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Use of SSU rDNA group-I intron to distinguish *Monilinia fructicola* from *M. laxa* and *M. fructigena*

Ciaran E. Fulton *, Averil E. Brown

Department of Applied Plant Science, The Queen's University of Belfast, Agriculture and Food Science Research Centre, Newforge Lane, Belfast BT9 5PX, UK

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Abstract

Monilinia fructicola, *M. laxa* and *M. fructigena* are the causal agents of brown rot of pome and stone fruits. *M. fructicola* is not present in Europe and is classed as a quarantine pathogen in EU countries. A 418-bp group-I intron has been located in the small subunit (SSU) rDNA gene of *M. fructicola* which is absent from *M. laxa* and *M. fructigena*. PCR primers specific to the 3′-region of the intron together with the SSU rDNA primer NS5 were able to amplify a 444-bp product from *M. fructicola* and fruit tissue infected with *M. fructicola* but not from the other two species. This allows for the rapid and sensitive detection of this pathogen in planta.

Keywords: *Monilinia fructicola*; *Monilinia laxa*; *Monilinia fructigena*; Brown rot; SSU rDNA group-I intron; Diagnostic

1. Introduction

Three species of *Monilinia* are currently recognised as causing brown rot in *Prunus* spp. (stone fruit) and *Malus* and *Pyrus* spp. (pome fruit). Of the three species, *M. laxa* and *M. fructigena* occur widely in EU countries, whilst *M. fructicola* occurs primarily in North and South America, South Africa and Australasia and is an EU quarantine listed organism. *M. fructicola* is particularly problematic in the USA where there are reports of fungicide resistance occurring in orchards [1] and it is believed to show more adaptability and variability possibly due to the more frequent occurrence of the sexual stage [2].

Currently, identification of *Monilinia* species relies on cultural and morphological characteristics; the procedures are slow and identification may be inconclusive, relying on often variable characters [3,4]. In this paper we describe the presence of a group-I intron, characterised by conserved core elements [5], in the SSU rDNA gene of *M. fructicola* and its exploitation as a diagnostic probe for the rapid detection of this pathogen.

2. Materials and methods

2.1. Fungal cultures

Fifty-six isolates of the three brown rot causing *Monilinia* species *M. fructicola, M. laxa* and *M. fructigena* were used in this study. Isolates were supplied

* Corresponding author. Tel.: +44 (1232) 255263; Fax: +44 (1232) 668375; E-mail: c.fulton@a1.qub.ac.uk

EMBL accession numbers: Y14210, Y14211.

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Table 1
Monilinia isolates used in this study

Isolate no.	Origin	Host	Date
Monilinia laxa			
USA 55	United States	*Prunus* sp.	?[a]
jap 1390	Japan	*P. mume*	1989[a]
es-12	Spain	*P. armeniaca*	1996[a]
dar 41474	Australia	*P. armeniaca*	1978[a]
pd1.96	The Netherlands	*Prunus* sp.	1996[a]
pd2.96	The Netherlands	*Prunus* sp.	1996[a]
pd17.96	The Netherlands	*Prunus* sp.	1996[a]
CBS 488.50	The Netherlands	*P. domestica*	1950[a]
cc 682	Italy	*P. persica*	1992[b]
cc 781	UK	*P. domestica*	1994[b]
cc 879	France	*P. persica* var. *nectarina*	1995[b]
cc 880	Cyprus	*P. persica* var. *nectarina*	1995[b]
cc 881	France	*P. persica*	1995[b]
cc 882	France	*P. persica*	1995[b]
cc 887	UK	*P. domestica*	1995[b]
cc 888	UK	*P. domestica*	1995[b]
cc 889	UK	*P. domestica*	1995[b]
cc 890	UK	*P. domestica*	1995[b]
cc 951	UK	*P. domestica*	1996[b]
cc 954	S. Africa	*P. persica*	1996[b]
M. fructigena			
cc 747	UK	*M. pumila*	1969[b]
cc 752	Poland	*P. domestica*	1993[b]
cc 783	UK	*Pyrus* sp.	1994[b]
jap 1145	Japan	*M. pumila*	1987[a]
jap 2317	Japan	*M. pumila*	1995[a]
pd3.96	The Netherlands	*M. pumila*	1996[a]
pd5.96	The Netherlands	*M. pumila*	1996[a]
pd8.96	The Netherlands	*P. persica*	1996[a]
pd16.96	The Netherlands	*P. communis*	1996[a]
utad B6	Portugal	*M. domestica*	1995[c]
utad L4-3	Portugal	*P. communis*	1995[c]
PtM2	Portugal	*Cydonia* sp.	?[a]
es-48	Spain	*P. domestica*	1996[a]
CF1	UK	*Malus* sp.	1996
Alistair	UK	*Malus* sp.	1996
fr-7	France	*M. pumila*	199?[a]
fr-9	France	*M. pumila*	199?[a]
ispave 920	Italy	?	1996[c]
PC 280	UK	*Malus* sp.	1996[d]
PC 315	UK	*Malus* sp.	1996[d]
M. fructicola			
cc 778	Australia	*Prunus* sp.	1971[b]
nz 2394	New Zealand	*Prunus* sp.	1994[a]
jap 1829	Japan	*P. persica*	1992[a]
cc 953	USA	*P. domestica*	1996[b]
cc 865	USA	*P. domestica*	1994[b]
cc 684	New Zealand	*P. domestica*	1992[b]
cc 748	New Zealand	*P. persica*	1968[b]

