European Journal of Plant Pathology **105:** 495–500, 1999.
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Genetic variation among and within *Monilinia* species causing brown rot of stone and pome fruits

C.E. Fulton[1,*], G.C.M. van Leeuwen[2] and Averil E. Brown[1]
[1]*Department of Applied Plant Science, The Queen's University of Belfast, Newforge Lane, Belfast, BT9 5PX, UK;*
[*]*Current address: Department of Plant Science, University College, Cork, Republic of Ireland;*
Phone: (00353 21)902104; E-mail: stp18024@bureau.ucc.ie); [2]*Plantenziektenkundige Dienst, Geertjesweg 15, P.O. Box 9102, NL-6700 HC, Wageningen, The Netherlands*

Accepted 18 May 1999

Key words: brown rot, *Monilinia laxa*, *M. fructigena*, *M. fructicola*, ITS sequence data, SSU rDNA group-I intron, RAPD-PCR

Abstract

Nucleotide sequence analysis of the internal transcribed spacer (ITS) regions 1 and 2 of the ribosomal DNA (rDNA) divided the three brown rot pathogens *Monilinia laxa*, *M. fructicola* and *M. fructigena* into four distinct groups. Isolates of *M. fructigena* received from Japan, which varied by 5 base substitutions in the ITS region from the European *M. fructigena* isolates, formed the fourth group. Four of five Japanese isolates of *M. fructicola* tested varied from the New World isolates in that they did not possess a group-I intron in the small subunit (SSU) rDNA. RAPD-PCR data indicated that isolates of *M. laxa* varied but were randomly distributed worldwide; ITS data indicated no apparent distinction between those from *Malus* spp. and those from *Prunus* spp. *M. fructigena* similarly did not cluster according to geographic origin. In contrast, *M. fructicola* isolates tended to be clustered according to their origin; Japanese isolates of *M. fructicola* clustered together and showed similarity to some of the New Zealand isolates. Isolates from USA and Australia were more variable.

Introduction

Three species of *Monilinia* cause brown rot in *Prunus* spp. (stone fruit) and *Malus* and *Pyrus* spp. (pome fruit) (Byrde and Willets, 1977). *M. fructigena* is mainly a fruit pathogen, *M. laxa* is considered to be more a pathogen of blossoms and twigs than of fruit and *M. fructicola* is a pathogen of blossom, twigs and fruit but mainly affects stone fruits. In several regions of the world two of the three species coexist. *M. fructigena* is found mainly in the 'Old World', notably Europe and Asia and coexists with *M. laxa*. *M. fructicola* and *M. laxa*, which produce similar disease symptoms and have common hosts, coexist in the 'New World', notably North America and Australia; these species show slight differences in their pathogenicity (Ogawa and English, 1960). Only in Central and Eastern Asia, where *Pyrus*, *Prunus* and *Malus* spp. originate, do all three species of *Monilinia* coexist (Wormald, 1927; Terui and Harada, 1966). It is suggested that the *Monilinia* spp. in an ancestral form first became associated with wild fruit trees in this region and it was postulated by Byrde and Willetts (1977) that an ancestral form of the brown rot fungi was similar to *M. laxa*. They speculated that *M. fructigena* and *M. fructicola* may have evolved from variants of the ancestral form, the latter in North America before the parental form lost the ability to produce apothecia. Phylogenetic evidence supports the view that *M. laxa* was derived first and possibly was more or less similar to the common ancestor of these *Monilinia* species (Holst-Jensen et al. 1997a). Morphological differences between Japanese isolates of *Monilinia* spp. and isolates from elsewhere have been observed and reviewed by Byrde and Willetts

(1977) and Batra and Harada (1986). In this paper we describe some genetic variation among isolates of all three species from different geographical regions, but with particular reference to variation in isolates of *M. fructigena* and *M. fructicola* from Japan.

Materials and methods

Fungal cultures

Seventy-one isolates of the three brown rot *Monilinia* species *M. laxa*, *M. fructigena* and *M. fructicola* were used in this study. Many of the isolates were included in a previous study and their origin and identification are given in Fulton and Brown (1997). Isolates cc 954 and ispave 926 previously tabulated were not used in the present study. The origin and identification of the remaining isolates are given in Table 1. *Monilinia* isolates were maintained on potato dextrose agar (PDA) (Oxoid) at 23 °C.

