Research Article

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Characterization of the *cytochrome b* (*cyt b*) gene from *Monilinia* species causing brown rot of stone and pome fruit and its significance in the development of QoI resistance

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Abstract

BACKGROUND: Quinone outside inhibitor (QoI) resistance as a consequence of point mutations in the *cytochrome b* (*cyt b*) gene has been reported in numerous plant pathogenic fungi. To examine the potential for QoI resistance development in those *Monilinia* species causing brown rot of stone and pome fruits [*Monilinia fructicola* (G Winter) Honey, *M. laxa* (Aderhold & Ruhland) Honey and *M. fructigena* (Aderhold & Ruhland) Honey], an examination was made of the sequence and exon/intron structure of their *cyt b* genes for the presence of any point mutations and/or introns commonly associated with resistance to QoIs in fungal plant pathogens.

RESULTS: None of the point mutations typically linked to QoI resistance was present in any of the *Monilinia* isolates examined. Furthermore, the *cyt b* genes from *M. fructicola* and *M. laxa*, but not *M. fructigena*, possessed a group-I-like intron directly after codon 143. Based on the results obtained, a simple PCR assay using a single primer pair was developed, allowing discrimination between the three *Monilinia* species without the need for culturing.

CONCLUSIONS: Results suggest that resistance to QoI fungicides based on the G143A mutation is not likely to occur in *M. fructicola* or *M. laxa*. Conversely, *M. fructigena* may be at higher risk for developing QoI resistance owing to the absence of a G143-associated intron.

Keywords: *cytochrome b Monilinia* spp.; brown rot; Qo inhibitor sensitivity; G143A; exon/intron organization

1 INTRODUCTION

Monilinia fructicola (G Winter) Honey, *M. laxa* (Aderhold & Ruhland) Honey and *M. fructigena* (Aderhold & Ruhland) Honey are fungal plant pathogens that cause brown rot disease of *Prunus* spp., as well as *Malus* and *Pyrus* spp., and result in significant pre- and post-harvest crop losses worldwide. *Monilinia fructicola* is the most prevalent species in the Americas, Australia and Japan,[1–4] although it has also recently emerged in several European countries.[5–7] *Monilinia fructigena* is currently believed to be limited to Europe and parts of Asia. By comparison, *M. laxa* has a more cosmopolitan distribution and often coexists with the other two *Monilinia* species responsible for brown rot.[3,8,9]

At present, single-site specific fungicides such as sterol demethylation inhibitor (DMI) and quinone outside inhibitor (QoI) fungicides are the most reliable means of managing brown rot epidemics.[10,11] However, owing to their highly specific mode of action, the repetitive use of site-specific fungicides fosters the development of resistant populations;[12,13] a consequence that could potentially obstruct the ability to manage brown rot of stone fruit. Indeed, resistance to DMI fungicides is becoming more prevalent in populations of *M. fructicola* throughout the eastern United States,[10,12,14–16] and QoI resistance has been reported in a broad range of other pathosystems, including *Mycosphaerella fijiensis* Morelet on banana,[17,18] *Podosphaera fusca* (Fr.) Braun & Shishkoff on cucurbit,[19] *Pyricularia grisea* (Sacc.) on ryegrass[20,21] and *Venturia inaequalis* (Cooke) Winter on apple.[22] In spite of several reports of commercial control failures using QoI fungicides and field isolates with reduced sensitivity to QoI fungicides in the eastern United States,[15] qualitative resistance has not yet been documented in any of the brown rot fungi. However, with the emergence of *Monilinia* populations exhibiting resistance to DMI fungicides and the limited number of viable fungicide alternatives to QoIs, selection pressure for the development of resistance to QoIs is almost certain to increase in the coming years.

QoI fungicides inhibit mitochondrial respiration by binding the outer quinone oxidizing pocket of the cytochrome bc_1 enzyme complex, which is encoded by the mitochondrial *cytochrome b* (*cyt b*) gene. This inhibition blocks the electron transfer process in the respiratory pathway and leads to an energy deficit due to a paucity of adenosine triphosphate.[23] A number of single nucleotide polymorphisms leading to amino acid substitutions

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in two conserved domains important for ligand binding within the cytochrome b transmembrane protein have been found to confer resistance to Qo inhibitors in a wide range of organisms, including bacteria, algae, yeast, protozoa and animals.[24–27] In most phytopathogenic fungi with reduced sensitivity to QoI fungicides recovered so far, resistance was conferred by single point mutations within the first of the two conserved domains (approximate amino acid positions 125–155). Isolates exhibiting the highest levels of resistance possessed a mutation that results in a switch from glycine to alanine at amino acid 143 (G143A).[17,28,29] While other amino acid substitutions, such as F129L[21,30–32] and G137R,[32] have also been found to impart reduced sensitivity to QoI fungicides, the magnitude of resistance that they confer appears to be lower than that generated by G143A (www.frac.info).

Interestingly, the presence of an intron immediately following the G143A site appears to prevent the development of the G143A mutation owing to its inhibition of intronic splicing in a number of species, including *Puccinia* spp., *Alternaria solani* Sorauer, *Pyrenophora teres* Dreschler and *Botrytis cinerea* Pers.; a trait that has been found to correlate with relatively low levels of resistance to QoI fungicides.[32–35] Therefore, not only the *cyt b* coding sequence but also the exon/intron structure appear to be important factors for predicting the evolution of resistance to QoI fungicides. To date, the *cyt b* genes from several pathogenic fungi have been sequenced and their exon/intron organizations have been characterized to evaluate the inherent risk of developing resistance to QoIs.[17,33,36,37] However, there is currently no information concerning this gene in *Monilinia* species. The aim of the present study was to analyze the sequence and structure of the *cyt b* genes from the three *Monilinia* species that cause brown rot of stone and pome fruit (*M. fructicola, M. laxa* and *M. fructigena*) in the context of QoI fungicide sensitivity. Furthermore, an attempt was made to develop a simple PCR assay based on the present results that distinguishes between the three *Monilinia* species examined by means of differences in intron lengths within the *cyt b* gene. The ability to quickly identify and differentiate between *M. fructicola, M. laxa* and *M. fructigena* could be used as a tool by researchers to indentify and map the prevalence of newly emerging species, and by inspection agencies to facilitate the screening of imported materials, thus preventing the introduction of invasive species. Additionally, this assay is vital to disease management programs in the northeastern United States, where recommended fungicides and application timings differ depending on the species present.

2 MATERIALS AND METHODS

2.1 Fungal isolates

Nine single-spore isolates of *M. fructicola* and six single-spore isolates of *M. laxa* collected from symptomatic fruit in 2006–2009 as part of a resistance monitoring effort[15] were used for experimentation (Table 1). Isolates were collected from sweet cherry (*Prunus avium* L.), tart cherry (*Prunus cerasus* L.) and peach [*Prunus persica* (L.) Batsch] orchards from four geographically separated populations within the primary stone fruit production regions of New York and Pennsylvania. All *Monilinia* isolates were selected from collections that represent the scope of QoI sensitivity and exposure for the region. Specifically, orchards and their corresponding *M. fructicola* and *M. laxa* isolates were classified into one of three categories: 'baseline', sensitive' or 'resistant' (Table 1). The 'baseline' isolates came from geographically isolated orchards with no history of exposure to fungicides. The 'sensitive' isolates were from orchards with previous exposure to QoI fungicides, but had achieved complete control of brown rot. The 'resistant' isolates were from orchards that developed more than 10% incidence of brown fruit rot following a successfully applied QoI fungicide. Isolates of other fungal species [*B. cinerea, V. inaequalis, Alternaria* sp., *Geotrichum candidum* Link, *Fusarium* sp., *Botryosphaeria obtusa* (Schwein.) Shoem. and *Penicillium expansum* Link] were collected locally from 2006–2009 at New York State Agricultural Experiment Station fruit research plantings in Geneva, New York.

All isolates were stored on silica gel (6–12 mesh) at 4 °C and maintained on 4% potato dextrose agar (PDA) at 25 °C in the dark as previously described.[11] Sensitivity of *M. fructicola* and *M. laxa* isolates to the QoI fungicide pyraclostrobin was confirmed by conducting mycelial relative growth assays.[11,38] Assays were carried out using PDA media amended with salicylhydroxamic acid (Acros Organics, Morris Plains, NJ) at 100 μg mL^{-1} and technical-grade pyraclostrobin (PESTANAL; Sigma-Aldrich Co., St Louis, MO) at a concentration 100 times the baseline EC_{50} (0.01 μg mL^{-1}) for New York.[15] Relative growth values were determined from two colonies of each isolate and three independent replicate plating events on different batches of media. Combined relative growth values for *M. fructicola* and *M. laxa* collections were analyzed for statistical differences using the Mann–Whitney rank sum test for non-parametric data with the statistical analysis components of SigmaPlot 11 (Systat Software Inc., San Jose, CA).

In addition to *M. fructicola* and *M. laxa* isolates from the United States, seven isolates of *M. fructigena* from orchards in the United Kingdom and continental Europe with no history of QoI insensitivity or control failures (Table 1) were also included in the study. Nucleic acids (DNA and RNA) for each isolate were kindly provided by Dr Antonieta De Cal (Department of Plant Protection, CIT-INIA, Madrid, Spain), Dr Themis Michailides (Department of Plant Pathology, University of California, Davis, CA), Dr Xiangming Xu (East Malling Research, East Malling, Kent, UK) and Dr Imre J Holb (Department of Plant Protection, University of Debrecen, Debrecen, Hungary).