Table 1 (*continued*).
Monilinia isolates used in this study

Isolate no.	Origin	Host	Date
cc 866	USA	*P. domestica*	1994[b]
cc 867	USA	*P. domestica*	1994[b]
CBS 203.25	USA	*M. sylvestris*	1925[a]
CBS 165.24	?	*C. vulgaris*	1924[a]
dar 27029	Australia	*P. persica*	1976[a]
dar 27036	Australia	*P. avium*	1976[a]
nz 2090	New Zealand	*P. domestica*	1990[a]
nz 1490	New Zealand	*P. persica*	1990[a]
ispave 926	Portugal	*M. domestica*	1995[c]

[a]Cultures supplied by Plantenziektenkundige Dienst (Plant Protection Service), Wageningen, The Netherlands.
[b]Cultures supplied by Central Science Laboratory, York, UK.
[c]Cultures supplied by Istituto Sperimentale per la Patologia Vegetale, Rome, Italy.
[d]Cultures supplied by Horticulture Research International, East Malling, UK.

by the Central Science Laboratory (CSL), York, UK, Plant Protection Service (PPS), Wageningen, The Netherlands and Istituto Sperimentale per la Patologia Vegetale (ISPAVE), Rome, Italy and Horticulture Research International (HRI), East Malling, UK. All isolates were grown on potato dextrose agar (PDA) at 23°C (Table 1).

2.2. DNA extraction

All fungal isolates were grown over sterile discs of cellophane on PDA plates to facilitate the subsequent removal of the mycelium from the agar. DNA was extracted using a phenol/chloroform extraction method derived from that of Raeder and Broda [6] but with an additional phenol/chloroform extraction. The genomic DNA pellet was resuspended in sterile distilled water to give a final concentration of approximately 20–50 ng μl^{-1}. DNA quality was assessed by gel electrophoresis on 1% TAE agarose gels stained with ethidium bromide [7].

Host plant DNA was extracted from lyophilised tissue using DNAzol™ reagent (Gibco BRL) according to the manufacturer's instructions and the DNA concentrations were adjusted to approximately 50 ng μl^{-1}.

2.3. PCR amplification and sequencing of SSU rDNA

PCR amplification of the SSU rDNA (18S) from *M. fructicola, M. laxa* and *M. fructigena* was performed on a Perkin Elmer 480 Thermal Cycler using the following programme: 1 min at 94°C, 1 min 30 s at 53°C, 2 min at 72°C, for 30 cycles. The reaction mixtures consisted of 50 ng template DNA, 1.5 mM $MgCl_2$, 1 unit Red Hot Taq polymerase (Advanced Biotechnologies), 200 μM dNTPs (Sigma Chemical Co.), 1×PCR buffer IV (75 mM Tris-Cl, pH 9.0, 20 mM $(NH_4)_2SO_4$, 0.1% (w/v) Tween) and 0.6 μM of each primer, NS1 and NS8 [8]. DNA in aliquots of the individual PCR reactions were quantified by gel electrophoresis as described in Section 2.2.

Individual PCR products were purified using the Wizard™ DNA Clean Up kit (Promega) according to the manufacturer's instructions. PCR products were sequenced using double-stranded template (200 ng μl^{-1}) and initially 1 μM of the NS1 (forward) and NS8 (reverse) primers, following the protocol supplied with the Prism™ Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems). Primers NS3, NS5 and NS7 [8] were used in the sequencing of the PCR products in the forward direction, whilst primers NS2, NS4 and NS6 were used to sequence the PCR products in the reverse orientation. In addition, internal primers were designed from the resulting data to complete the sequencing in both directions, these were: *mfrg 3′* (CAT AGG CCT GCT TTG AA), *mfruct 3′* (TCT CTC GAC GTT ATG TCC), *mfructn3+* (ACG GAA AGG CAC CAC CAG).

