Development of new PCR primers for identification of *Monilinia* species*

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New polymerase chain reaction (PCR) primers were developed for the identification of the EU quarantine pest *Monilinia fructicola*. These allowed nine *M. fructicola* isolates to be distinguished from other fungi, including six isolates of *M. laxa* and six isolates of *M. fructigena* (which also cause brown rot of stone and pome fruit). Three *M. fructicola* isolates from Japan and one from Australia did not react with a primer set previously published for *M. fructicola*. *M. fructicola* isolates could be subdivided into three groups based on the size of their nuclear rDNA small subunit. The subunits from the four non-reactive isolates were either smaller or larger than the reactive group. Further primers were developed which were specific for either *M. laxa* or *M. fructigena*. Another new primer identified both *M. laxa* and *M. fructigena*, and yet another *M. laxa* and *M. fructicola*. When used in combination, these primers specific for two species correctly identified unknown isolates of all three *Monilinia* species. The new primers designed in this study have been used to identify, rapidly and correctly, pustules taken directly from infected plum fruits, thus demonstrating their diagnostic potential.

Introduction

Monilinia fructicola, *Monilinia laxa* and *Monilinia fructigena* cause fungal brown rot disease of *Prunus*, *Malus* and *Pyrus* spp. Identification is difficult as these fungi are morphologically similar. However, accurate and rapid identification is necessary to prevent the introduction of *M. fructicola* which is not present in Europe (EPPO A1 quarantine pest) while the other two species are indigenous to Europe. Several methods exist for identification of *M. fructicola* (Fulton & Brown, 1997; Corazza *et al.*, 1998; Hughes *et al.*, 1988; Snyder & Jones, 1999). Despite being both rapid and sensitive, the PCR method developed by Fulton & Brown (1997) has recently been shown not to detect some *M. fructicola* isolates from Japan. These isolates do not contain a 418-bp group-I intron in their nuclear rDNA small subunit (Fulton *et al.*, 1999), which Fulton and Brown's primer combination NS 5 and *Mfs-3* acts upon.

Although direct trade in stone and pome fruits between Japan and Europe is not extensive, *M. fructicola* isolates lacking the 418-bp group-I intron could be imported via a third country or be present in other countries. It was therefore imperative to develop PCR methods to detect all known isolates of *M. fructicola*. New primers based on subtle DNA sequence differences in the internal transcribed spacer (ITS) 1 and 2 regions of the nuclear rRNA gene repeat were therefore produced (Table 1). The effectiveness of these primers and the occurrence of group-I introns in isolates of *M. fructicola* is discussed.

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Method

Growth of fungal cultures and extraction of DNA [adapted from Lodhi *et al.* (1994)]

Twenty-one *Monilinia* isolates representing all three brown-rot species were previously identified using a cultural protocol (Corazza *et al.*, 1998). These isolates and four non-*Monilinia* isolates (Table 2) were grown on 4% potato dextrose agar for 10 days at 22 °C, 12 h light (near UV)/12 h dark. Following incubation, the isolates were scraped from the surface of the inoculated plates using sterile microscope slides into 2-mL microcentrifuge tubes. Each tube was filled a quarter full, then a similar volume of 0.5 mm glass beads (Biospec Products) was added along with 100 µL aliquots of 1% Na_2SO_3, 1% polyvinylpolyrrolidone, 4% bovine serum albumin (BSA), and 1 mL extraction buffer [20 mM sodium EDTA, 100 mM Tris-HCl adjusted to pH 8.0 with HCl, 1.4 M NaCl and 2% CTAB (cetyltrimethyl ammonium bromide)]. Each tube was sealed with a screw-cap lid containing an o-ring and these were shaken in a MiniBeadbeater-8 homogenizer (Biospec Products) for 5 min on quarter power to macerate each sample. The macerated samples were then incubated at

Table 1 Primers used in this study

Primer name	Primer sequence 5′ to 3′	Reference
Mfc-F1	TAT GCT CGC CAG AGG ATA ATT A	Unpublished
Mfc-R1	GAT TTT AGA GCC TGC CAT TA	Unpublished
Ml-Mfg-F2	GCT CGC CAG AGA ATA ATC	Unpublished
Ml-Mfc-R1	GAT TTT AGA GCC TGC CAT TG	Unpublished
Mfg-R2	GGT CAA CCA TAG AAA ATT GGT	Unpublished
Mfs-3	CAC TCG AAA GCA TTG AGT TG	Fulton & Brown (1997)
NS 1	GTA GTC ATA TGC TTG TCT C	White *et al.* (1990)
NS 5	AAC TTA AAG GAA TTG ACG GAA G	White *et al.* (1990)
NS 8	TCC GCA GGT TCA CCT ACG GA	White *et al.* (1990)
ITS 1	TCC GTA GGT GAA CCT GCG G	White *et al.* (1990)
ITS 4	TCC TCC GCT TAT TGA TAT GC	White *et al.* (1990)

