	+	−	
M. fructigena	POM1	*Malus pumila*	1995	SRPV Lyon	S-E France	−	+	−	
M. fructigena	ABRI2F	*Prunus armeniaca*	1995	SRPV Lyon	S-E France	−	+	−	
M. fructigena	ABRI3	*Prunus armeniaca*	1995	SRPV Lyon	S-E France	−	+	−	
M. fructicola	CC 953	*Prunus domestica*	1996	P.D. Wageningen***	USA	−	−	+	
M. fructicola	NZ2090	*Prunus domestica*	1990	P.D. Wageningen	New Zealand	−	−	+	
M. fructicola	NZ2394	*Prunus armeniaca*	1994	P.D. Wageningen	New Zealand	−	−	+	
M. fructicola	JAP 1829	*Prunus persica*	1992	P.D. Wageningen	Japan	−	−	+	
M. fructicola	DAR 27029	*Prunus persica*	1976	P.D. Wageningen	Australia	−	−	+	
M. fructicola	DAR 27036	*Prunus avium*	1976	P.D. Wageningen	Australia	−	−	+	

*: LNPV, Unité de Mycologie Agricole et Forestière, Nancy, France.
**: Service Régional de la Protection des Végétaux, Lyon, France.
***: Plantenziektenkundige Dienst (Plant protection service),Wageningen, The Netherlands.

pathogens commonly associated with brown rot on trees or fruits: *Penicillium* sp., *Phoma* sp., *Sclerotium rolfsii*, *Alternaria* sp., *Fusarium lateritium*, *Fusarium culmorum*, *Fusarium oxysporum*, *Gonatobotryum* sp., *Nectria ditissima*, *Diplodia mutila*, *Pythium* sp., and *Phytophthora cactorum*. No amplification was obtained with any of the fungal species tested.

In order to test the ability of the species-specific PCR to detect different species present in a mixture, genomic DNA from isolates of the three *Monilinia* species were mixed two by two in different proportions (1 : 3, 1 : 1 and 3 : 1). Whatever the two species and whatever the proportion in the DNA mixture, PCR with the two primer pairs corresponding to the two components in the mixture resulted in the expected amplification products, while PCR with the third primer pair yielded no amplification product.

Direct detection of the pathogens in naturally infected fruits (peach, pear, cherry) was also assessed. Approximatively 0.1 g of infected fruit tissue (either with or without *Monilinia* fructifications) was cut and transferred into a 1.5 ml microcentrifuge tube. Total fungal and plant DNAs were extracted following the protocol of Henrion et al. (1994), modified with an extra phenol–chloroform extraction. The number of PCR cycles was raised to 35. DNA from infected fruit tissue was simultaneously tested with the three species-specific primer pairs (Figure 3). Specific PCR detection was obtained with peach (*M. laxa*, 6 samples: PE1 to PE6), cherry (*M. laxa*, 2 samples CE1 and CE2), and pear (*M. fructigena*, 3 samples: PO1 to PO3). For all the naturally infected fruits that were tested, only one species of *Monilinia* was detected. No diseased fruit was simultaneously infected by two or three *Monilinia* species.

In conclusion, species-specific primers have been developed in order to amplify a DNA fragment from each of the three *Monilinia* species infecting fruit trees. With this method, unidentified samples were subjected to DNA extraction and to an unique PCR

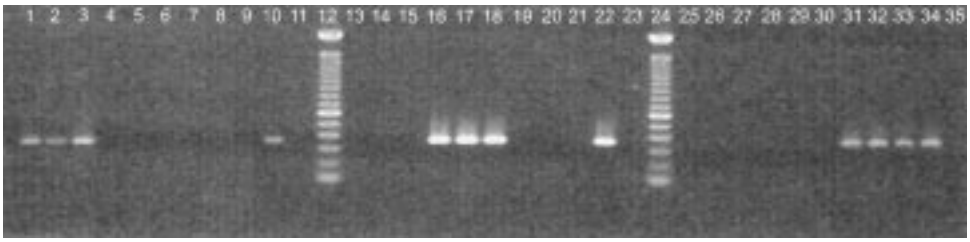

Figure 2. Amplification products generated with species-specific pairs of primers ITS1Mlx/ITS4Mlx (lanes 1–11), ITS1Mfgn/ITS4Mfgn (lanes 13–23) and ITS1Mfcl/ITS4Mfcl (lanes 25–35). Lanes 1, 13, 25: *M. laxa* isolate CERI4; lanes 2, 14, 26: *M. laxa* isolate ABRI1; lanes 3, 15, 27: *M. laxa* isolate PRUN4; lanes 4, 16, 28: *M. fructigena* isolate POM2; lanes 5, 17, 29: *M. fructigena* isolate POIR4; lanes 6, 18, 30: *M. fructigena* isolate PRUN1; lanes 7, 19, 31: *M. fructicola* isolate DAR207029; lanes 8, 20, 32: *M. fructicola* isolate CC953; lanes 9, 21, 33: *M. fructicola* isolate NZ2090; lane 10: positive control for *M. laxa* (isolate LUC1A); lane 22: positive control for *M. fructigena* (isolate LAI1B); lane 34: positive control for *M. fructicola* (isolate CC953); lanes 11, 23, 35: negative control (water); lanes 12, 24: 100 bp DNA ladder.

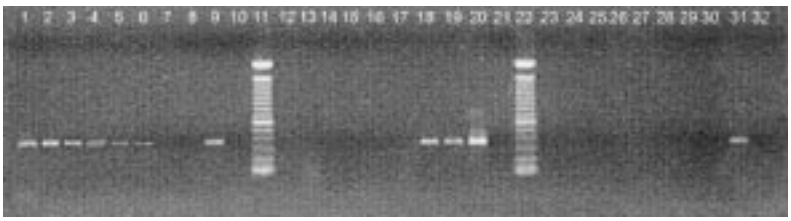

Figure 3. Amplification of DNA from fruit samples using species-specific pairs of primers ITS1Mlx/ITS4Mlx (lanes 1–10), ITS1Mfgn/ITS4Mfgn (lanes 12–21) and ITS1Mfcl/ITS4Mfcl (lanes 23–32). Lanes 1, 12, 23: PE1; lanes 2, 13, 24: PE2; lanes 3, 14, 25: PE3; lanes 4, 15, 26: PE4; lanes 5, 16, 27: CE1; lanes 6, 17, 28: CE2; lanes 7, 18, 29: PO1; lanes 8, 19, 30: PO2; lane 9: positive control for *M. laxa* (isolate LUC1A); lane 20: positive control for *M. fructigena* (isolate LAI1B); lane 31: positive control for *M. fructicola* (Isolate CC953); lanes 10, 21, 32: negative control (healthy fruit DNA); lanes 11, 22: 100 bp DNA ladder.

assay, performed with each of the three species-specific primers pairs, at the same annealing temperature. The 39 *Monilinia* isolates were easily and unequivocally identified, and the identifications confirmed those obtained with microbiological features. The specificity of the primers used with stringent conditions (annealing temperature: 62.5 °C) was high enough to prevent closely related fungi from being amplified. This PCR-based method may be particularly useful both for routine detection and identification of *M. laxa*, *M. fructigena* and *M. fructicola* from cultures and from infected fruits. Direct detection *in planta* is of particular interest because it avoids the prior isolation of the pathogen before analysis, which is a time-consuming step. Furthermore, it should allow the detection of several *Monilinia* spp. present simultaneously on infected fruits. This reliable and rapid method could be of particular interest for quarantine fungi surveys, as for *M. fructicola* in the European Countries and for *M. fructigena* in Australia.

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