Genetic variation between strains of *Monilinia fructicola* and *Monilinia laxa* isolated from cherries in Michigan

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Abstract: Polymerase chain reaction (PCR)-mediated analysis of rDNA from isolates of *Monilinia fructicola* and *Monilinia laxa* from Michigan cherry orchards revealed interspecies restriction site variation in the internal transcribed spacer 1 (ITS1) region and length variation in the small subunit (SSU) rRNA gene. ITS1 sequences from both species were 146 bp long; however, the ITS1 of *M. laxa* differed at three positions from the ITS1 of *M. fructicola*. Although the sequences of the ITS1 regions from both species were nearly identical, the enzyme *Mse*I cuts the PCR-amplified ITS1 region of the two species differentially. PCR amplification of the 3′ end of the SSU rRNA gene yielded products of approximately 940 and 520 bp from *M. fructicola* and *M. laxa*, respectively. A 421-bp group I intron was detected by PCR within the SSU rDNA of 32 isolates of *M. fructicola* but not in the eight isolates of *M. laxa*. Intron sequences from each of four isolates of *M. fructicola* were identical, and the SSU rDNA flanking sequences from these isolates and from two isolates of *M. laxa* were nearly identical. Arbitrarily primed PCR analysis of genomic DNA with microsatellite primers $(GACA)_4$ and $(GTG)_5$ revealed that the number and size of the amplification products were characteristic for each species. Distinctive and reproducible sets of amplification products were obtained from 32 isolates of *M. fructicola* and the eight isolates of *M. laxa*. Our results illustrate the potential of PCR amplification of ribosomal and genomic DNA for differentiating these tree-fruit-infecting brown rot fungi.

Key words: 18S rDNA, American brown rot, European brown rot.

Résumé : Faite par réaction en chaîne catalysée par la polymérase (« PCR »), une analyse de l'ADNr d'isolats du *Monilinia fructicola* et du *Monilinia laxa*, provenant de vergers à cerisiers du Michigan, a révélé des variations interspécifiques des sites de restriction de la région de l'espaceur interne transcrit 1 (ITS1) et des variations dans la longueur du gène de l'ARNr de la petite sous-unité (PSU). Les séquences des ITS1 des deux espèces avaient une longueur de 146 pb; par contre, l'ITS1 du *M. laxa* différait de celui du *M. fructicola* à trois positions. Malgré la quasi-similitude des séquences de la région de l'ITS1 des deux espèces, l'enzyme *Mse*I coupait différemment la région de l'ITS1 amplifiée par PCR. L'amplification par PCR de l'extrémité 3′ du gène de l'ARNr de la PSU a généré des produits d'environ 940 et 520 pb pour le *M. fructicola* et le *M. laxa* respectivement. Un intron de 421 pb, appartenant au groupe I, a été détecté par PCR dans l'ADNr de la PSU des 32 isolats testés du *M. fructicola*, mais pas dans celui des huit isolats testés du *M. laxa*. La séquence de l'intron était identique pour quatre isolats du *M. fructicola* examinés, et les séquences adjacentes de l'ADNr de la PSU de ces isolats et de deux isolats du *M. laxa* étaient presque identiques. L'analyse par PCR à l'aide d'amorces arbitraires, les microsatellites $(GACA)_4$ et $(GTG)_5$, a révélé que le nombre et le dimension des produits d'amplification pouvaient être caractéristiques de chaque espèce. Des ensembles distinctifs et reproductibles de produits d'amplification ont été obtenus pour les 32 isolats de *M. fructicola* et les huit isolats de *M. laxa*. Nos résultats démontrent le potentiel de l'amplification par PCR des ADN ribosomiques et génomiques pour la distinction entre ces champignons de la pourriture brune des arbres fruitiers.

Mots clés : ADNr 18S, pourriture brune américaine, pourriture brune européenne.

Introduction

Brown rot diseases, caused by various species of *Monilinia*, are economically important on stone and pome fruit crops worldwide. In North America, the American brown rot fungus, *Monilinia fructicola* (Wint.) Honey, is common in all areas where stone fruits are grown, while the related European brown rot fungus, *Monilinia laxa* Aderhold & Ruhland, is common on stone fruit crops almost exclusively in the West of the continent (13). Outbreaks of *M. laxa* also have been reported on sour cherry (*Prunus cerasus* L.) from Michigan (4), New York (18), and Wisconsin (19). *Monilinia laxa* had not been reported in the Great Lakes states since 1949, but in 1993 an outbreak occurred on sour cherry in Michigan and Wisconsin. A third brown rot pathogen, *Monilinia fructigena* Honey, is not known to occur in North America, although it may have been introduced to North America and then eradicated (1). *Monilinia fructicola* is distributed globally except in

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Europe, where the related species *M. laxa* and *M. fructigena* are the predominant species (2).