Prior to experimentation, the species identity of each isolate included in the study was verified by DNA sequencing of the two internal transcribed spacer (ITS) regions and 5.8S gene in the nuclear ribosomal repeat using primers ITS1-F[39] and ITS4[40] as described previously.[41]

2.2 DNA and RNA extraction from fungal isolates

Genomic DNA was extracted from one-week-old mycelial cultures of *M. fructicola* and *M. laxa* isolates, as well as a selection of other fungi (e.g. *B. cinerea, V. inaequalis, Alternaria* sp., *G. candidum, Fusarium* sp., *B. obtusa* and *P. expansum*), using the MasterPure Yeast DNA Extraction Kit according to the manufacturer's instructions (Epicentre Biotechnologies, Madison, WI). Additionally, *M. fructicola* and *M. laxa* DNA extractions were performed directly from brown rot lesions on both naturally infected and artificially inoculated fruit. In the latter case, individual sweet cherries (*P. avium*) were artificially inoculated with *M. fructicola* (isolate MBH24A) and *M. laxa* (isolate MbhMF08-14B) as described previously.[11] Naturally infected 'Montmorency' tart cherry fruit (*P. cerasus*) were collected from a commercial production orchard in Ontario County, New York. Sporulating lesions were scraped from the fruit surface using a scalpel, and genomic DNA was extracted directly from resulting tissues using the Ultraclean Soil DNA Isolation Kit (MO BIO Laboratories Inc.,

Table 1. *Monilinia fructicola, M. laxa* and *M. fructigena* isolates utilized in this study and their relative growth rates on medium amended with pyraclostrobin

Species	Isolate	Orchard designation	Relative growth (%) (±SEM)[a]	Resistance Status[b]	Origin[c]	Year[d]
M. fructicola	MBH13B	MBH	25.6(±0.2)	Baseline	Sweet cherry 'Black Gold'/ Wayne County, NY	2008
	MBH24A	MBH	22.4(±0.1)	Baseline	Sweet cherry 'Black Gold'/ Wayne County, NY	2008
	MBH3B	MBH	26.6(±0.2)	Baseline	Sweet cherry 'Black Gold'/ Wayne County, NY	2008
	MBH12B	MBH	16.8(±0.1)	Baseline	Sweet cherry 'Black Gold'/ Wayne County, NY	2008
	ChocMF17	ChocMF	40.9(±0.1)	Resistant	Peach 'PF-25'/Lancaster County, PA	2009
	ChocMF56	ChocMF	44.7(±0.0)	Resistant	Peach 'Redhaven'/ Adams County, PA	2009
	ChocMF63	ChocMF	42.6(±0.3)	Resistant	Peach 'PF-25'/Lancaster County, PA	2009
	BitEBR08-1-1	EBR	36.9(±0.1)	Resistant	Tart cherry 'Surefire'/ Niagara County, NY	2008
	Peach7c	Peach7	22.7(±0.3)	Baseline	Peach Baby Gold #5/ Ontario County, NY	2006
M. laxa	EBRBa11b	EBR	14.4(±0.2)	Sensitive	Tart cherry 'Montmorency'/ Niagara County, NY	2008
	MbhMF08-14B	MBH	9.7(±0.3)	Baseline	Sweet cherry 'Black Gold'/ Wayne County, NY	2008
	TLRS3	TLR	0	Baseline	Sweet cherry 'Hedelfingen'/ Ontario County, NY	2006
	B.Sch#1	B.Sch	12.3(±0.1)	Sensitive	Tart cherry 'Balaton'/ Niagara County, NY	2009
	B.Sch#2	B.Sch	9.3(±0.2)	Sensitive	Tart cherry 'Balaton'/ Niagara County, NY	2009
	B.Sch#4	B.Sch	8.5(±0.1)	Sensitive	Tart cherry 'Balaton'/ Niagara County, NY	2009
M. fructigena	ES41	ES	N/A	N/A	Apple/Spain	1996
	ES49	ES	N/A	N/A	Plum/Spain	1996
	apple7	appleE	N/A	N/A	Apple/UK	1999
	apple13	appleE	N/A	N/A	Apple/UK	1999
	apple15	appleE	N/A	N/A	Apple/UK	1999
	Mfg4-GY-A	MFG	N/A	N/A	Apple/Hungary	2009
	Mfg5-SP-A	MFG	N/A	N/A	Apple/Hungary	2009

[a] Sensitivity is expressed as percentage colony growth on medium amended with analytical-grade pyraclostrobin (1.0 μg mL^{-1}) relative to that on non-amended medium. Values are the means and standard errors with measurements made from two colonies for each isolate and three independent replicate plates from different batches of media.
[b] Practical resistance status for *M. fructicola* and *M. laxa* isolates on which sensitivity testing was performed. The category 'baseline' indicates that the isolate came from a geographically isolated orchard with no history of exposure to fungicides. The category 'sensitive' (% relative growth <30) indicates that the isolate had come from an orchard with past exposure to QoI fungicides, but complete control of brown fruit rot was achieved. The category 'resistant' (% relative growth >30) indicates that isolates were collected from an orchard that developed more than 10% incidence of brown rot following an successful application of a QoI fungicide.
[c] Indicates the stone or pome fruit host from which the isolates were harvested, as well as the county and state (or country) from which each isolate was collected.
[d] Indicates the year in which each fungal isolate was collected.

Carlsbad, CA) according to the manufacturer's directions. As a control, DNA was also extracted from the flesh of uninfected cherry fruit in the same manner.

Total RNA was isolated from five-day-old mycelial cultures of *M. fructicola* and *M. laxa* isolates using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. RNA integrity was verified by resolving on a 1% agarose gel. Quantification of all nucleic acids was carried out using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA).

2.3 Cloning of the full-length *cyt b* coding sequence

First-strand cDNA synthesis was carried out using 1 μg of total RNA from *M. fructicola* (isolates MBH12B and BitEBR08-1-1), *M. laxa* (isolates EBRBa11b and MbhMF08-14B) and *M. fructigena* (isolate Mfg4-GY-A) with an oligo-dT primer and Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's

Table 2. Primer sequences

Primer name	Sequence (5′ to 3′)
MoniliniaP450ATG-fwd	ATGAGAATTTTTAAAAGTCATCCCT
MoniliniaP450STOP-rev	TTATCTACTAGGCTTTTCTTTAGTTAATAC
B.fuckeliana321-fwd	ACATAGGAAGAGGTTTATATTATGG
B.fuckeliana559-rev	AGTTGCGTTATTAACAGAGAAACC
Laxa-exon3-fwd	TTACCTTACGGTCAAATGTCGCTA
Gena-4430-rev	CAAATAAATTTGGATGGACTCTCTG
P450exon6-1-rev	CTGTTTAAAGTTGCGTTATTAACAGAG
P450exon4-1-fwd	TGAATTGGAGGTGATATTGTTGAG
colaexon3-fwd	TTTACCTTACGGTCAAATGAGCCT
colaexon4-rev	AACTCAACAATATCACCTCCAATTCAT
laxaexon3-fwd	TTACCTTACGGTCAAATGTCGCTA
laxaexon4-rev	CAACAATATCTTGTCCAATTCATGGT
P450intron6-2-fwd	AGGTGAGTAGGAAATACAGATAAATG
P450intron6-2-rev	AGTTCAACTCAGATCTAAAGATACCTC

recommendations. Subsequent amplification of *cyt b*-specific cDNA was performed using 1.5 µL of cDNA and the Platinum PCR Supermix High Fidelity system in a final volume of 25 µL. Primers MoniliniaP450ATG-fwd and MoniliniaP450STOP-rev (see Table 2 for a full list of primer sequences) were designed to amplify the *cyt b* sequence from translational start codon to translational stop codon on the basis of the previously published *cyt b* sequence from the closely related *Botryotinia fuckeliana* Whet. (GenBank accession AB262970). In each case, reactions were incubated at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 50 °C for 30 s and 68 °C for 2 min, with a final elongation step of 68 °C for 4 min.

2.4 Screening of *cyt b* sequences for the presence of typical mutations conferring resistance to QoI fungicides

To determine whether the *cyt b* sequences contained typical mutations associated with QoI resistance in the remaining *Monilinia* isolates not utilized in the cloning of the full-length mRNA sequence (Table 1), cDNA was generated from total RNA as described in Section 2.3, and nucleotides 321–559 of *cyt b*-specific cDNA (corresponding to amino acids 107 to 186 and spanning the highly conserved region in which the G143A, F129L and G137R mutations occur) were amplified using primers B.fuckeliana321-fwd and B.fuckeliana559-rev. Alternatively, in the case of *M. fructigena* isolates for which RNA was not available, approximately 200 ng of genomic DNA was utilized as a template in place of cDNA in PCR assays with primers Laxa-exon3-fwd and Gena-4430-rev, which amplify a fragment spanning nucleotides 6971 to 7725 from the genomic *cyt b* sequence (including codons 133–163). Thermal cycling parameters were identical to those described in the previous section in each case.

2.5 Cloning of the full-length *cyt b* gene sequence

The full-length *cyt b* gene (from translational start codon to translational stop codon) was amplified from genomic DNA of *M. fructicola* (isolate Peach7c) and *M. fructigena* (isolate ES41) in a single PCR reaction using primers MoniliniaP450ATG-fwd and MoniliniaP450STOP-rev. In the case of *M. laxa* (isolate EBRBa11b), two successive PCR reactions were performed to retrieve the full-length *cyt b* gene sequence. Primers P450exon6-1-rev and P450exon4-1-fwd were designed on the basis of the *M. laxa cyt b* mRNA sequence and were utilized in conjunction with primers MoniliniaP450ATG-fwd and MoniliniaP450STOP-rev, respectively, to allow the generation of overlapping amplicons. All PCR amplifications were carried out with 200 ng of template DNA using the Platinum PCR Supermix High Fidelity system according to the manufacturer's recommendations, with the exception that each reaction was supplemented with 1 unit of Platinum *Taq* DNA polymerase High Fidelity (Invitrogen). Thermal parameters utilized were identical to those described for the cloning of *cyt b* cDNA, except for the elongation time, which was increased to 8 min, and the final elongation step, which was increased to 10 min.