Sequencing was conducted on an Applied Biosystems Model 373A DNA sequencer and the sequence

data were compiled and edited using Sequence Navigator™ software (Applied Biosystems).

2.4. Detection of *M. fructicola* in vitro and in planta

PCR primers *mfs-3* (CAC TCG AAA GCA TTG AGT TG), designed from the 3′-end of the *M. fructicola* intron sequence, and NS5 (AAC TTA AAG GAA TTG ACG GAA G) were used to specifically amplify a 444-bp PCR product corresponding to the entire 18S group-I intron plus an additional 26 bp upstream from the intron start. PCR amplification conditions were as follows: 1 min at 94°C, 1 min at 58°C and 1 min 30 s at 72°C. The PCR products were visualised and purified as described in Section 2.3.

The ability of the primers to detect the pathogen in planta was also assessed by inoculating surface sterilised plums with isolates of the three *Monilinia* species. Agar plugs of isolates es-12 (*M. laxa*), cc 747 (*M. fructigena*) and cc 684 (*M. fructicola*) were aseptically placed onto the fruit tissue, sealed in bags and then incubated for several days in the dark at 23°C until signs of infection were visible. Infected fruit tissue (2 cm^2), remote from the inoculation site, was removed and DNA extracted using DNAzol™ as described in Section 2.2. The DNA preparations required a 100-fold dilution prior to PCR amplification to overcome the effects of plant based inhibitors. Universal primers ITS 1 and ITS 4 [8] were used to verify the suitability of the DNA preparations for PCR and primers NS 5 and *mfs-3* to detect *M. fructicola* in planta.

3. Results and discussion

3.1. Amplification of 18S rDNA and isolation of the *M. fructicola* intron

The PCR products generated using the NS1 and NS8 primers were approximately 1.7 kb in size for the *M. laxa* and *M. fructigena* isolates tested whilst the *M. fructicola* isolates gave a product of approximately 2.1 kb (Fig. 1).

The nucleotide sequence data of the SSU rDNA PCR products from the *M. fructicola* isolates revealed the presence of a 418-bp insert, located near the 3′-end of the 18S gene (nt 1098–1515), which was not present in any of the *M. laxa* or *M. fructigena* isolates. With the exception of this intron, the 18S nucleotide sequence is highly conserved among the three *Monilinia* species (accession numbers: *M. fructicola* Y14210; *M. laxa*/*M. fructigena* Y14211). FASTA analysis [9] of the 418-bp intron sequence with the plant/fungal EMBL databases revealed homology with other fungal 18S group-I introns, including the closely related *S. sclerotiorum* [10]. The conserved sequence elements P, Q, R and S could be identified at base positions 1192–1202, 1303–1312, 1345–1358 and 1422–1433, respectively (Fig. 2).

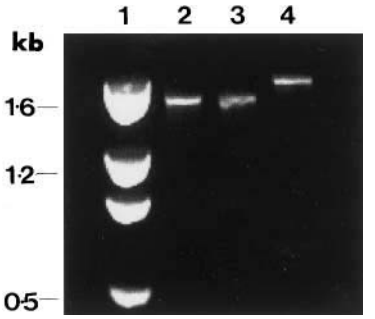

Fig. 1. Amplification products generated using primers NS1 and NS8. Lane 1, digested pGEM used as molecular size marker; lane 2, *M. laxa* (cc 682); lane 3, *M. fructigena* (cc 747); lane 4, *M. fructicola* (cc 778).