All three species, *M. fructicola*, *M. laxa*, and *M. fructigena*, belong to the section Junctoriae (2, 15). The species are usually distinguished on colony morphology and variations in sporulation on potato dextrose agar (PDA), as reported by Hewitt and Leach (13) nearly 60 years ago. *Monilinia laxa* generally produces lobed, slow-growing colonies with poor conidial production, while *M. fructicola* generally produces colonies with smooth margins and abundant conidial production. Identification by morphology is sometimes uncertain because isolate characteristics may change with repeated subculturing and because of some overlap in colony morphology between species (25, 26). Electrophoretic patterns (26), germ-tube branching (16), hyphal anastomoses between germinating conidia (26), and formation of black lines (barrages) between colony margins of the two species (31) have been used for differentiating isolates where morphological characters are ambiguous or atypical. However, these methods are not sufficiently specific to be used routinely. Highly specific tools for differentiating between the closely related brown rot pathogens would be particularly important where quarantine regulations for *M. fructicola* have been established (29).

Molecular tools for rapidly distinguishing between the tree-fruit infecting species of *Monilinia* are currently quite limited. Sequencing of the noncoding internal transcribed spacer 1 (ITS1) region was used by Carbone and Kohn (3) to establish a phylogenetic framework for 27 species within the Sclerotiniaceae. Recently, noncoding and coding rDNA sequences were used to establish the phylogenetic relationship and evolution of 17 species of *Monilinia* and eight closely related species (15). However, molecular methods are not yet sufficiently automated and available for routinely distinguishing large numbers of isolates. The polymerase chain reaction (PCR) coupled to restriction analysis is more feasible and is currently widely used to identify fungi (12). Other potential methods for distinguishing the two species may include variation in the length of the small subunit (SSU) rRNA gene and arbitrarily primed PCR (ap-PCR) of genomic DNA. In the absence of specific nucleotide sequence information, species-specific DNA polymorphism can often be detected by ap-PCR using microsatellite primers (33, 35). Recently, ap-PCR analysis was used to distinguish between closely related *Colletotrichum* species isolated from a range of plants (9, 10). Ap-PCR analysis may also be useful for distinguishing *M. laxa* from *M. fructicola*.

The objective of this study was to determine whether isolates of *M. fructicola* could be distinguished from isolates of *M. laxa* on the basis of sequence variation in their ITS1 region. In addition, we determined whether there were differences between *M. laxa* and *M. fructicola* in the length of the SSU rRNA gene and in the pattern of ap-PCR products. Preliminary results of this research have appeared in abstract form (30).

Materials and methods

Isolates

The isolates of *M. fructicola* were obtained from sweet cherry fruit collected at random from two cherry orchards (designated JB and KK) near Traverse City, Michigan. Additional isolates were obtained from sour cherry fruit collected randomly from an orchard (SC) near Paw Paw, Michigan. Orchard SC was located about 200 and 250 km from orchards JB and KK, respectively. A benomyl-resistant isolate collected in Michigan in 1975 (17) was included with the isolates collected in 1993 for orchards SC, KK, and JB. The isolates of *M. laxa* were from infected spurs of sour cherry collected at three sites in Michigan and one site in Wisconsin in 1993 and 1994. Colony morphology, whether margins were entire or lobed, whether conidia were present or absent, whether the stroma was dark, and whether the colony nearly filled the Petri dish, was determined after 7 days for several isolates of *M. fructicola* and *M. laxa* (Table 1).

DNA extraction

Each isolate was grown in 125 mL of potato dextrose broth (Difco Laboratories, Detroit, Mich.) at room temperature for 7–10 days. Using sterile forceps, mycelium was transferred from liquid culture to a 1.5-mL microcentrifuge tube and centrifuged for 1 min. After the supernatant was poured off, additional mycelium was transferred to the tube and it was centrifuged again. This process was repeated until the tube contained approximately 500 μL of mycelium. The mycelium was washed with 600 μL of Tris-EDTA (TE) buffer, centrifuged for 5 min, and the supernatant poured off (6). Then 600 μL of extraction buffer (200 mM Tris-HCl (pH 8.5), 200 mM NaCl, 25 mM EDTA, 0.5% SDS (27)) was added and the mycelium was macerated using a disposable pellet pestle. DNA was purified by phenol/chloroform extraction, precipitated with two volumes ethanol and 0.1 volume 3 M sodium acetate overnight at –20°C, pelleted, rinsed twice with 70% ethanol, vacuum dried, and suspended in 100 μL of TE buffer (22). DNA was stored at –20°C.