2.6 Sequencing and bioinformatic analyses

All PCR products were gel purified using the Wizard SV Gel and PCR Clean-Up system (Promega, Madison, WI) and were either cloned into the pGEM-T easy vector (Promega) or sequenced directly. All sequencing was carried out using Big Dye Terminator chemistry and AmpliTaq-FS DNA Polymerase (Applied Biosytems, Foster City, CA) using the Applied Biosystems Automated 3730xl DNA Analyzer at the Cornell University DNA Sequencing facility in Ithaca, New York. In the case of the *cyt b* gene sequences, a primer walking strategy was utilized to sequence the respective clones owing to their large size (a complete listing of the primer sequences are available upon request). All nucleotide sequences were aligned manually using ClustalW[42] and BioEdit version 7.0.8.0,[43] and were compared with previously reported sequences using BLAST.[44] Coding regions were virtually translated using the EMBOSS Transeq program (http://www.ebi.ac.uk/Tools/emboss/transeq/index.html) with the yeast mitochondrial genetic code. The ORF Finder program (http://www.ncbi.nlm.nih.gov/projects/gorf/) was utilized with the yeast mitochondrial genetic code to predict open reading frames of nucleotide sequences within intronic regions; protein motifs were then deduced using the Conserved Domains program (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). Prediction of transmembrane domains within the cyt b amino acid sequence was carried out using the 'DAS' Transmembrane Prediction server (http://www.sbc.su.se/~miklos/DAS/)[45] with a strict cut-off of 2.2.

2.7 Analysis of the *cyt b* G143 genomic region for the presence of an intron in *Monilinia* isolates

To determine whether an intron was present directly following the G143 site in the remaining *Monilinia* isolates not utilized for cloning the full-length gene sequence (Table 1), primers were designed to anneal on either side of the G143 codon in each of the three species. Primers colaexon3-fwd and colaexon4-rev were utilized to amplify the G143 region from *M. fructicola* genomic DNA, while primers laxaexon3-fwd and laxaexon4-rev were used to amplify the same region from *M. laxa* and *M. fructigena* genomic DNA. PCR amplifications were carried out using 200 ng template DNA with the Platinum PCR Supermix system and the following thermal parameters: 94 °C for 2 min, 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 68 °C for 2.5 min, and a final elongation step of 68 °C for 4 min. Control reactions lacking template DNA were performed in each case. PCR products were resolved on a 1.5% agarose gel and visualized with ethidium bromide staining and UV illumination.

2.8 Development of PCR primers to discriminate between brown rot pathogens

Based on the sequence and exon/intron structure of the *cyt b* genes from *M. fructicola, M. laxa* and *M. fructigena*, the authors designed a pair of primers spanning differential insertion/deletion events in the three species, which are surrounded by highly conserved sequences. Primers P450intron6-2-fwd and P450intron6-2-rev anneal on either side of a variable region located in the sixth intron of *M. fructicola* (5′ primer annealing sites of 7465 and 8085, where 1 is the first nucleotide of the ATG codon of the *cyt b* gene) and *M. laxa* (5′ primer annealing sites of 9390 and 9890) and the seventh intron of *M. fructigena* (5′ primer annealing sites of 11 141 and 11 923) to amplify fragments of 621 bp from *M. fructicola*, 501 bp from *M. laxa* and 783 bp from *M. fructigena*. PCR assays were carried out using 200 ng of template DNA from six isolates of each species, respectively, with the Platinum PCR Supermix system and a thermal program of 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s and 68 °C for 1.5 min, with a final elongation step of 68 °C for 2 min. In each case, 2 µL of the PCR products was resolved on a 1% agarose gel and visualized using UV illumination. Three sets of primers shown in previous studies to distinguish between various *Monilinia* species were utilized as controls: primers MO368-5, MO368-8R, MO368-10R and Laxa-R2[46] were designed to be used in a multiplex reaction to generate differentially sized products for *M. fructicola* (537 bp), *M. laxa* (351 bp) and *M. fructigena* (402 bp); primers MLF2 and MLR2[47] were designed specifically to amplify a 375 bp fragment from *M. laxa*; primers IGenaS and IGenaAS[48] were designed exclusively to amplify a 500 bp product from *M. fructigena*.

To ascertain that the primers designed in this study were in fact specific to *Monilinia* species, PCR assays were also carried out using 200 ng of genomic DNA from other fungi commonly associated with fruit plantings in North America. Reactions utilizing primers ITS1-F[39] and ITS4,[40] which amplify products of similar size comprising the two internal transcribed spacer (ITS) regions and the 5.8S gene in the nuclear ribosomal repeat, were carried out as positive controls for DNA quality.

To determine whether primers P450intron6-2fwd and P450intron6-2-rev could be utilized rapidly to differentiate between *Monilinia* species without the need for culturing of the fungi, PCR assays were also carried out using 200 ng of DNA extracted directly from sporulating fruit lesions. *M. fructigena* fungal isolates were not accessible owing to the absence of the pathogen in the United States; therefore, rapid detection of this pathogen was instead evaluated by conducting PCR assays utilizing a 1 : 1 mixture of genomic DNA from cherry fruit (100 ng) and *M. fructigena* (100 ng) as template. To evaluate whether species differentiation was possible using this technique in the case of combined populations of *M. fructicola* and *M. laxa*, which have been found to coexist within the same brown rot population in New York,[9] PCR amplification was executed using DNA extracted from a cherry co-inoculated with *M. fructicola* (isolate MBH24A) and *M. laxa* (isolate MbhMF08-14B) as template. Amplification utilizing DNA extracted from uninfected cherry flesh was included as a negative control. The assay was also conducted on naturally infected 'Montmorency' cherry fruit to ensure that artificial inoculation did not confound the presence of fruit surface microbes. In each case, the PCR parameters were identical to those described for use with DNA isolated from pure mycelial cultures.

3 RESULTS

3.1 Sensitivity of *Monilinia fructicola* and *Monilinia laxa* isolates to pyraclostrobin

Mean relative growth values for *M. fructicola* isolates on media amended with 1.0 µg mL^{-1} of pyraclostrobin ranged from 16.8 to 44.7%, whereas values for *M. laxa* isolates ranged from 0 to 14.4% (Table 1). These differences between the combined relative growth values of *M. fructicola* and *M. laxa* isolates were significant ($P = 0.002$). Moreover, in orchards that contained both *M. fructicola* and *M. laxa* isolates (e.g. the MBH orchard in Wayne County and the EBR orchard in Niagara County), the relative growth values of *M. laxa* isolates were consistently lower than those of *M. fructicola*.

3.2 Analysis and comparison of the *cyt b* coding sequences from *Monilinia fructicola, Monilinia laxa* and *Monilinia fructigena*

The full-length coding regions of the *cyt b* genes from *M. fructicola* isolates MBH12B (GenBank accession GU952 814) and BitEBR08-1-1 (GenBank accession GQ423059), *M. laxa* isolates EBRBa11b (GenBank accession GU952 816) and MbhMF08-14B (GenBank accession GQ423060) and *M. fructigena* isolate Mfg4-GY-A (GenBank accession GU952 818) were obtained by PCR amplifying *cyt b*-specific cDNA utilizing primers designed to anneal at the translational start and stop codons, respectively, based on the same gene from the closely related species *B. fuckeliana* (GenBank accession AB262970). All of the *cyt b* coding sequences from the three *Monilinia* species tested were 1176 bp in length and exhibited a minimum of 96.1% identity (*M. fructicola*) at the nucleotide level with the *cyt b* coding region of *B. fuckeliana* (Table 3). In addition, the *cyt b* nucleotide sequences were highly conserved among the three *Monilinia* species. While those from *M. fructicola* and *M. fructigena* exhibited the least identity (97.5%), those of *M. laxa* and *M. fructigena* displayed the highest degree of similarity, with 99.1% identity at the nucleotide level (Table 3).

Deduced cyt b protein sequences from all three *Monilinia* species were 391 amino acids in length and conformed to the genetic code for mitochondrial genes in other fungi.[49,50] While the cyt b protein sequence of *M. fructicola* displayed the least identity with that of *B. fuckeliana* (99.5%), those of *M. fructigena* and *B. fuckeliana* were identical (Table 3). Among the three *Monilinia* species, *M. laxa* and *M. fructigena* shared the highest level of identity at the amino acid level (99.7%) while *M. fructicola* and *M. laxa* shared the lowest (99.2%) (Table 3), with only a total of three amino acid substitutions between the three protein sequences (Fig. 1).

In silico analyses of the hydropathy profiles for each of the three *Monilinia* cyt b amino acid sequences indicated that their predicted transmembrane folding patterns were very similar to those of *cyt b* genes in other organisms.[25,51] Each protein was predicted to comprise eight transmembrane helices connected by seven extramembrane loops. To date, the majority of mutations affecting QoI sensitivity have been found to be located within transmembrane helix C and the cd loop of the cyt b protein between amino acids 125–155.[25,52] Interestingly, two of the three amino acid substitutions (species specific) observed between the three *Monilinia* cyt b protein sequences were located in very close proximity to this region (amino acids 154 and 159) (Fig. 1).

To determine whether this region of the three *Monilinia cyt b* genes possessed any of the typical mutations associated with the development of QoI resistance in other fungal species, fragments including codons 129, 137 and 143 were examined from a selection of *Monilinia* isolates from different geographical locations (a total

Table 3. Nucleotide and amino acid identities between *cyt b* coding regions and putative proteins from *Monilinia fructicola, M. laxa*, and *M. fructigena*, as well as the closely related reference fungus *Botryotinia fuckeliana*

		Nucleotide identity (%)			
	Species	*M. fructicola*	*M. laxa*	*M. fructigena*	*B. fuckeliana*
Amino acid identity	*M. fructicola*	–	98.0[a]	97.5	96.1
	M. laxa	**99.2**[b]	–	99.1	97.2
	M. fructigena	**99.5**	**99.7**	–	96.7
	B. fuckeliana	**99.5**	**99.7**	**100**	–

[a] Numbers in regular type indicate the percentage nucleotide identity between the 1176 bp mRNA sequences.
[b] Numbers in bold type indicate the percentage amino acid identity between the 391 amino acid protein sequences.