The homology of the intron to other fungal and plant group-I intron sequences prevented the intron from being used directly as a diagnostic marker. Indeed, primers designed solely from the 5′- and 3′-ends of the *M. fructicola* intron sequence data

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        10         20         30         40         50         60
TAACTGCAGT AACTCTGCGC CAAAAAGCAG CCCGTAAGGG TGAGGTGGTT CGCCTTCAAC

        70         80         90        100        110        120
TTAAATGCTA GTCTATTATA AGGCTACATT CCCAAATTGC GGGAACACCC TAATGCTCTC
                                      P
       130        140        150        160        170        180
ACTTCCAAGC TGACATTTGA AAGAATGCAG TGGCCAGGCT AATCACCTGG GTATGGACAT

       190        200        210        220        230        240
AACGTCGAGA GATGATACAA TGGGCTATCC GCATCCTTTC CCTTCATACG CATAGTATAT
                                 Q
       250        260        270        280        290        300
GGAAAAGGTT CAGAGACTAA ATGGGAATGG CTAGTTTATT AAATTCAACG TTTTAGATGC
             R
       310        320        330        340        350        360
GTACTGAAGT TAATGATACT AGTTAAGATA TAGTCCGTCG GTAGGTGAAA ACTTACGGTT
                                 S
       370        380        390        400        410
CAAAGCTTGA AGCTTGACCC TTGGAAGCAA CTCAATGCTT TCGAGTGAAT ATAAAACG
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Fig. 2. Nucleotide sequence of the *M. fructicola* group-I intron and positions of the conserved core elements, P, Q, R and S (denoted underlined).

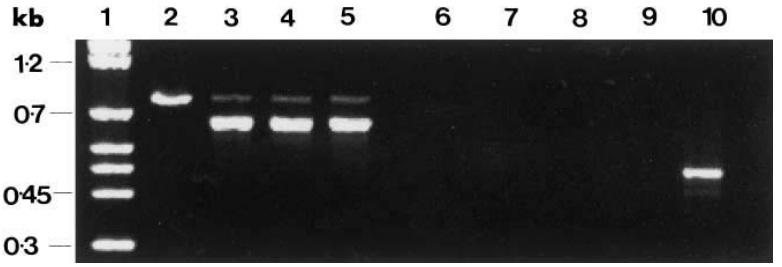

Fig. 3. Amplification products generated using primers ITS 1 and ITS 4 (lanes 2–5): lane 2, from healthy plum tissue; lane 3, *M. laxa*-infected plum; lane 4, *M. fructigena*-infected plum; lane 5, *M. fructicola*-infected plum. Amplification products generated using primers NS5 and *mfs-3* (lanes 6–10): lane 6, water (negative control); lane 7, from healthy plum tissue; lane 8, from *M. laxa*-infected plum; lane 9, *M. fructigena*-infected plum; lane 10, *M. fructicola*-infected plum. (Lane 1, pGEM-digested DNA as molecular size marker.)

were able to amplify group-I introns from the other two *Monilinia* species at lower annealing temperatures. Subsequent dot blot analysis of the fungal genomic DNA revealed that these introns exist elsewhere within the genome of some of the isolates of the other two species, albeit present as a single copy (data not shown). Therefore, the primer NS5, together with *mfs-3*, was used to amplify the 444-bp product. All the *M. fructicola* isolates produced amplifproducts, 444-bp in size; none of the *M. laxa* or *M. fructigena* isolates yielded a PCR product using these primers.

DNA from *Monilinia*-infected and healthy plums was used to determine if the pathogen could be detected in planta by PCR. Only DNA from *M. fructicola*-infected fruit produced a band corresponding to the 18S intron. To ensure that this result was not due to the quality of the template, the universal primers ITS 1 and 4 were used to verify that the DNA was amplifiable. One band, approximately 800 bp in size, corresponding to the plant ITS 1, 5.8S rDNA and ITS 2 region was produced in the healthy (control) DNA sample. The 'infected' plum DNA yielded two bands with each of the *Monilinia* spp. relating to the plum and the pathogen ITS 1, 5.8S and ITS 2 regions (800 and 550 bp, respectively, Fig. 3).

Many group-I introns have been reported from the nuclear genomes of fungi, the majority from the SSU rDNA gene [11–14]. They are characterised by conserved core elements [5] and other secondary features. Their evolutionary distribution in fungi has been speculated upon with suggestions that, other than resulting from an evolutionarily divergent event, they may be the result of lateral transfer from closely related species or possibly from host to pathogen [15]. Byrde and Willetts [16] first suggested that *M. fructicola* and *M. fructigena* might both have arisen from an ancestral form of *M. laxa*. The significance, therefore, of *M. fructicola* possessing an intron lacking in the other brown rot causing *Monilinia*s may indicate a more recent evolutionary divergence possibly arising from its establishment in the 'New World'.

The sensitivity of PCR enables the direct detection of the pathogen in the host without the prior need for isolating and culturing the pathogen. The technique described here uses PCR to provide a fast, simple, non-isotopic means of detecting a quarantine organism.

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and in no way anticipates the Commission's future policy in this area.

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