Amplification and sequencing

All primers were synthesized by the Macromolecular Structure Facility at Michigan State University. Primers for the ITS1 region (ITS1F 5′-CTTGGTCATTTAGAGGAAGTAA-3′ and ITS2 5′-GCTGCGTTCTTCATCGATGC-3′) and for the 3′ end of the SSU rDNA (NS7 5′-GAGGCAATAACAGGTCTGTGATGC-3′ and NS8 5′-TCCGCAGGTTCACCTACGGA-3′) were taken from Gardes and Bruns (12) and White et al. (34). Primers AJ139 (5′-GAAGACTAACTACTGCGAAAGC-3′) and AJ140 (5′-ACCTGTTATTGCCTCAAACTT-3′) were developed using Oligo primer analysis software (National Biosciences, Inc., Plymouth, Minn.) based on the SSU rDNA sequence of *Sclerotinia sclerotiorum* (GenBank accession number X69850). These primers were used to amplify the part of the SSU rDNA just upstream from primer NS7. The following simple repeat sequences were used as single primers to amplify microsatellite regions in the genome: 5′-GACAGACAGACAGACA-3′ (10) and 5′-GTGGTGGTGGTGGTG-3′ (21). All polymerase chain reactions (PCR) were performed in MJ Research thermocycler model PTC-150 (MJ Research, Inc., Watertown, Mass.).

PCR reactions for amplification of the ITS1 region and the SSU rRNA gene were carried out in a 50-μL volume containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl,

Table 1. Isolates of *Monilinia fructicola* and *Monilinia laxa*, their origin, colony morphology, and results of polymerase chain reaction (PCR) assays using primers for the internal transcribed spacer 1 (ITS1) region, small subunit (SSU) rRNA gene, and arbitrarily primed PCR (ap-PCR) with microsatellite primers $(GACA)_4$ and $(GTG)_5$.

Isolate	Year of isolation	Locality	Host	Colony type[a]	PCR-ITS1 fragment (bp)[b]	PCR-SSU fragment (bp)[c]	ap-PCR band patterns[d]
Monilinia fructicola							
SC-5	1993	Paw Paw, Mich.	Montmorency sour cherry	1	250	nd	2, 4
SC-41	1993	Paw Paw, Mich.	Montmorency sour cherry	1	250 (146)	940 (421)[e]	nd
SC-44	1993	Paw Paw, Mich.	Montmorency sour cherry	1	250 (146)	940	nd
SC-49	1993	Paw Paw, Mich.	Montmorency sour cherry	1	250 (146)	940	nd
SC-55	1993	Paw Paw, Mich.	Montmorency sour cherry	1	250	940	nd
SC-57	1993	Paw Paw, Mich.	Montmorency sour cherry	1	250 (146)	940	2, 4
SC-59	1993	Paw Paw, Mich.	Montmorency sour cherry	1	nd	940	2, 4
SC-70	1993	Paw Paw, Mich.	Montmorency sour cherry	1	250	940	2, 4
SC-75	1993	Paw Paw, Mich.	Montmorency sour cherry	1	nd	940	2, 4
KK-11	1993	Kewadin, Mich.	Sweet cherry	1	250	940	2, 4
KK-15	1993	Kewadin, Mich.	Sweet cherry	1	250	940	2, 4
KK-20	1993	Kewadin, Mich.	Sweet cherry	1	250 (146)	940 (421)[e]	2, 4
KK-22	1993	Kewadin, Mich.	Sweet cherry	1	250	940	2, 4
KK-27	1993	Kewadin, Mich.	Sweet cherry	1	nd	940	nd
KK-29	1993	Kewadin, Mich.	Sweet cherry	1	nd	nd	2, 4
KK-31	1993	Kewadin, Mich.	Sweet cherry	1	250	940	2, 4
KK-33	1993	Kewadin, Mich.	Sweet cherry	1	nd	940 (412)[e]	nd
KK-45	1993	Kewadin, Mich.	Sweet cherry	1	250	940	2, 4
KK-48	1993	Kewadin, Mich.	Sweet cherry	1	250 (146)	940	2, 4
KK-49	1993	Kewadin, Mich.	Sweet cherry	1	250	nd	2, 4
KK-50	1993	Kewadin, Mich.	Sweet cherry	1	nd	nd	2, 4
KK-54	1993	Kewadin, Mich.	Sweet cherry	1	250 (146)	940	2, 4
JB-2	1993	Empire, Mich.	Sweet cherry	1	250	940	2, 4
JB-4	1993	Empire, Mich.	Sweet cherry	1	250	940	2, 4
JB-6	1993	Empire, Mich.	Sweet cherry	1	250	940	nd
JB-8	1993	Empire, Mich.	Sweet cherry	1	250	940	2, 4
JB-9	1993	Empire, Mich.	Sweet cherry	1	250 (146)	940	2, 4
JB-10	1993	Empire, Mich.	Sweet cherry	1	250	940	2, 4
JB-11	1993	Empire, Mich.	Sweet cherry	1	250 (146)	940	2, 4
JB-12	1993	Empire, Mich.	Sweet cherry	1	250	940	2, 4
JB-13	1993	Empire, Mich.	Sweet cherry	1	250 (146)	940	2, 4
JB-14	1993	Empire, Mich.	Sweet cherry	1	250	940	2, 4
JB-26	1993	Empire, Mich.	Sweet cherry	1	250 (146)	940 (421)[e]	nd
JB-62	1993	Empire, Mich.	Sweet cherry	1	250	940	2, 4
JB-65	1993	Empire, Mich.	Sweet cherry	1	250	940	2, 4
CB-2	1975	Hart, Mich.	Montmorency sour cherry	V	250 (146)	940	3, 4
Monilinia laxa							
DG-7	1993	Suttons Bay, Mich.	Meteor sour cherry	2	250	520	1, 5
DG-8	1993	Suttons Bay, Mich.	Meteor sour cherry	2	250 (146)	520	1, 5
NP-1	1993	Northport, Mich.	Montmorency sour cherry	2	250	520	1, 5
NP-2	1993	Northport, Mich.	Montmorency sour cherry	2	250 (146)	520 (469)[f]	1, 5
WI-2	1993	Sturgeon Bay, Wis.	Montmorency sour cherry	2	250 (146)	520 (469)[f]	1, 5
WI-3	1993	Sturgeon Bay, Wis.	Montmorency sour cherry	2	250	520	1, 5
Grants	1994	Shelby, Mich.	Erdi Botermo sour cherry	2	250 (146)	520	1, 5
Meteor	1994	Suttons Bay, Mich.	Meteor sour cherry	2	250	520	1, 5