65 °C in a heating block (Grant Instruments,) for 20 min before being cooled on ice for 2 min. Each sample was then centrifuged at about 11 000 *g* for 5 min before 500 μL of the upper (aqueous) phase was pipetted off into a fresh 2-mL microcentrifuge tube. This sample was purified by combining it with 400 μL of a 24:1 mixture of chloroform/isoamyl alcohol. The tubes were sealed and the contents mixed 20 times by inversion, then centrifuged as before. The aqueous phases were again purified as described above. Then in new microcentrifuge tubes, 400 μL of each doubly purified aqueous phase was combined with 200 μL of 5 M NaCl and 600 μL isopropanol. The sealed tubes were inverted 20 times, then cooled at −20 °C for 15 min to precipitate DNA. The DNA was pelleted by centrifugation at about 11 000 *g* for 5 min and the supernatant discarded. Each pellet was resuspended in 400 μL of 70% ethanol, then the DNA re-pelleted and separated as described above. Finally the pellet was dried in a vacuum centrifuge (DNA Speed Vac), then resuspended in 100 μL TE buffer (10 mM Tris-HCl and 1 mM EDTA adjusted to pH 8.0). Aliquots (20 μL) from the resuspended pellet extracts were then diluted 10-fold using further TE buffer.

PCR amplification

Amplifications were performed in 200-μL tubes containing a reaction master mix consisting of 0.5 μM of each primer (Table 1), 200 μM dNTPs (Sigma), 2.5 μL 10× PCR buffer (GeneAmp, Perkin Elmer), 2.5 μL BSA (10 mg mL^{-1}), 1.25 units AmpliTaq (Perkin Elmer). Each master mix was made up to 24.5 μL with sterile distilled water. The master mixes were loaded with 0.5-μL aliquots from the 10-fold-diluted DNA extracts, apart from *M. fructigena* isolate cc 747 when the undiluted extract was used as the DNA yield from this isolate was low. The concentration of DNA used was < 2 ng μL^{-1}; TE buffer was used as a negative control. A Perkin Elmer 9700 Thermal Cycler was used with the cycling conditions as summarized in Table 2.

Isolates including *M. fructicola* (Aust 5 & 6) were also amplified as 100-μL volumes using the master mix described above with the universal primers ITS1 and ITS4 but no BSA. This reaction amplified the ITS1, 5.8 S and ITS2 regions for each isolate which were sequenced as described in Fulton & Brown (1997). Sequences were then compared with information stored on the EMBL/GenBank database. Five new primers were designed from the sequence information and used together or in combination with published primers (Table 1).

Results

Results are summarized in Table 2. Fulton & Brown's *M. fructicola*-specific primer set NS 5 and *Mfs*-3 produced a diagnostic amplicon (about 440 bp) for five *M. fructicola* isolates but no band for three from Japan and one from Australia. However, when the new *M. fructicola* primer set *Mfc*-F1 and *Mfc*-R1 was used, all nine *M. fructicola* isolates were correctly identified producing a diagnostic band of about 280 bp. This primer set did not cross-react with any of the other 16 isolates.

Primer set *Ml-Mfg*-F2 and *Ml-Mfc*-R1 only amplified a DNA band from *M. laxa* (about 280-bp), while set ITS1 and *Mfg*-R2 only amplified a DNA band from isolates of *M. fructigena* (about 460 bp). Other primer sets were less specific: primer set ITS1 and *Ml-Mfc*-R1 amplified a band (about 380-bp) only from the 15 isolates of *M. laxa* and *M. fructicola*, while primers *Ml-Mfg*-F2 and ITS4 amplified a band about 420 bp only from the 12 isolates of *M. laxa* and *M. fructigena*. All the samples except the TE negative control amplified a band (about 520 bp) using the universal primers ITS1 and ITS4 which showed that amplifiable DNA was extracted from all the fungal cultures. DNA was also amplified for all the fungal isolates using the universal primers NS1 and NS8 which amplified the nuclear rDNA small subunit. These primers produced three different-sized amplicons of about 1.7 kb, 2.1 kb or 2.4 kb with *M. fructicola* while the other isolates produced only products of about 1.7 kb.