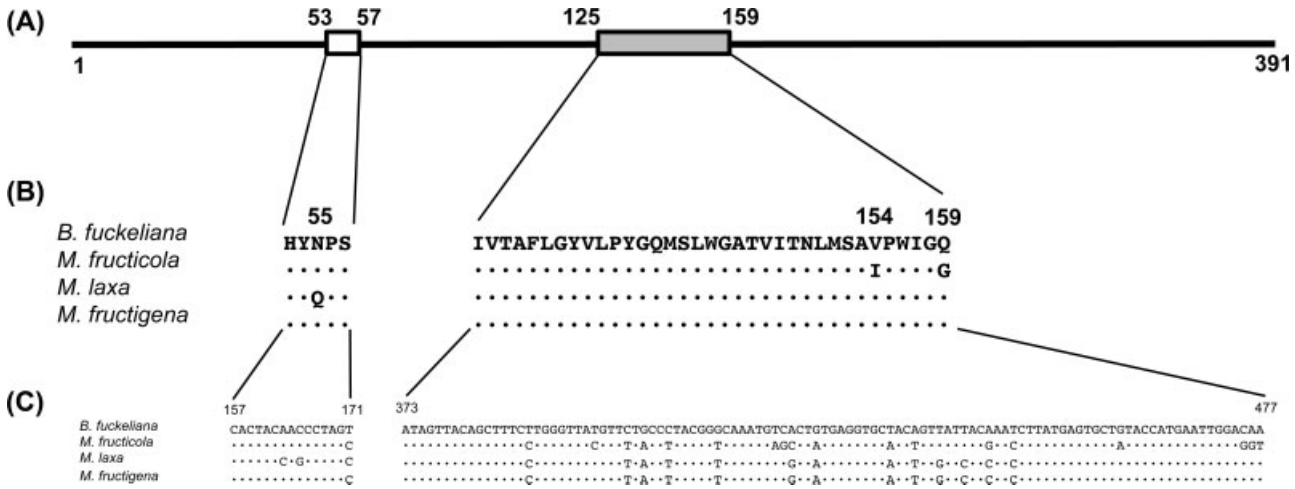

Figure 1. Species-specific amino acid substitutions and corresponding nucleotide differences in *cyt b* sequences from *Monilinia fructicola, M. laxa* and *M. fructigena*, as well as the closely related reference fungus *Botryotinia fuckeliana*. A schematic diagram of the cyt b protein with boxes denoting regions containing amino acid substitutions between the various species is shown in (**A**). Amino acid (**B**) and nucleotide (**C**) residues are shown within regions in which species-specific variations at the amino acid level were observed between the four species analyzed, including the region in which the majority of amino acid substitutions conferring resistance to QoI fungicides are known to occur (amino acid positions 125–159; gray box). Species-specific amino acid variations were observed at positions 55 (*M. laxa*), as well as positions 154 and 159 (*M. fructicola*). Variations in nucleic acid residues were far more numerous, especially in the region corresponding to amino acids 125–159; however, in the majority of cases they did not translate into amino acid substitutions. Amino acid positions are in bold, while nucleotide residues are in regular type. Dots represent residues that are identical to the corresponding residue in *B. fuckeliana*.

of nine *M. fructicola*, six *M. laxa* and seven *M. fructigena*). In every case, the G143A, F129L and G137R mutations were absent from the isolates tested (data not shown).

3.3 Analysis and comparison of *cyt b* exon/intron organization in *Monilinia fructicola, Monilinia laxa* and *Monilinia fructigena*

To enable a comparison of the *cyt b* exon/intron structure in *M. fructicola, M. laxa* and *M. fructigena*, the authors cloned full-length gene fragments (from the translational start codon to the translational stop codon) and determined their sequences utilizing a primer walking strategy. Fragments of 11 951 bp, 13 505 bp and 13 556 bp in length were successfully amplified from *M. fructicola* (GenBank accession GU952 815), *M. laxa* (GenBank accession GU952 817) and *M. fructigena* (GenBank accession HM149254) genomic DNA respectively. In each case, the intron/exon junctions were identified through comparison with their corresponding mRNA sequences (Fig. 2).

The *cyt b* gene sequences from *M. fructicola* and *M. laxa* were very similar to one another in their exon/intron organization; however, they differed more dramatically from those of both *M. fructigena* and the phylogenetically related *B. fuckeliana* (Fig. 2). While the *cyt b* gene from *B. fuckeliana* was composed of five exons and four introns, those of the three *Monilinia* species included eight exons and seven introns. Although 5′ portions of each *cyt b* gene examined were relatively similar in sequence and structure, the vast difference in their lengths (approximately 6.6 kb for *B. fuckeliana* and over 11.9 kb for the three *Monilinia* species) was mainly the result of structural variations near their 3′ termini. While the most 3′ 690 bp of the *B. fuckeliana cyt b* gene sequence comprised a single exon, this same region was divided into four exons and three introns in *M. fructicola* and *M. laxa*, or three exons and two introns in *M. fructigena*, with corresponding nucleotide lengths of 6.1 kb (*M. fructicola*), 5.7 kb (*M. laxa*) and 4.3 kb (*M. fructigena*).

In the case of *M. fructicola* and *M. laxa*, two regions of very high nucleotide identity (>97%) were present in the *cyt b* genes between nucleotides 1–3342 and 6904–9343 in *M. fructicola* and nucleotides 1–4321 and 8826–11 140 in *M. laxa* respectively. However, in spite of the considerable levels of homology in these two regions, both included various differential insertions/deletions. For example, in the most 5′ conserved region,

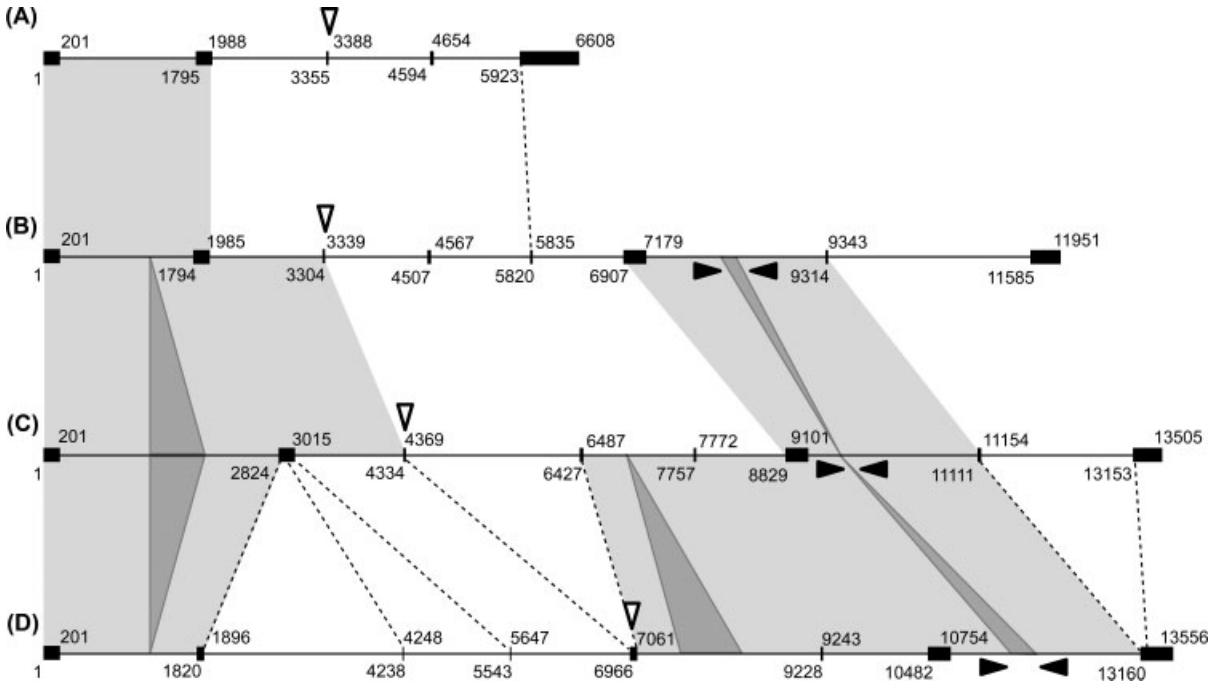

Figure 2. Genetic structures of the *cyt b* gene sequences from *Monilinia fructicola, M. laxa* and *M. fructigena*, as well as the closely related reference fungus *Botryotinia fuckeliana*. Structural representations of the *cyt b* genes from *B. fuckeliana* (**A**), *M. fructicola* (**B**), *M. laxa* (**C**) and *M. fructigena* (**D**) are shown. Boxes denote exonic sequences, while lines signify introns. Numbers indicate nucleotide positions of exons. Light-gray areas indicate regions of homology (exhibiting >97% nucleotide identity) between the different species. Dark-gray areas indicate relative insertion/deletion events between the various species. Dashed lines denote differences in exon/intron organization between the various species. Vertical open arrows show sites of the G143 codon, while horizontal solid arrows indicate the annealing sites of primers P450intron6-2-fwd and P450intron6-2-rev.

a 1028 nucleotide insert was present at position 1273 in *M. laxa*, while, in the most 3′ conserved region, 18 and 101 nucleotide inserts were present at positions 7550 and 7652 in *M. fructicola*. In addition, the lengths of exons 7 and 8 differed between the two *Monilinia* species (Figs 2B and C Table 4).