[a]1, colony margins entire, conidia usually formed, colony nearly fills Petri dish in 7 days; 2, colony margins lobed, no conidia formed, colony darkly pigmented, colony never fills Petri dish even after 14 days. V, variable: slow growing, pigmented, sparse conidial formation.

[b]Length of the amplification fragments obtained with primers ITS1F and ITS2. Number in parentheses indicates the actual length of the ITS1 region alone, determined by sequence analysis and confirmed by comparison with sequences retrieved from GenBank. nd, not done.

[c]Length of the SSU DNA fragment amplified with primers AJ139 and AJ140.

[d]Band patterns 1, 2, and 3 were obtained using microsatellite primer $(GACA)_4$. Pattern 3 shares six bands in common with pattern 2 and one in common with pattern 1. Band patterns 4 and 5, obtained with primer $(GTG)_5$, share no bands in common.

[e]Number in parentheses indicates the intron length determined by sequence analysis.

[f]Number in parentheses indicates the region of the SSU rDNA confirmed by sequence analysis.

1.5 mM $MgCl_2$, 0.1% Triton X-100, 160 µM each deoxynucleotide-tri-phosphate (dNTP), 50 pmol of each primer, 2 U *Taq* DNA polymerase (Gibco/BRL, Gaithersburg, Md.), and 10–100 ng of template DNA and overlaid with two drops of mineral oil (20). A negative control containing no template was also included. The thermal program consisted of an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 3 min, followed with a final extension at 72°C for 7 min. PCR amplifications were performed two or three times from each isolate.

PCR products were observed by electrophoresis on a 1% agarose gel (SeaKem LE, FMC BioProducts, Rockland, Maine) in 1 × Tris-acetate (TAE) buffer. Products were purified from the PCR reaction mix using a DNA binding resin (Promega Wizard PCR Preps DNA purification system, Promega, Madison, Wis.) and sequenced at the Michigan State University Plant Research Laboratory Sequencing Facility. Products were sequenced from both strands. Sequences of the two strands were used to generate a consensus sequence for each isolate. Ambiguities, revealed by alignment of the two complementary sequences, were resolved by sequencing that region of DNA again. Sequences obtained from additional sequencing were added to the original two to generate a consensus. Identity of the ITS1 sequence was confirmed by aligning the consensus sequence from PCR products with ITS1 sequences retrieved from GenBank: *M. fructicola* (accession number U21815), *M. fructigena* (U21825), *Monilinia oxycocci* (U21833), and *Monilinia megalospora* (U21834), *S. sclerotiorum* (U21810), and *Sclerotinia minor* (U21818). The SSU rDNA sequence was confirmed by aligning the consensus sequence from PCR products with the SSU rDNA sequence for *S. sclerotiorum* (X69850) retrieved from GenBank. Sequence comparison was performed by MegAlign (DNASTAR, Inc., Madison, Wis.) using the Clustal V method to examine sequence distances for all sequence pairs and then align sequence groups (14).