Table 1. Seventeen of the 71 isolates of *Monilinia laxa*, *M. fructigena* and *M. fructicola* used in this study (the remaining 54 isolates are listed in Fulton and Brown, 1997)

Isolate no.	Origin	Host	Date
Monilinia laxa			
cc 952	UK	*P. domestica*	1996[B]
PC 239	UK	*Malus* spp.	1996[C]
PC 261	UK	*Malus* spp.	1996[C]
PC 264	UK	*Malus* spp.	1996[C]
PC 271	UK	*Malus* spp.	1996[C]
M. fructigena			
cc 782	UK	*Malus* spp.	1994[B]
jap 1815	Japan	*M. pumila*	1991[A]
jap 2314	Japan	*M. pumila*	1994[A]
jap 2315	Japan	*M. pumila*	1994[A]
jap 2316	Japan	*M. pumila*	1994[A]
pd 27.96	Netherlands	*Pyrus domestica*	1996[A]
M. fructicola			
mf-2	USA	*Prunus* sp.	?[A]
mf-74	USA	*Prunus* sp.	?[A]
jap 1438	Japan	*M. pumila*	1989[A]
jap 1535	Japan	*Prunus sp.*	1990[A]
jap 2636	Japan	*P. cerasus/avium*	1995[A]
jap 2810	Japan	*Prunus sp.*	1996[A]

[A]Cultures supplied by Plantenziektenkundige Dienst (Plant Protection Service), Wageningen, NL.
[B]Cultures supplied by Central Science Laboratory, York, UK.
[C]Cultures supplied by Horticulture Research International, East Malling, UK.

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 based on that of Raeder and Broda (1985) but with a third additional phenol/chloroform extraction. The genomic DNA pellet was re-suspended 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% (w/v) TBE agarose gels stained with ethidium bromide.

PCR amplification and sequencing of ITS regions

The ITS 1, 5.8S and ITS 2 regions of the fungal rDNA were amplified using the primers ITS 1 ext (GTA ACA AGG TTT CCG TAG GTG, an extended primer of ITS 1, White et al., 1990) and ITS 4 (White et al., 1990). The reactions were 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 primers ITS 1 ext and ITS 4. DNA in aliquots of the individual PCR reactions were quantified by gel electrophoresis as previously described.

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 1 μM of the ITS 1 ext (forward) and ITS 4 (reverse) primers, following the protocol supplied with the Prism™ Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems). 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). Phylogenetic analyses were conducted using the Molecular Evolutionary Genetics Analysis program (Kumar et al., 1993).

PCR amplification and sequencing of SSU rDNA

The SSU rDNA region from the *M. fructicola* isolates listed in Table 1 was amplified using the universal primers NS1 and NS8 (White et al., 1990) and the PCR products sequenced as previously described (Fulton and Brown, 1997).

RAPD-PCR amplification and data analysis

For RAPD analysis, primers A03 (AGT CAG CCA C), A11 (CAA TCG CCG T), A13 (CAG CAC CCA C), B06 (TGC TCT GCC C), B07 (GGT GAC GCA G) and B10 (CTG CTG GGA C), obtained from Operon Technologies, CA, USA, were used after they were shown to produce clear, reproducible, polymorphic banding patterns with the isolates used in this study. The reactions were again performed on a Perkin Elmer 480 Thermal Cycler using the following programme conditions – 1 min 10 s at 94 °C, 2 min at 30 °C and 2 min at 72 °C, with 1 °C s^{-1} ramping to the extension step, for 45 cycles. The reaction mixtures were as above, except that 1 µM of the random primer was used per reaction. Ten µl aliquots of the RAPD-PCR products were run on 1% (w/v) TBE high resolution agarose (Sigma Chemical Co.) and the gel images were scanned using the Grab-It (ver. 2.5, Synoptic Ltd., 1993–97) software package. The banding patterns were analysed using the Phoretix 1D Advanced (ver. 3.01, Non Linear Dynamics Ltd., 1996–97) software package and a similarity matrix produced from simple matching coefficients. Statistical analyses were performed with PHYLIP 3.57 (Felsenstein, 1993) and the trees constructed using the Neighbor-Joining method (Saitou and Nei, 1987).