In contrast, the exon/intron organization of the *M. fructigena cyt b* gene sequence differed considerably from those of *M. fructicola* and *M. laxa* (Fig. 2; Table 4). While it comprised the same number of exons and introns as the *cyt b* genes from the other two *Monilinia* species tested and contained similar highly conserved 5′ (spanning nucleotides 1–1896) and 3′ (spanning nucleotides 7002–13 204 and 6428–11 155 in *M. fructigena* and *M. laxa* respectively, or nucleotides 10 479–13 193 and 6904–9347 in *M. fructigena* and *M. fructicola* respectively) regions, the positioning of intronic sequences was distinct (Fig. 2). For instance, the *cyt b* gene from *M. fructigena* consisted of three separate exons (exons 2–4) in the region corresponding to the single exon 2 in *M. fructicola* and *M. laxa*, while the region corresponding to exons 7 and 8 in *M. fructicola* and *M. laxa* were combined into a single exon in *M. fructigena* (exon 8). Furthermore, the region corresponding to exons 3 and 4 in *M. fructicola* and *M. laxa* were combined into a single exon in *M. fructigena* (exon 5). Intriguingly, while exons 3 and 4 were separated by an intron (intron III) directly after codon 143 in both *M. fructicola* and *M. laxa*, as well as *B. fuckeliana*, this same intron was lacking in *M. fructigena* (Fig. 2; Table 4).

To determine whether an intron was present immediately following the G143 site in a selection of *Monilinia* isolates from different geographical locations, PCR amplifications were carried out with genomic DNA from the remaining isolates utilized in this study using primers that anneal on either side of the G143 site. In the absence of an intron, a 92 bp fragment from *M. fructicola* and 87 bp fragments from *M. laxa* and *M. fructigena* would be expected. However, all *M. fructicola* and *M. laxa* isolates tested generated 1258 bp and 2144 bp products (Figs 3A and B) respectively, which corresponded in size to the presence of their respective

Table 4. Locations of exonic regions within *cyt b* gene sequences in *Monilinia fructicola, M. laxa* and *M. fructicola*, as well as the closely related reference fungus *Botryotinia fuckeliana*

	M. fructicola	*M. laxa*	*M. fructigena*	*B. fuckeliana*
Exon 1	1–201[a]	1–201	1–201	1–201
	1–67[b]	**1–67**	**1–67**	**1–67**
Exon 2	1794–1985	2824–3015	1820–1896	1795–1988
	68–131	**68–131**	**68–93**	**68–131**
Exon 3	3304–3339	4334–4369	4238–4248	3355–3388
	132–143	**132–143**	**93–96**	**132–143**
Exon 4	4507–4567	6427–6487	5543–5647	4594–4654
	144–164	**144–164**	**96–131**	**144–164**
Exon 5	5820–5835	7757–7772	6966–7061	5923–6608
	164–169	**164–169**	**131–164**	**164–391**
Exon 6	6907–7179	8829–9101	9228–9243	–
	169–260	**169–260**	**164–169**	
Exon 7	9314–9343	11 111–11 154	10 482–10 754	–
	260–270	**260–275**	**169–260**	
Exon 8	11 585–11 951	13 153–13 505	13 160–13 556	–
	270–391	**275–391**	**260–391**	

[a] Numbers in regular type indicate the nucleotide locations of exonic regions.
[b] Numbers in bold type indicate the corresponding codon positions of exonic regions.

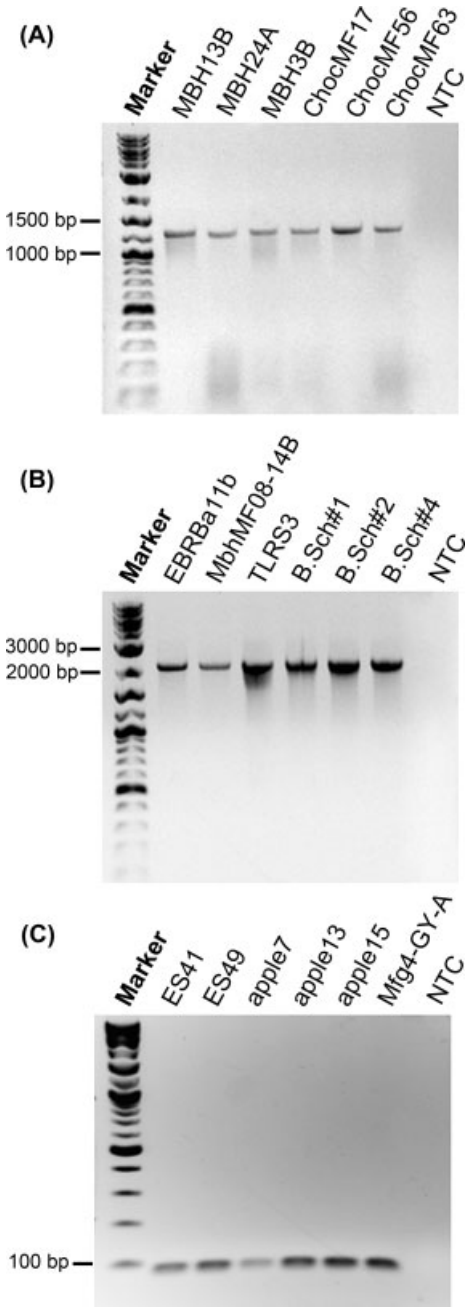

Figure 3. Analysis of *cyt b* gene sequences for the presence of G143-associated introns in *Monilinia fructicola, M. laxa* and *M. fructigena*. Amplicons from PCR assays utilizing primers that anneal on either side of the G143 codon are shown for six representative isolates of *M. fructicola* (**A**), *M. laxa* (**B**) and *M. fructigena* (**C**) respectively. All *M. fructicola* and *M. laxa* isolates tested yielded bands consistent in size with fragments including the G143-associated intron. Conversely, all *M. fructigena* isolates analyzed were found to lack this intron. No template controls (NTCs) were included in each case.

G143-associated intronic sequences (1168 bp and 2058 bp). Conversely, amplicons derived from *M. fructigena* isolates were consistent in size with fragments lacking this intron (Fig. 3C), which confirmed original analyses of the *cyt b* gene sequence from this species (Fig. 2D; Table 4).

Sequence analyses of intron III from *M. fructicola* and *M. laxa* indicated that in both cases they contained open reading frames corresponding to 259 and 212 amino acids, respectively, bearing regions (nucleotides 3529–4050 in *M. fructicola* and 5480–5917 in *M. laxa*) with homology to domains present in mRNA maturase/endonuclease proteins. Putative start codons were located 14 nucleotides upstream (*M. fructicola*) and 997 nucleotides downstream (*M. laxa*) of the 5′ splice sites of intron III in each case.

3.4 Development of PCR primers for the discrimination of *Monilinia fructicola, Monilinia laxa* and *Monilinia fructigena*

Based on the *cyt b* sequence and exon/intron structure of the three *Monilinia* species tested in this study, a pair of primers were designed that annealed to highly conserved regions spanning insertion/deletion events within the sixth intron of *M. fructicola* and *M. laxa*, or the seventh intron of *M. fructigena* (Fig. 2). For every isolate tested, this primer pair allowed discrimination between *M. fructicola, M. laxa* and *M. fructigena* in a single PCR amplification through the generation of 621 bp, 501 bp and 783 bp fragments respectively (Fig. 4A). Three sets of additional primers shown previously to distinguish between *Monilinia* species were utilized as controls: primers MO368-5, MO368-8R, MO368-10R and Laxa-R2[46] were utilized in a multiplex reaction to discriminate between *M. fructicola, M. laxa* and *M. fructigena*; primers MLF2 and MLR2[47] were used specifically to amplify a 375 bp product from *M. laxa*; primers IGenaS and IGenaAS[48] were used exclusively to amplify a 500 bp product from *M. fructigena*. Interestingly, while control primers IgenaS and IgenaAS were successful in their ability to amplify an appropriately sized product solely from *M. fructigena*, primers MLF2 and MLR2 amplified a 375 bp fragment not only from the *M. laxa* isolates but also from all six isolates of *M. fructigena* tested in this study. Furthermore, control primers utilized in the multiplex reaction[46] amplified a 351 bp product from *M. laxa* isolates, a 402 bp product from *M. fructigena* isolates and a 537 bp fragment from the three *M. fructicola* isolates harvested from diseased cherries in New York. However, the same multiplex reaction generated no products from any of the three *M. fructicola* isolates collected from a peach orchard in Pennsylvania (Fig. 4A).

To ascertain that the present primers were specific to *Monilinia* species, PCR amplifications were also carried out using template DNA from a variety of other fungal pathogens commonly found in the primary types of fruit production operations in this region of the United States (e.g. apples and stone fruit). While amplification products of the expected size were observed for the three *Monilinia* species tested, no amplification products were produced for any of the other fungal species analyzed. Conversely, positive control amplifications of the rDNA cluster containing the ITS region and the 5.8S gene resulted in the generation of appropriately sized products in all instances (Fig. 4B), confirming the integrity of the template DNA.