Ap-PCR amplification of genomic DNA

Random amplifications using microsatellite primers $(GACA)_4$ and $(GTG)_5$ were performed on 36 isolates following the protocol of Freeman et al. (10). Each reaction was performed in a 50-µL volume containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 160 µM each dNTP, 50 pmol of primer, 2 U *Taq* DNA polymerase, and 10–100 ng of template DNA and overlaid with two drops of mineral oil. A negative control containing no template was also included. The thermal program consisted of an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 48°C for 30 s, and extension at 72°C, for 1.5 min, with a final extension at 72°C for 4 min. PCR amplifications were performed two or three times per isolate. PCR products were separated in 1.5% agarose gel in TAE buffer at 80 V for 2 h.

Restriction digestion

The ITS1 region from isolates of both species were amplified and purified as described above. The purified product from each reaction was digested with 5 U of *Mse*I (ICN Pharmaceuticals Inc., Costa Mesa, Calif.) at 37°C for 3 h. Restriction fragments were separated in 2% agarose gel in TAE buffer.

Nucleotide sequence accession numbers

DNA sequences for all the strains of *Monilinia* examined by sequence analysis in this study are available in GenBank under accession numbers AF010500 to AF010506.

Results

Sequence analysis of the ITS1 region

Based on colony morphology, 36 and eight isolates were classified as putative *M. fruticola* and *M. laxa* isolates, respectively (Table 1). PCR with primers ITS1F and ITS2 yielded a product of approximately 250 bp from 30 isolates of *M. fructicola* and the eight isolates of *M. laxa* (Table 1). Sequencing of amplified fragments from 12 isolates of *M. fructicola* and four isolates of *M. laxa* revealed the 3′ end of the SSU rRNA gene, a 146-bp ITS1 region, and the 5′ end of the 5.8S rRNA gene. Alignment of ITS1 sequences for Michigan isolates of *M. fructicola* revealed that 10 isolates had ITS1 sequences identical with an authentic strain of *M. fructicola* U21815 (3), and that isolates SC-49 and SC-57 differed from the sequence for the authentic strain by 1 and 3 bp, respectively.

Alignment of ITS1 sequences from four isolates of *M. laxa* indicated that there was very little intraspecific sequence variation, but that the ITS1 sequence for *M. laxa* differed by 3 bp from the ITS1 sequence for *M. fructicola*. The base pair difference at position 105 removed a *Mse*I site from *M. laxa*; therefore, the *Mse*I restriction fragment for isolates of *M. laxa* was 14 bp larger than the corresponding fragment for isolates of *M. fructicola* (Fig. 1). Finally, the consensus ITS1 sequence for *M. laxa* showed 98, 96, 83, 74, 97, and 97% nucleotide similarity with *M. fructicola*, *M. fructigena* U21825, *M. megalospora* U21834, *M. oxycocci* U21833, *S. sclerotiorum* U21810, and *S. minor* U21818, respectively.

Identification of an intron in *M. fructicola*

PCR amplification of the 3′ end of the SSU rDNA with primers NS7 and NS8 yielded a product of approximately 380 bp from both *M. fructicola* and *M. laxa*. Amplification using primers AJ139 and AJ140 yielded a product of approximately 940 bp from 32 isolates of *M. fructicola* and of 520 bp from the eight isolates of *M. laxa* (Table 1, Fig. 2). Sequencing of the PCR products from four isolates of *M. fructicola* and two of *M. laxa* revealed that the length variation was due to a 421-bp insertion sequence in *M. fructicola*. However, the flanking SSU rDNA sequences (460 bp) from the two species exhibited 99% similarity. Alignment of the SSU rDNA sequences for *M. fructicola* and *S. sclerotiorum* (X69850) showed that the insert in *M. fructicola* was located at the same position as a group I intron in *S. sclerotiorum* (36). However, the insertion sequence from *M. fructicola* showed only 54% nucleotide similarity to the intron from *S. sclerotiorum*.

Analysis of the sequence and secondary structure of the 421-bp insertion sequence from *M. fructicola* demonstrated

Fig. 1. Length variation in ITS1 DNA fragments PCR amplified from *Monilinia laxa* and *Monilinia fructicola* following *Mse*I digestion analysis. Lanes 1–4, *M. laxa* isolates DG-8, NP-1, WI-2, and Grants, respectively; lanes 5–8, *M. fructicola* isolates SC-41, KK-20, JB-13, and CB-2, respectively. The size of restriction fragments is calculated from a 100-bp ladder (lane M).