Discussion

Fulton *et al.* (1999) noted that some Japanese *M. fructicola* isolates lack a group-I intron in their nuclear rDNA small subunit, so that DNA extracted from these isolates does not amplify using primer NS 5 and *Mfs*-3. In our study, we

Table 2 Provenance of isolates and PCR results

Sample	Reference	Country of origin	Amplification by primer set; positive (+) or negative (−) and the approximate molecular weight of the amplicon produced							
			MFs 3 & NS 5* 444 bp	*Mfc*-F1 & *Mfc*-R1† 280 bp	*Ml-Mfg*-F2 & *Ml-Mfc*-R1‡ 280 bp	ITSI & *Mfg*-R2‡ 460 bp	*Ml-Mfg*-F2 & ITS 4‡ 420 bp	ITS 1 & *Ml-Mfc*-R1‡ 380 bp	ITS 1 & ITS 4* 520 bp	NS-1 & NS-8§ (for bp see below)
M. fructicola	NZ 23. 94	New Zealand	+	+	−	−	−	+	+	B
M. fructicola	cc 865	USA	+	+	−	−	−	+	+	B
M. fructicola	cc 778	Australia	+	+	−	−	−	+	+	B
M. fructicola	cc 953	USA	+	+	−	−	−	+	+	B
M. fructicola	Jap 1829	Japan	−	+	−	−	−	+	+	C
M. fructicola	Jap 1438	Japan	−	+	−	−	−	+	+	A
M. fructicola	Jap 1535	Japan	−	+	−	−	−	+	+	A
M. fructicola	Aust 5	Australia	−	+	−	−	−	+	+	C
M. fructicola	Aust 6	Australia	+	+	−	−	−	+	+	B
M. laxa	cc 682	Italy	−	−	+	−	+	+	+	A
M. laxa	Es – 12	Spain	−	−	+	−	+	+	+	A
M. laxa	USA 55	USA	−	−	+	−	+	+	+	A
M. laxa	dar 41474	Australia	−	−	+	−	+	+	+	A
M. laxa	Jap 1390	Japan	−	−	+	−	+	+	+	A
M. laxa	Cult D	Australia	−	−	+	−	+	+	+	A
M. fructigena	Es – 48	Spain	−	−	−	+	+	−	+	A
M. fructigena	cc 747	UK	−	−	−	+	+	−	+	A
M. fructigena	Pt M2	Portugal	−	−	−	+	+	−	+	A
M. fructigena	Jap 2317	Japan	−	−	−	+	+	−	+	A
M. fructigena	cc 752	Poland	−	−	−	+	+	−	+	A
M. fructigena	pd 8. 96	Netherlands	−	−	−	+	+	−	+	A
Fusarium sp.	cc 27	UK	−	−	−	−	−	−	+	A
Colletotrichum sp.	cc 530	UK	−	−	−	−	−	−	+	A
Lambertella sp.	cc 677	USA	−	−	−	−	−	−	+	A
Alternaria sp.	cc 743	USA	−	−	−	−	−	−	+	A
TE buffer	None	None	−	−	−	−	−	−	−	A

*2 min at 94°C, then 30 cycles of 1 min at 94°C, 1 min at 53°C and 1.5 min at 72°C followed 10 min at 72°C.
†2 min at 94°C, then 27 cycles of 1 min at 94°C, 1 min at 59°C and 1.5 min at 72°C followed 10 min at 72°C.
‡2 min at 94°C, then 30 cycles of 1 min at 94°C, 1 min at 59°C and 1.5 min at 72°C followed 10 min at 72°C.
§2 min at 94°C, then 27 cycles of 1 min at 94°C, 1 min at 56°C and 1.5 min at 72°C followed 10 min at 72°C.
A,B,C Molecular weight of amplicons about 1.7 kb, 2.1 kb and 2.4 kb respectively.

showed that DNA from three *M. fructicola* isolates from Japan (Jap 1829, Jap 1438 and Jap 1535) and an Australian isolate (Aust 5) were not amplifiable using this primer set. By amplifying the whole nuclear rDNA small subunit from these and other *M. fructicola* isolates, we observed that *M. fructicola* could be split into three groups (A, B and C) based on the molecular weight of the amplicon. Group A, containing isolates Jap 1438 and Jap 1535, had a molecular weight similar to that of isolates of *M. laxa*, *M. fructigena* and the non-*Monilinia* isolates (about 1.7 kb). The size of this amplicon indicates a lack of the group-I intron in these isolates and this explains why they were not identified as *M. fructicola* using primers NS 5 and *Mfs*-3. Group B isolates, however, with amplicons of about 2.1 kb, were amplified using primers NS 5 and *Mfs*-3. These isolates therefore contained the group-I intron as described by Fulton & Brown (1997). Group C, containing isolates Jap, 1829 and Aust 5, had an amplicon of about 2.4 kb indicating an insert of about 700 bp in their small subunit.