PCR assays using primers P450intron6-2-fwd and P450intron6-2-rev were also conducted using *M. fructicola* and *M. laxa* genomic DNA extracted directly from infected fruit as template, whereas *M. fructigena* DNA was mixed in a 1 : 1 ratio with genomic DNA from cherry fruit owing to the absence of the pathogen in the United States. Amplicons identical in size to those generated from genomic DNA isolated from mycelial cultures were produced for all three *Monilinia* species tested, while genomic DNA extracted from uninfected cherry fruit yielded no amplification product (Fig. 4C). Similarly, DNA extracted from naturally infected fruit produced amplicons identical to those obtained from cultures and artificially infected fruit (data not shown). Furthermore, the same primers were found to be capable of successfully discriminating between

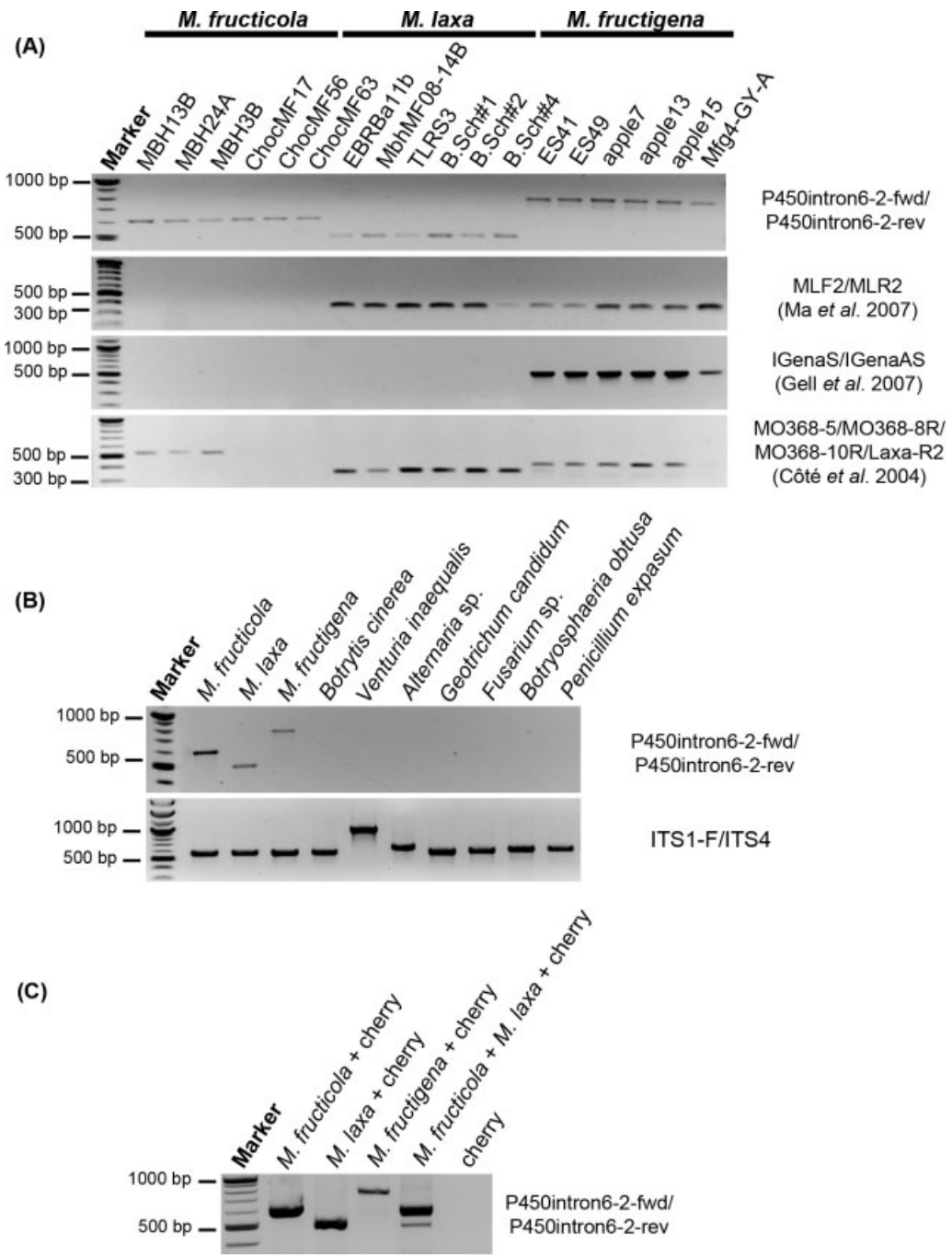

Figure 4. Ability of a novel set of primers to identify and differentiate between *Monilinia fructicola, M. laxa* and *M. fructigena*. (**A**) Primers P450intron6-2-fwd and P450intron6-2-rev, which anneal to highly conserved regions on either side of deletion/insertion events located within the sixth intron of the *cyt b* gene from *M. fructicola* and *M. laxa*, and the seventh intron of *M. fructigena*, were utilized to amplify fragments of 621 bp from *M. fructicola*, 501 bp from *M. laxa* and 783 bp from *M. fructigena*. Three sets of primers shown in previous studies to distinguish between various *Monilinia* species were utilized as controls. (**B**) The specificity of primers P450intron6-2-fwd and P450intron6-2-rev was tested by conducting PCR assays on a variety of other fungi commonly associated with stone and pome fruit. (**C**) To assess whether primers P450intron6-2-fwd and P450intron6-2-rev could be utilized to differentiate between *Monilinia* species without the need for culturing of the fungi, PCR tests were performed using *M. fructicola* and *M. laxa* DNA extracted directly from sporulating lesions on sweet cherries as template. *M. fructigena* DNA was combined in a 1 : 1 ratio with cherry DNA to achieve the same effect. Mixed infections containing both *M. fructicola* and *M. laxa* were also amplified directly from lesions on sweet cherries, simultaneously generating both 621 bp and 501 bp bands. DNA from uninfected sweet cherry flesh was utilized as a negative control.

M. fructicola and *M. laxa* in a single reaction, as would be the case in a mixed infection. In this instance, fragments of 621 bp and 501 bp (corresponding to *M. fructicola*- and *M. laxa*-specific products respectively) were simultaneously amplified when DNA from co-inoculated fruit was utilized as template (Fig. 4C).

4 DISCUSSION

In recent years, reports of QoI fungicide resistance resulting from point mutations in the *cyt b* gene have been described for an increasing number of phytopathogenic fungi.[17–22,31] In an effort to predict the potential for QoI resistance development in the

three most common *Monilinia* species causing brown rot of stone and pome fruits (*M. fructicola, M. laxa* and *M. fructigena*) in North America and Europe, the authors analyzed both the sequence and exon/intron structure of the *cyt b* genes from each species to reveal the presence of any typical point mutations and/or introns commonly associated with sensitivity to QoI fungicides.

In all three *Monilinia* species tested, the *cyt b* mRNA sequences were highly conserved, with *M. laxa* and *M. fructigena* exhibiting the most identity at the nucleotide level (99.1%) (Table 3). Similarly, comparison of deduced amino acid sequences indicated that *M. laxa* and *M. fructigena* shared the highest degree of identity (99.7%) (Table 3). Sequence analyses of the *cyt b* coding regions from all of the *M. fructicola, M. laxa* and *M. fructigena* isolates utilized in this study (Table 1) indicated that in no instance were mutations leading to the replacement of glycine with alanine at amino acid 143 (G143A), which has been found to confer strong resistance to QoI fungicides in a number of plant pathogens,[17,28,29] present. In addition, all of the isolates tested lacked mutations that would result in the F129L[21,30–32] and G137R[32] substitutions, which have been suggested to confer a lower level of QoI resistance than the G143A mutation. These findings were not completely unexpected, as none of the *M. fructicola* and *M. laxa* isolates utilized in this study exhibited a qualitative resistance QoI phenotype (i.e. equivalent growth on amended and non-amended media) towards the fungicide pyraclostrobin (Table 1). Indeed, qualitative resistance to QoI fungicides has not been reported for any *Monilinia* species to date. However, several isolates of *M. fructicola* (e.g. those from the ChocMF and EBR orchards) demonstrated reduced sensitivity towards pyraclostrobin; a response associated with a quantitative phase of QoI resistance previously described in both *Monilinia*[15,53] and the causal agent of apple scab, *Venturia inaequalis*.[54]

Structurally, the genomic *cyt b* sequence of the three *Monilinia* species (eight exons and seven introns) differed considerably from that of the closely related species *B. fuckeliana* (five exons and four introns) (Fig. 2). While the exon/intron organization of the *M. fructicola* and *M. laxa cyt b* genes were similar to each other, that of *M. fructigena* was unique (Fig. 2). Interestingly, both *M. fructicola* and *M. laxa* included an intron (intron III) directly following the G143 site in the *cyt b* sequence (Figs 2, 3A and 3B; Table 4). In both instances, the exonic base preceding the 5′ splice site was a T, and the intronic base immediately upstream of the 3′ splice site was a G, as is characteristic for group I introns in other organisms.[55] In addition, the third intron of both *M. fructicola* and *M. laxa* contained open reading frames exhibiting high levels of homology with an mRNA maturase; a trait that is commonly associated with group I introns and is essential for splicing.[56] It has been suggested previously that the presence of a group I intron directly after the codon for glycine at amino acid position 143 affects the occurrence of point mutations leading to the G143A mutation, and hence the development of qualitative resistance to QoI fungicides.[33] As exonic sequences near the splice sites of group I introns play a crucial role in mRNA splicing and subsequent translation,[55–58] codon 143 from the *cyt b* gene of species containing the intron is very likely required for intronic splicing. Therefore, it has been suggested that the nucleotide substitution necessary for the G143A mutation to occur would have serious consequences on intronic excision, and would almost certainly be lethal.[33] Accordingly, there appears to be a negative correlation between the presence of this intron and the likelihood of evolving resistance to QoI fungicides, which is the case in a number of species, including *Puccinia* spp., *Alternaria solani, Pyrenophora teres* and *Botrytis cinerea*.[32–35] In contrast, pathogens such as *Alternaria alternate* (Fr.) Keissler, *Blumeria graminis* Speer, *Magnaporthe grisea* (Hebert) Barr., *Mycosphaerella fijiensis, M. graminicola* (Fuckel) Schroter, *V. inaequalis* and *Plasmopara viticola* Berl. & de Toni, for which resistance to QoI fungicides caused by the G143A mutation is well documented, do not bear an intron at this site.[33] This is also the case for *M. fructigena*, in which there is no intron within 35 bp of codon 143 (Figs 2 and 3C; Table 4), suggesting that this species may be at risk of evolving the G143A mutation and associated high levels of resistance to QoI fungicides.