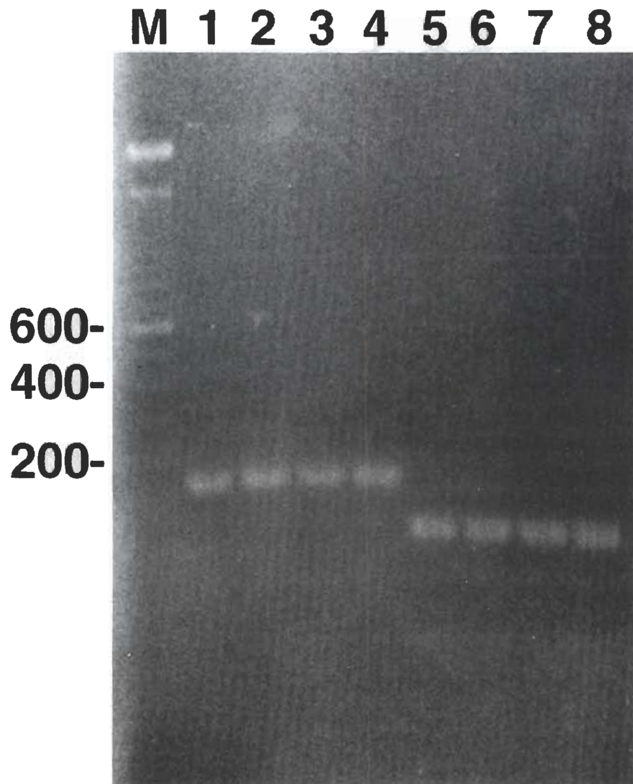
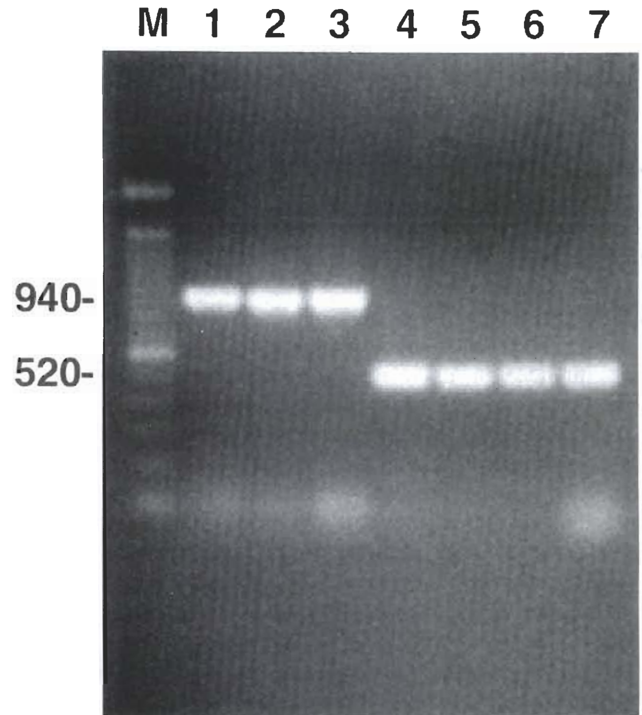


Fig. 2. Comparison of polymerase chain reaction (PCR) assays for a region of the SSU (18S) rRNA gene of *Monilinia fructicola* and of *Monilinia laxa*. PCR products were amplified using primers AJ139 and AJ140 and separated by electrophoresis through 1% agarose gels. Lane M, 100-bp ladder; lanes 1–3, *M. fructicola* isolates SC-41, KK-20, and JB-13, respectively; and lanes 4–7, *M. laxa* isolates DG-8, NP-1, WI-2, and Grants, respectively.



that it possessed the characteristic features known to be conserved among group I introns (Fig. 3). The conserved GU immediately preceding the 5′ splice site and the G at the 3′ splice site were present. The sequence also contained the four conserved sequence elements P, Q, R, and S and the order of their occurrence in the sequence (5′-P-Q-R-S-3′) necessary for the formation of the group I intron core structure (5). Conserved sequence elements P and Q base-paired to form helix P4, and elements R and S paired to form helix P7. Also, the 3′ end of element Q was base-paired with the 5′ end of element R to begin helix P6. Helices P1 to P9, including a pseudoknot formed by P3 and P7, were also identified. Five fungi containing introns in the SSU rDNA at the same location as the insert in *M. fructicola* were selected from GenBank for comparison of the intron region (7, 23, 24, 32) (Fig. 3). All contained the conserved sequence elements characteristic of group I introns, but the sequences varied in length from 310 to 453 bp and showed little sequence similarity outside of the conserved sequence elements (Fig. 3).

Comparison of *M. fructicola* and *M. laxa* by ap-PCR

The microsatellite primer $(GACA)_4$ generated six PCR products from *M. fructicola* genomic DNA that ranged in length from 200 to 1500 bp (Fig. 4A). Isolate CB-2 showed a distinct seventh band of approximately 1500 bp that was not observed for any of the other 27 isolates of *M. fructicola*. Ap-PCR amplification of DNA from eight isolates of *M. laxa* generated three PCR products of approximately 410, 1150, and 1500 bp in length. The patterns for isolates of the two species generally did not show any bands of common size. A 1500-bp product was present in *M. laxa* and in *M. fructicola* isolate CB-2, but not in other isolates of *M. fructicola*.