Results

ITS nucleotide sequence analysis

Nucleotide sequence data were obtained for the ITS 1–5.8S–ITS 2 region of all 71 *Monilinia* isolates and, with the exception of the Japanese isolates of *M. fructigena*, concurred with previously published data (representative EMBL accession nos. *M. laxa*, Z73784; *M. fructigena*, Z73779; *M. fructicola*, Z73777; Holst-Jensen et al., 1997a). The Japanese isolates of *M. fructigena* (EMBL accession no. Y17876) differed from the previously reported sequence for *M. fructigena*, by four transitions within the ITS 1 region and one transition in the ITS 2 region. Both *M. laxa* and *M. fructicola* differed from the Japanese *M. fructigena* sequences by ten variable sites within the ITS1/2 region. Phylogenetic analyses of the ITS nucleotide sequences, including *Sclerotinia sclerotiorum* as an outgroup (EMBL accession no. Z73799), using the Kimura 2-Parameter method (Kimura, 1980) to calculate distance and the Neighbor-Joining method (Saitou and Nei, 1987) to construct the tree revealed that the Japanese *M. fructigena* isolates were more closely related to the non-Japanese *M. fructigena* isolates than to the other two species (Figure 1).

SSU rDNA sequence analysis

The non-Japanese *M. fructicola* isolates tested revealed the presence of the group-I intron at position 943 in the SSU rDNA (Fulton and Brown, 1997). However, the Japanese *M. fructicola* isolates, with the exception of isolate jap 1829, did not possess an intron in the SSU rDNA.

RAPD-PCR data

Total DNA from 58 *Monilinia* isolates was assessed for polymorphism using 6 random 10-base primers. Each of the primers amplified reproducible fragments with faint or ambiguous bands being excluded from the analyses. A total of 138 bands across 20 *M. laxa* isolates, 139 bands across 20 *M. fructigena* isolates and 90 bands across 18 *M. fructicola* isolates were analysed.

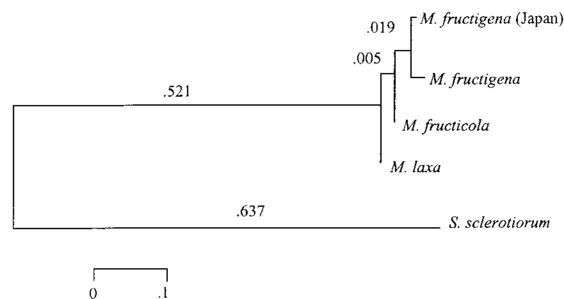

Figure 1. Neighbor-Joining phylogenetic tree produced from nucleotide sequence analysis of the ITS 1, 5.8S gene and ITS 2 region from isolates of *Monilinia laxa*, *M. fructigena* and *M. fructicola*, using the MEGA statistical package (Kumar et al., 1993). *Sclerotinia sclerotiorum* was included as an outgroup. The pairwise genetic distance values were produced using the Kimura 2-Parameter algorithm.