Fulton *et al.* (1999) noted that isolate Jap 1829 contained a group-I intron, but this present study showed that the insert was approximately 260 bp greater in size than the previously reported group-I intron for other *M. fructicola* isolates (Fulton & Brown, 1997). Our study also revealed that a similar-sized insert was also present in Australian isolate Aust 5 but not in another two Australian isolates, indicating that at least two genetically different *M. fructicola* groups exist in Australia.

The discovery that primer set NS 5 and *Mfs*-3 did not amplify DNA from some Japanese isolates and one Australian isolate confirmed that a more accurate *M. fructicola* primer set was needed for diagnostic purposes. This was achieved by designing new PCR primers based on subtle differences in the ITS1 and ITS2 regions of the nuclear rRNA gene. Using the new primers (*Mfc*-F1 and *Mfc*-R1), it was possible to identify all *M. fructicola* isolates tested from a wide range of geographical locations. Other primers that specifically identified only isolates of *M. laxa* or *M. fructigena* were also developed. Less specific primer sets were also produced; these included primers ITS1 and *Ml-Mfc*-R1 which identified only isolates of *M. laxa* and *M. fructicola* and *Ml-Mfg*-F2 and ITS4 which identified only isolates of *M. laxa* and *M. fructigena*. This second set is very similar to one designed by Hilber *et al.* (1997) and could be useful for distinguishing *M. fructicola* from indigenous *Monilinia* species. It is envisaged that the two dual-species primer sets could be used in combination to identify unknown *Monilinia* brown-rot species.

Preliminary studies show that the newly designed primers produce similar results whether applied to pustules taken from artificially infected plum fruits or to *in vitro* cultures of the fungus. Thus, the newly developed primers besides accurately identifying *Monilinia* species *in vitro*, show great potential for rapid diagnosis of brown-rot infected fruits.

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Mise au point de nouvelles amorces de PCR pour l'identification des *Monilinia*

De nouvelles amorces de PCR ont été développées pour identifier l'organisme de quarantaine de l'UE *Monilinia fructicola*. Elles ont permis de distinguer neuf isolats de *M. fructicola* d'autres champignons, dont six isolats de *Monilinia laxa* et six isolats de *Monilinia fructigena* (qui sont également responsables d'une pourriture brune des arbres fruitiers à noyau et à pépins). Trois isolats de *M. fructicola* du Japon et un isolat d'Australie n'ont pas réagi avec une série d'amorces précédemment publiée pour *M. fructicola*. Les isolats de *M. fructicola* ont pu être séparés en trois groupes selon la taille de la petite sous-unité de leur ADNr. Les sous-unités des quatre isolats non réactifs étaient plus petites ou plus grandes que pour le groupe réactif. D'autres amorces spécifiques à *M. laxa* ou à *M. fructigena* ont également été développées. Une autre amorce nouvelle identifiait *M. laxa* et *M. fructigena*, et une autre *M. laxa* et *M. fructicola*. Utilisées en combinaison, ces amorces spécifiques à deux espèces identifiaient correctement les isolats inconnus des trois espèces de *Monilinia*. Les nouvelles amorces conçues dans cette étude ont été utilisées pour identifier, rapidement et correctement, des pustules prises directement sur des prunes infectées, démontrant ainsi leur potentiel pour le diagnostic.

Разработка новых праймеров PCR для идентификации видов *Monilinia*

Разработаны новые праймеры PCR для идентификации карантинного для ЕС вредного организма *Monilinia fructicola*. Они позволяют отличать девять изолятов *M. fructicola* от других грибов, в том числе от шести изолятов *Monilinia laxa* и шести изолятов *Monilinia fructigena* (который также вызывает бурую гниль косточковых и семечковых плодов). Три изолята *M. fructicola* из Японии и один из Австралии не прореагировали с комплектом праймера, ранее опубликованным для *M. fructicola*. Изоляты *M. fructicola* могут быть разделены на три группы по размеру малой субъединицы их рДНК. Субъединицы четырех нереагирующих изолятов были либо меньше, либо больше, чем реактивная группа. Разработаны также праймеры, специфичные для *M. laxa* и *M. fructigena*. Еще один новый праймер определял как *M. laxa*, так и *M. fructigena*, а другой определял и *M. laxa* и *M. fructicola*.

При использовании их в сочетании, эти праймеры, специфичные для двух видов, правильно определяли неизвестные изоляты всех трех видов *Monilinia*. Новые праймеры, разработка которых описана в данном исследовании, использовались для оперативного и правильного определения пустул, собранных непосредственно с зараженных плодов сливы, тем самым демонстрируя их диагностический потенциал.

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