Amplification of *M. fructicola* DNA with the microsatellite primer $(GTG)_5$ also generated six PCR products that ranged in length from 300 to 1500 bp (Fig. 4B). Amplification of DNA from eight isolates of *M. laxa* generated four PCR products of approximately 350, 500, 900, and 1000 bp in length. No bands of common size were present between the two species.

Discussion

Three molecular methods can now be used to separate isolates of *M. fructicola* from *M. laxa* based on interspecific genetic variation. Enzymatic digestion of the ITS1 region after PCR amplification, length variation in PCR-amplification products using new SSU rDNA intron-spanning primers, and ap-PCR phenotypes using two microsatellite primers were used to differentiate isolates of

Fig. 3. Alignment of the conserved sequence elements of a group I intron found within the SSU (18S) rRNA gene of *Monilinia fructicola* with group I introns from the SSU rRNA gene of five other fungi. The conserved GU immediately preceding the 5′ splice site and the conserved G preceding the 3′ splice site are underlined. Fungi (GenBank accession number, reference) are abbreviated as follows: Mf, *Monilinia fructicola* (AF010505, this study); Ss, *Sclerotinia sclerotiorum* (X69850, 35); Um, *Ustilago maydis* (X62396, 7); By, *Bensingtonia yamatoana* (D38239, 31); Pi, *Protomyces inouyei* (D11377, 22), and Pl, *Protomyces lactucae-debilis* (D14164, 23).

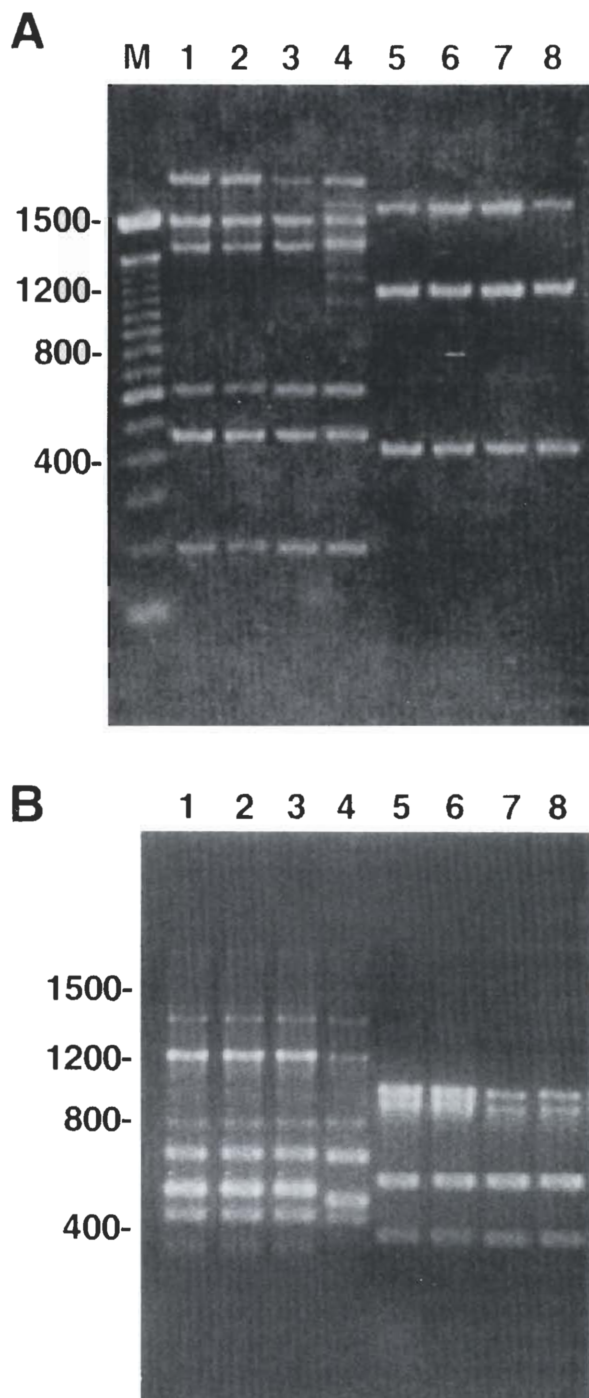
	Intron size	SSU gene	5′ splice site ↓	P	Q
Mf	420	5′---ACCACCAGGC	GU	----AAUUGCGGG–AA----	UAUCCGCAUC----
Ss	310	5′---ACCACCAGGC	GU	----AACUGCGGGGAA----	AAUCCGCAUC----
Um	410	5′---ACCACCAGGA	GU	----AAUUGCGGGAAA----	AAUCCGCAGC----
By	453	5′---ACCACCAGGU	GU	----AACUGCGGGAAA----	UAUCCGCAUC----
Pi	339	5′---ACCACCAGGA	GU	----AAUUGCGGGAAA----	AAUCCGCAGC----
Pl	339	5′---ACCACCAGGA	GU	----AAUUGCGGGAAA----	AAUCCGCAGC----