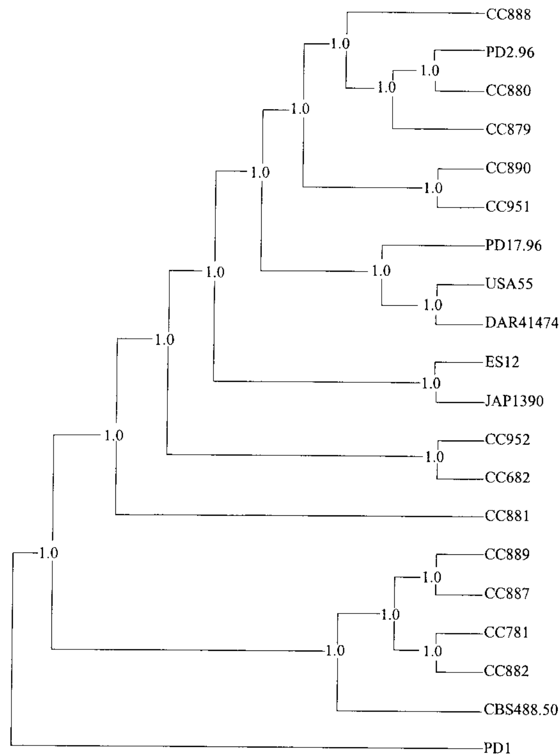

Figure 2. Clustering of individual isolates of *Monilinia laxa* from pairwise comparison of RAPD-PCR data using PHYLIP (Felsenstein, 1993) (host and origin of isolates are given in Table 1 and Fulton and Brown, 1997).

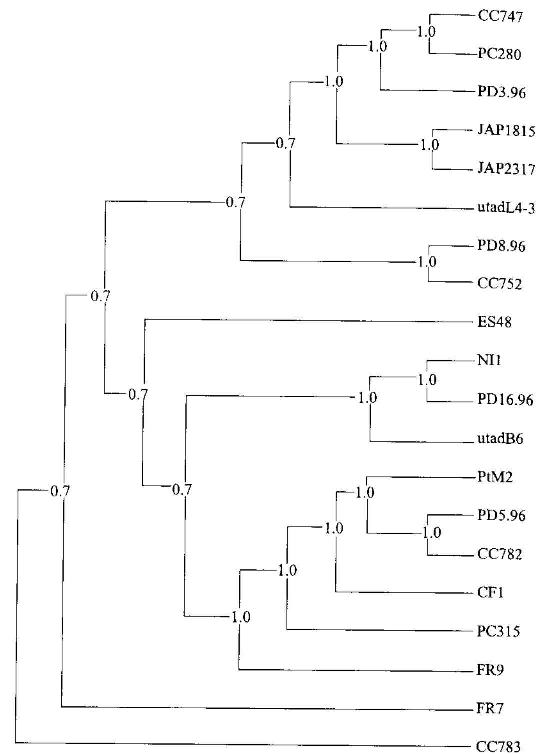

Figure 3. Clustering of individual isolates of *Monilinia fructigena* from pairwise comparison of RAPD-PCR data using PHYLIP (Felsenstein, 1993) (host and origin of isolates are given in Table 1 and Fulton and Brown, 1997).

M. laxa isolates from *Prunus* hosts were randomly distributed throughout the dendrogram (Figure 2) and there was no suggestion of geographic groupings. Similarly, RAPD-PCR analysis revealed no suggestion of geographic grouping among *M. fructigena* isolates (Figure 3) despite the ITS sequence differences between European and Japanese isolates. With *M. fructicola*, however, there was clustering of isolates from different geographic regions; the Japanese isolates clustered together but formed two subgroups. The New Zealand isolates also tended to cluster together while isolates from USA and Australia showed greater variation (Figure 4). The Japanese isolates appeared more closely related to some of the New Zealand isolates than to the Australian or USA isolates.

Discussion

Genetic information has already been published on *Monilinia* species that cause brown rot of pome and stone fruits (Carbone and Kohn, 1993; Holst-Jensen et al., 1997a,b). In this paper, however, we present some new information on the molecular identification of isolates of *M. fructigena* and *M. fructicola* obtained from Japan. No variation has been observed in the ITS nucleotide sequence of isolates of *M. laxa* from *Malus* and *Prunus* spp. and from worldwide sources (Holst-Jensen, 1997a). On the basis of RAPD-PCR data, variation in *M. laxa* appears unrelated to geographic origin. This suggests that *M. laxa* has been randomly distributed worldwide and appears to have readily adapted to its different hosts.