The presence of a group I intron immediately following codon 143 in *M. fructicola* and *M. laxa* implies that these species may be less prone to developing resistance to QoI fungicides than *M. fructigena*. However, it has been shown previously that populations of *B. cinerea* and *P. teres* may possess two different *cyt b* genotypes: one with and one without the G143-associated group I intron.[32–35,59] While the present analyses indicated no such variations in the presence of intron III in *M. fructicola* and *M. laxa* isolates obtained from various orchards in New York and Pennsylvania, the existence of such disparities in other geographical regions cannot be precluded for certain. Furthermore, the development of distinct mechanisms that confer qualitative resistance to QoIs cannot be ruled out at this point. For example, while the F129L and G137R mutations were not observed in any of the isolates tested in this study, the possibility exists for their evolution, as has been the case with the F129L mutation in *A. solani*.[31] Moreover, the activation of an alternative oxidase (AOX) has been shown to be responsible for a reduction in sensitivity of *M. graminicola* to QoI fungicides,[60] while an intraspecific polymorphism in the *cyt b* gene has been described in *Saccharomyces cerevisiae* Meyer ex Hansen;[61] mechanisms that both have the potential to occur in *M. fructicola* and *M. laxa*.

With the amount of fresh fruit imported and exported worldwide, it is becoming a challenge to prevent the spread of brown rot pathogens. Indeed, while *M. fructicola* is considered a quarantined species in Europe, it has now been detected in various European countries.[5–7] To the best of the authors' knowledge, North America remains free of *M. fructigena* as of yet, but the development of expeditious methods for the identification and differentiation of *M. fructicola, M. laxa* and *M. fructigena* will be critical for the prevention of further spread of these pathogens. Methods based on molecular biology techniques are becoming the industry standard for this purpose,[62] as identification of these species on the basis of morphological characteristics is difficult.[63] In the present study, a primer pair (P450intron6-2-fwd and P450intron6-2-rev) was designed that annealed to highly conserved regions within the sixth intron in *M. fructicola* and *M. laxa* and the seventh intron in *M. fructigena*, which contain various differential insertion/deletion events. These primers allowed discrimination between the three species in a single PCR amplification through the generation of 621 bp (*M. fructicola*), 501 bp (*M. laxa*) and 783 bp (*M. fructigena*) fragments (Fig. 4A), and were specific to *Monilinia* species in that they did not generate amplification products from other fungi commonly found in fruit production operations in this region (e.g. apples and stone fruit) or from the flesh of stone fruit itself (Figs 4B and C). Consequently, the primers described here did not require the isolation of the fungal pathogen prior to DNA extraction, which is essential for a rapid diagnostic assay.

To date, several other molecular assays have been developed to identify brown rot pathogens, but the majority of them have either been tested with only two of the three *Monilinia* species of concern or identify only one of the three species per reaction.[64–68] To validate the present assay, *M. fructicola, M. laxa* and *M. fructigena*

isolates were also tested with three additional sets of primers previously designed for the identification of *Monilinia* species. While primers IgenaS and IgenaAS[48] successfully amplified an appropriately sized product exclusively from *M. fructigena*, primers MLF2 and MLR2[47] amplified a 375 bp product not only from *M. laxa* isolates but also from all *M. fructigena* isolates tested in this study. Moreover, the method described by Côté *et al*.,[46] in which a multiplex PCR assay was utilized to discriminate between the three *Monilinia* species on the basis of differences in amplicon size, yielded appropriately sized products for all of the *M. laxa* and *M. fructigena* isolates in this study. However, only those three *M. fructicola* isolates collected from cherries in Wayne County, New York, resulted in a fragment of the expected size, while the three *M. fructicola* isolates collected from peaches in Adams County, Pennsylvania, yielded no amplification products (Fig. 4A). These results suggest that none of the previously developed methods tested here can distinguish between these three *Monilinia* species on their own. Consequently, several different methods should be utilized in conjunction when identifying *Monilinia* species from a wide range of geographic locations.

In conclusion, it was found that the *cyt b* genes from both *M. fructicola* and *M. laxa* possessed an intron directly after codon 143, suggesting that resistance to QoI fungicides based on the G143A mutation is not likely to occur in these pathogens. Conversely, this intron was lacking in *M. fructigena* isolates, which implies that the risk of developing QoI resistance conferred by the G143A mutation is high in this species, and possibly to a level that could be detected in an *in vitro* assay. Furthermore, a rapid and reliable PCR assay has been developed for the identification and differentiation of brown rot pathogens that occur in Europe and North America, which has the potential to be of use in preventing the further spread of invasive *Monilinia* species causing brown rot of fruit. These two discoveries are particularly relevant to the stone fruit producers in the northeastern United States where QoI use has increased in response to region-wide DMI resistance[15] and other *Monilinia* species are becoming more prevalent.[9] Therefore, strategies such as the limitation of QoI treatments, as well as the use of mixed and alternating fungicides, will be especially important for delaying the development of resistance in *M. fructigena* for as long as possible.

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REFERENCES

1 Willetts HJ, Byrde RJW and Fielding AH, The taxonomy of the brown rot fungi (*Monilinia* spp.) related to their extracellular cell wall-degrading enzymes. *J Gen Microbiol* **103**:77–83 (1977).
2 Landgraf FA and Zehr E, Inoculum sources of *Monilinia fructicola* in South Carolina peach orchards. *Phytopathology* **72**:185–190 (1982).
3 Batra LR, World species of *Monilinia* (fungi): their ecology, biosystematics and control. *Mycol Mem* **16**:1–246 (1991).
4 Ogawa JM and English H, *Diseases of Temperate Zone Tree Fruit and Nut Crops.* Publ. 3345, University of California, Division of Agricultural and Natural Resources, Oakland, CA (1991).
5 Bosshard E, Hilber-Bodmer M, Schärer HJ, Bünter M and Duffy B, First report of quarantine brown rot pathogen *Monilinia fructicola* on imported stone fruits in Switzerland. *Plant Dis* **90**:1554 (2006).
6 Petróczy M and Palkovics L, First report of brown rot caused by *Monilinia fructicola* on imported peach in Hungary. *Plant Dis* **90**:375 (2006).
7 Duchoslavová J, Širucková I, Zapletalová E, Navrátil M and Šafárová D, First report of brown rot caused by *Monilinia fructicola* on various stone and pome fruits in the Czech Republic. *Plant Dis* **91**:907 (2007).
8 Byrde RJW and Willetts HJ, *The Brown Rot Fungi of Fruit: Their Biology and Control*. Pergamon Press, Oxford, UK (1977).
9 Villani SM and Cox KD, Confirmation of European brown rot caused by *Monilinia laxa* on tart cherry, *Prunus cerasus*, in Western New York. *Plant Dis* **94**:783 (2010).
10 Schnabel G, Bryson PK, Bridges WC and Brannen P, Reduced sensitivity in *Monilinia fructicola* to propiconazole in Georgia and implications for disease management. *Plant Dis* **88**:1000–1004 (2004).
11 Cox KD, Bryson PK and Schnabel G, Instability of propiconazole resistance and fitness in *Monilinia fructicola*. *Phytopathology* **97**:448–453 (2007).
12 Zehr E, Luszcz LA, Olien WC, Newall WC and Toler JE, Reduced sensitivity in *Monilinia fructicola* to propiconazole following prolonged exposure in peach orchards. *Plant Dis* **83**:913–916 (1999).
13 Ishii H, QoI fungicide resistance: current status and the problems associated with DNA-based monitoring, in *Recent Developments in Management of Plant Diseases*, ed. by Gisi U, Chet I and Gullino L. Springer, Dordrecht, The Netherlands, pp. 37–45 (2010).
14 Luo CX, Cox KD, Amiri A and Schnabel G, Occurrence and detection of the DMI resistance-associated genetic element 'Mona' in *Monilinia fructicola*. *Plant Dis* **92**:1099–1103 (2008).
15 Villani SM and Cox KD, QoI sensitivity and the prevalence of DMI resistance in NY populations of *Monilinia fructicola*. *Phytopathology* **98**:S153 (2008).
16 Burnett A, Lalancette N and McFarland K, First report of the peach brown rot fungus *Monilinia fructicola* resistant to demethylation inhibitor fungicides in New Jersey. *Plant Dis* **94**:126 (2010).
17 Sierotzki H, Parisi S, Steinfeld U, Tenzer I, Poirey S and Gisi U, Mode of resistance to respiration inhibitors at the cytochrome bc_1 enzyme complex of *Mycosphaerella fijiensis* field isolates. *Pest Manag Sci* **56**:833–841 (2000).
18 Chin KM, Wirz M and Laird D, Sensitivity of *Mycosphaerella fijiensis* from banana to trifloxystrobin. *Plant Dis* **85**:1264–1270 (2001).
19 Fernández-Ortuño D, Pérez-García A, López-Ruiz R, Romero D, de Vicente A and Torés JA, Occurrence and distribution of resistance to QoI fungicides in populations of *Podosphaera fusca* in south central Spain. *Eur J Plant Pathol* **115**:215–222 (2006).
20 Vincelli P and Dixon E, Resistance to QoI (strobilurin-like) fungicides in isolates of *Pyricularia grisea* from perennial ryegrass. *Plant Dis* **86**:235–240 (2002).
21 Kim YS, Dixon EW, Vincessi P and Farman ML, Field resistance to strobilurin (QoI) fungicides in *Pyricularia grisea* caused by mutations in the mitochondrial cytochrome *b* gene. *Phytopathology* **93**:891–900 (2003).
22 Küng-Färber RB, Chin KM and Leadbitter N, Sensitivity of *Venturia inaequalis* to trifloxystrobin. *Pest Manag Sci* **58**:261–267 (2002).
23 Bartlett DW, Clough JM, Godwin JR, Hall AA, Hamer M and Parr-Dobrzanski B, The strobilurin fungicides. *Pest Manag Sci* **58**:649–662 (2002).
24 Di Rago JP, Coppee JY and Colson AM, Molecular basis for resistance to myxothiazol, mucidin (strobilurin A), and stigatellin. Cytochrome *b* inhibitors acting at the center of the mitochondrial ubiquinol-cytochrome *c* reductase in *Saccharomyces cerevisiae*. *J Biol Chem* **264**:14 543–14 548 (1989).
25 Degli Esposti M, De Vries S, Crimi M, Ghelli A, Patarnello T and Meyer A, Mitochondrial cytochrome b: evolution and structure of the protein. *Biochim Biophys Acta* **1143**:243–271 (1993).
26 Brasseur G, Saribas AS and Daldal F, A compilation of mutations located in the cytochrome *b* subunit of the bacterial and