	R	S	3′ splice site ↓	SSU gene
Mf	---GUUCAGAGACUAAA----	AAGAUAUAGUCC----	ACG	GGAGCCUGCG---3′
Ss	---GUUCAGAGACUAAA----	AAGAUAUAGUCC----	AUG	GGAGCCUGCG---3′
Um	---GUUCAGAGACUAAA----	AAGAUAUAGUCC----	UCG	GGAGCCUGCG---3′
By	---GUUCAGAGACUAGA----	AAGGUAUAGUCC----	AAG	GGAGCCUGCG---3′
Pi	---GUUCAGAGACUAGA----	AAGAUAUAGUCC----	AUG	GGAGCCUGCG---3′
Pl	---GUUCAGAGACUAGA----	AAGAUAUAGUCC----	AUG	GGAGCCUGCG---3′

the two *Monilinia* species responsible for brown rot of stone fruit crops in Michigan. *Monilinia laxa* and *M. fructicola* have been differentiated for nearly 60 years based on colony morphology when grown on PDA (13). However, not all isolates can be reliably identified based on colony morphology alone (25, 26), and alternative criteria have been developed to distinguish atypical isolates (16, 26, 31). Although our study only included isolates from Michigan and a few from Wisconsin, it illustrates the potential value of these techniques for evaluating *Monilinia* species on tree fruit crops in other regions. An advantage of these PCR-based techniques is that they permit the identification of isolates with altered morphology and growth characteristics, while confirming the identity of typical isolates.

Based on our examination of ITS1 sequences from local isolates of *Monilinia*, there can be little doubt that *M. laxa* as well as *M. fructicola* occur in Michigan and Wisconsin cherry orchards. Our finding of limited intraspecific variation in the ITS1 of *M. laxa* and of *M. fructicola* is consistent with data recently reported for *M. fructicola* from Canada, *M. laxa* from Italy and Norway, and *M. fructigena* from Denmark and Norway (15). Our sequence analysis confirms that only a few nucleotide differences exist between these species (3, 15). Although ITS1 sequences for *M. laxa* and *M. fructicola* differed by only three nucleotides, we were able to exploit this variation to differentiate the two species after *Mse*I digestion of PCR amplification products. We have examined available ITS1 sequence data for *M. fructigena* (3, 15) and found that ITS1 DNA from this species should yield a longer *Mse*I restriction fragment than either *M. laxa* or *M. fructicola*. Therefore, *Mse*I analysis should differentiate the three brown rot fungi in the section Junctoriae found on stone and pome fruits worldwide.

Fig. 4. Arbitrarily primed PCR amplification of genomic DNA from *Monilinia fructicola* and *Monilinia laxa* using microsatellite primers $(GACA)_4$ (A) and $(GTG)_5$ (B). Lane M, 100-bp ladder or empty; lanes 1–4, *M. fructicola* isolates SC-41, KK-20, JB-13, and CB-2, respectively; and lanes 5–8, *M. laxa* isolates DG-8, NP-1, WI-2, and Grants, respectively. PCR products in Figs. 4A and 4B were separated by electrophoresis through 1.5% agarose gels.



Group I introns occur in rDNA of a diverse range of fungi and other organisms. Their discovery is relatively new and their relationship to one another is poorly known. The identification of a group I intron in the SSU rRNA gene of all isolates of *M. fructicola* regardless of their geographic origin in Michigan indicates that it is a highly conserved character for *M. fructicola*. Its detection in an isolate collected in 1975 also suggests that it is a stable molecular marker. Although the presence of this intron appears to be a specific character for the identification of *M. fructicola* from Michigan stone fruit orchards, our study needs to be extended to isolates from other regions to establish its presence in populations of *M. fructicola* worldwide. Coincident with our earlier report (30), the intron was reported from isolates of *M. fructicola* from Australia, New Zealand, Japan, Portugal, and the United States (11). As more isolates are examined, it is possible that a population of *M. fructicola* lacking the intron may be found.

The ap-PCR data presented here indicate that *M. fructicola* may possess little intraspecific variation; however, this result was unexpected because of the ability of *M. fructicola* to outcross (8, 28). The finding of genetically identical individuals by ap-PCR suggests that either asexual propagation is occurring in Michigan or our sample was not adequate to detect occasional instances of sexual reproduction. Better genetic markers, markers that might reveal considerable variation within orchard populations of *M. fructicola*, will be needed to obtain more detailed knowledge about genetic diversity and population structure of *M. fructicola* in Michigan orchards.

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