It was suggested that variant forms of a *M. laxa*-like ancestor (Holst-Jensen, 1997a) may have evolved in Central or Eastern Asia to give new subspecies or species related to *M. fructigena* that became pathogens of *Malus* spp. (Byrde and Willetts, 1977). The isolates from Japan, designated *M. fructigena*, were however, on the basis of ITS nucleotide sequence data, distinct from European isolates. Isolates with the same ITS sequence as the European *M. fructigena* isolates

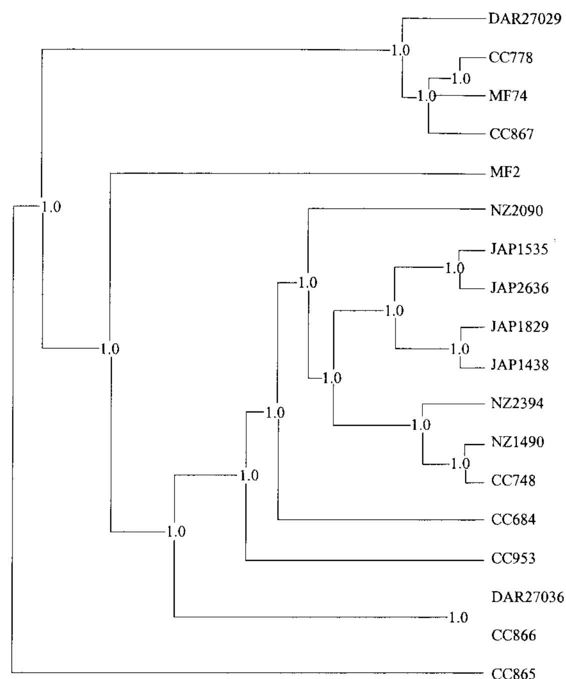

Figure 4. Clustering of individual isolates of *Monilinia fructicola* from pairwise comparison of RAPD-PCR data using PHYLIP (Felsenstein, 1993) (host and origin of isolates are given in Table 1 and Fulton and Brown, 1997).

were not found among *Monilinia* isolates from Japan suggesting that they may not have evolved in Eastern Asia. Although ITS sequence data indicated that the Japanese *M. fructigena* isolates were more similar to the European *M. fructigena* isolates than to either *M. laxa* or *M. fructicola* they were, by comparison with other species of Sclerotiniaceae (Carbone and Kohn, 1993; Holst-Jensen et al., 1997a,b), sufficiently different so as to be possibly regarded as a separate species. The faster growth rate of the Japanese *M. fructigena* isolates and the production of smaller conidia compared with European isolates (van Leeuwen, unpublished, 1998) and morphological variation described by Batra and Harada (1986) could support separation of the distinct ITS rDNA genotypes.

In a previous study (Fulton and Brown, 1997), a group-I intron was located in the SSU rDNA of *M. fructicola* isolates (a 943 SSU group-I intron; Gargas et al., 1995) but not in *M. fructigena* or *M. laxa*. On the basis of this, PCR primers were designed to distingitish *M. fructicola*, which is an EU quarantine pathogen, from the other two brown rot species and for its rapid detection in the host tissues. Since that report further isolates of *M. fructicola* have been analysed and those received from Japan did not contain the intron. This inconsistency renders this rapid detection method somewhat less useful than had been thought previously. *M. fructicola* was first reported in Japan only in 1963 (Terui and Harada, 1966) and could be a relatively recent introduction. Similarities in the RAPD-PCR banding patterns between the Japanese *M. fructicola* isolates and those from New Zealand might suggest that they had a similar origin, Japanese isolates having either lost the intron from the SSU rDNA or developed from an isolate which never possessed one. We do not know whether or not isolate jap 1829, which possessed the group-I intron, was native to Japan or had been isolated from imported fruit. It would be of considerable benefit to investigate further East Asian isolates to obtain a better understanding of the distribution and genetic variation within the brown rot *Monilinia* species.

Acknowledgements

The authors would like to thank Roger Cook, Central Science Laboratory, UK and Angela Berrie, Horticulture Research International, UK for providing some of the *Monilinia* isolates used in this study. We would also like to thank Douglas McReynolds, Department of Agriculture for Northern Ireland, for assistance with the automated sequencing.

This work has been carried out with the financial support from the Commission of the European Communities, Agriculture and Fisheries (FAIR) specific RTD programme, Fair 1-0725, 'Development of diagnostic methods and a rapid field kit for monitoring *Monilinia* rot of stone and pome fruit, especially *M. fructicola*'. It does not necessarily reflect its views and in no way anticipates the Commission's future policy in this area.

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