mitochondrial bc_1 complex. *Biochim Biophys Acta* **1275**:61–69 (1996).
27 Kraiczy P, Haase U, Gencic S, Flindt S, Anke T, Brandt U, *et al*, The molecular basis for the natural resistance of the cytochrome bc_1 complex from strobilurin-producing Basidiomycetes to center Q_P inhibitors. *Eur J Biochem* **235**:54–63 (1996).
28 Sierotzki H, Wullschleger J and Gisi U, Point mutation in cytochrome *b* gene conferring resistance to strobilurin fungicides in *Erysiphe graminis* f. sp. *tritici* field isolates. *Pestic Biochem Physiol* **68**:107–112 (2000).
29 Ishii H, Fraaije BA, Sugiyama T, Noguchi K, Nishimura K, Takeda T, *et al*, Occurrence and molecular characterization of strobilurin resistance in cucumber powdery mildew and downy mildew. *Phytopathology* **91**:1166–1171 (2001).
30 Gisi U, Sierotzki H, Cook A and McCaffery A, Mechanisms influencing the evolution of resistance to QoI inhibitor fungicides. *Pest Manag Sci* **58**:859–867 (2002).
31 Pasche JS, Piche LM and Gudmestad ND, Effect of the F129L mutation in *Alternaria solani* on fungicides affecting mitochondrial respiration. *Plant Dis* **89**:269–278 (2005).
32 Sierotzki H, Frey R, Wullschleger J, Palermo S, Karlin S, Godwin J, *et al*, Cytochrome b gene sequence and structure of *Pyrenophora teres* and *P. tritici-repentis* and implications for QoI resistance. *Pest Manag Sci* **63**:225–233 (2007).
33 Grasso V, Palermo S, Sierotzki H, Garibaldi A and Gisi U, Cytochrome b gene structure and consequences for resistance to Qo inhibitor fungicides in plant pathogens. *Pest Manag Sci* **62**:465–472 (2006).
34 Banno S, Yamashita K, Fukumori F, Okada K, Uekusa H, Takagaki M, *et al*, Characterization of QoI resistance in *Botrytis cinerea* and identification of two types of mitochondrial cytochrome *b* gene. *Plant Pathol* **58**:120–129 (2009).
35 Jiang J, Ding L, Michailides TJ, Li H and Ma Z, Molecular characterization of field azoxystrobin-resistant isolates of *Botrytis cinerea*. *Pestic Biochem Physiol* **93**:72–76 (2009).
36 Zheng D and Köller W, Characterization of the mitochondrial cytochrome *b* gene from *Venturia inaequalis*. *Curr Genet* **32**:361–366 (1997).
37 Grasso V, Sierotzki H, Garibaldi A and Gisi U, Characterization of the cytochrome *b* gene fragment of *Puccinia* species responsible for the binding site of QoI fungicides. *Pestic Biochem Physiol* **84**:72–82 (2000).
38 Luo C-X and Schnabel G, Adaptation to fungicides in *Monilinia fructicola* isolates with different fungicide resistance phenotypes. *Phytopathology* **98**:230–238 (2008).
39 Gardes M and Bruns TD, ITS primers with enhanced specificity for basidiomycetes: application to the identification of mycorrhizae and rusts. *Mol Ecol* **2**:113–118 (1993).
40 White TJ, Bruns T, Lee S and Taylor J, Amplification and direct sequencing of fungal and ribosomal RNA genes for phylogenetics, in *PCR Protocols: A Guide to Methods and Applications*, ed. by Innis MAD, Gelfand H, Sninsky JJ and White TJ. Academic Press, Inc., New York, NY, pp. 315–322 (1990).
41 Singer SD and Cox KD, The re-emergence and management of currant cane dieback in the Northeastern United States. *Plant Dis* **94**:1283–1289 (2010).
42 Thompson JD, Higgins DJ and Gibson TJ, CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**:4673–4680 (1994).
43 Hall TA, BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl Acids Symp Ser* **41**:95–98 (1999).
44 Altschul SF, Gish W, Miller W, Myers EW and Lipman DJ, Basic local alignment search tool. *J Mol Biol* **215**:403–410 (1990).
45 Cserzö M, Wallin E, Simon I, von Heijne G and Elofsson A, Prediction of transmembrane alpha-helices in prokaryotic membrane proteins: the dense alignment surface method. *Protein Eng* **10**:673–676 (1997).
46 Côté M-J, Tardif M-C and Meldrum AJ, Identification of *Monilinia fructigena, M. fructicola, M. laxa*, and *Monilia polystroma* on inoculated and naturally infected fruit using multiplex PCR. *Plant Dis* **88**:1219–1225 (2004).
47 Ma Z and Michailides TJ, Approaches for eliminating PCR inhibitors and designing PCR primers for the detection of phytopathogenic fungi. *Crop Prot* **26**:145–161 (2007).
48 Gell I, Larena I and Melgarejo P, Genetic diversity in *Monilinia laxa* populations in peach orchards in Spain. *J Phytopathol* **155**:549–556 (2007).
49 Hudspeth MES, The fungal mitochondrial genome – a broader perspective, in *Fungal Biotechnology*, ed. by Arora DK, Elander RP and Mukerji KG. Marcel Decker, New York, NY, pp. 213–242 (1992).
50 Woo PCY, Zhen H, Cai JJ, Yu J, Lau SKP, Wang J, *et al*, The mitochondrial genome of the thermal dimorphic fungus *Penicillium marneffei* is more closely related to those of molds than yeasts. *FEBS Lett* **555**:469–477 (2003).
51 Brasseur R, Calculation of the three-dimensional structure of *Saccharomyces cerevisiae* cytochrome b inserted in a lipid matrix. *J Biol Chem* **263**:12 571–12 575 (1988).
52 Fisher N, Brown AC, Sexton G, Cook A, Windass J and Meunier B, Modeling the Qo site of crop pathogens in *Saccharomyces cerevisiae* cytochrome *b*. *Eur J Biochem* **271**:2264–2271 (2004).
53 Amiri A, Brannen PM and Schnabel G, Reduced sensitivity in *Monilinia fructicola* field isolates from South Carolina and Georgia to respiration inhibitor fungicides. *Plant Dis* **94**:737–743 (2010).
54 Köller W, Parker DM, Turechek WW, Avila-Adame C and Cronshaw K, A two phase resistance response of *Venturia inaequalis* populations to the QoI fungicides kresoximmethyl and trifloxystrobin. *Plant Dis* **88**:537–544 (2004).
55 Cech TR, Conserved sequences and structures of group I introns: building an active site for RNA catalysis – a review. *Gene* **73**:259–271 (1988).
56 Burke JM, Molecular genetics of group I introns: RNA structure and protein factors required for splicing – a review. *Gene* **73**:273–294 (1988).
57 Lambowitz AL and Belfort M, Introns as mobile genetic elements. *Annu Rev Biochem* **62**:587–622 (1993).
58 Bolduc JM, Spiegel PC, Chatterjee P, Brady KL, Downing ME, Caprara MG, *et al*, Structural and biochemical analyses of DNA and RNA binding by a bifunctional homing endonuclease and group I intron splicing factor. *Gene Dev* **17**:2875–2888 (2003).
59 Kianianmomeni A, Schwarz G, Felsenstein FG and Wenzel G, Validation of a real-time PCR for the quantitative estimation of a G143A mutation in the cytochrome *bc*1 gene of *Pyrenophora teres*. *Pest Manag Sci* **63**:1046 (2007).
60 Miguez M, Reeve C, Wood PM and Hollomon DW, Alternative oxidase reduces the sensitivity of *Mycosphaerella graminicola* to QoI fungicides. *Pest Manag Sci* **60**:3–7 (2004).
61 Foury F, Roganti T, Lecrenier N and Purnelle B, The complete sequence of the mitochondrial genome of *Saccharomyces cerevisiae*. *FEBS Lett* **440**:325–331 (1998).
62 Martin RR, Delano J and Lévesque CA, Impacts of molecular diagnostic technologies on plant disease management. *Annu Rev Phytopathol* **38**:207–239 (2000).
63 Penrose LJ, Tarran J and Wong A-L, First record of *Sclerotinia laxa* Aderh. & Ruhl. New South Wales: differentiation from *S. fructicola* (Wint.) Rehm. by cultural characteristics and electrophoresis. *Aust J Agr Res* **27**:547–556 (1976).
64 Snyder CL and Jones AL, Genetic variation between strains of *Monilinia fructicola* and *Monilinia laxa* isolated from cherries in Michigan. *Can J Plant Pathol* **21**:70–77 (1999).
65 Förster H and Adaskaveg JE, Early brown rot infections in sweet cherry fruit are detected by *Monilinia*-specific DNA primers. *Phytopathology* **90**:171–178 (2000).
66 Ioos R and Frey P, Genomic variation within *Monilinia laxa, M. fructigena* and *M. fructicola*, and application to species identification by PCR. *Eur J Plant Pathol* **106**:373–378 (2000).
67 Boehm EWA, Ma Z and Michailides T, Species-specific detection of *Monilinia fructicola* from California stone fruits and flowers. *Phytopathology* **91**:428–439 (2001).
68 Ma Z, Luo Y and Michailides TJ, Nested PCR assays for detection of *Monilinia fructicola* in stone fruit orchards and *Botryosphaeria dothidea* from pistachios in California. *J Phytopathol* **151**:312–322 (2003).

