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Monilinia laxa, *M. fructigena* and *M. fructicola*: Risk estimation of resistance to QoI fungicides and identification of species with cytochrome *b* gene sequences

Monilinia laxa, *M. fructigena* und *M. fructicola*: Resistenzrisiko gegenüber QoI Fungiziden und Artidentifizierung anhand von Cytochrom *b* Sequenzen

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Abstract

Studies on the gene of the target protein of QoI fungicides (cytochrome *b*) in *Monilinia laxa*, *M. fructigena* and *M. fructicola* showed that the occurrence of the most important resistance mechanism to QoI fungicides, the G143A mutation, is relatively unlikely in *M. laxa* and *M. fructicola*. This is due to the presence of an intron sequence directly after codon 143. A mutation in codon 143 would presumably lead to incorrect mRNA maturation and consequently to a non-functional protein. No introns were found directly before or after codon 143 in *M. fructigena*, therefore the G143A mutation may be possible. Intron sequences have not been detected in immediate vicinity to the codons 129 and 137 in all three species, so the occurrence of the mutations F129L and G137R could be possible. Based on the differences in the intron-exon organization of the cytochrome *b* gene, a rapid, sensitive and reliable PCR assay for identification and differentiation of the 3 *Monilinia* species was developed.

Key words: fungicide resistance, *Monilinia fructicola*, *Monilinia fructigena*, *Monilinia laxa*, PCR detection assay, QoI fungicides

Zusammenfassung

Untersuchungen zum Gen des Zielproteins der QoI-Fungizide (Cytochrom *b*) in *Monilinia laxa*, *M. fructigena* und *M. fructicola* ergaben eine geringe Wahrscheinlichkeit, dass sich der wichtigste Resistenzmechanismus gegenüber QoI-Fungiziden, die Mutation G143A, in *M. laxa* und *M. fructicola* ausprägt. Dies ist durch eine Intronsequenz bedingt, die direkt nach dem Codon 143 beginnt. Eine Mutation im Codon 143 würde vermutlich zu einer fehlerhaften mRNA-Reifung und konsequenterweise zu einem nicht funktionellen Protein führen. In *M. fructigena* wurde keine Intron-Sequenz direkt vor oder nach dem Codon 143 gefunden, daher ist eine G143A Mutation in *M. fructigena* möglich. In unmittelbarer Nähe der Codons 129 und 137 sind in allen 3 *Monilinia*-Arten keine Intron-Sequenzen vorhanden, weswegen die Mutationen F129L und G137R auftreten können. Die Unterschiede in der Intron-Exon-Organisation ermöglichten die Entwicklung eines PCR-Verfahrens zur schnellen, sensitiven und sicheren Identifizierung und Unterscheidung dieser 3 *Monilinia*-Arten.

Stichwörter: Fungizid-Resistenz, *Monilinia fructicola*, *Monilinia fructigena*, *Monilinia laxa*, PCR-Nachweisverfahren, QoI-Fungizide

1 Introduction

The most important *Monilinia* species on pome and stone fruits worldwide are *Monilinia laxa*, *Monilinia fructigena* and *Monilinia fructicola*. *M. laxa* infects blossoms, spurs and fruits of various stone fruits throughout the world. *M. fructigena* is an important pathogen on fruits of pome and stone fruits in Europe and Asia and plays no role in infections of blossoms. Typical symptoms of both species are shown in Figures 1–3. *M. fructicola* is mainly a pathogen in the New World, i.e. North and Latin America, Australia and New Zealand, but has also been found in Europe in recent years (Mercier et al. 2009) and is listed on the EPPO A2 quarantine list, which means that *M. fructicola* is locally present in the EPPO region (OEPP/EPPO 2009). This species mainly infects the blossoms and fruits of stone fruits (Byrde and Willetts 1977). Infections of blossoms are called blossom blight; infections of fruits, brown rot.

Blossom blight and subsequent twig infections (caused by *M. laxa* and *M. fruticola*) are largely a problem of sour cherries, but outbreaks on sweet cherries, plums, peach and apricot also occur (Byrde and Willetts 1977; Stammler and Zeller 1989) and even pome fruit blossoms can be infected by *M. laxa* f.sp. *mali* (Byrde and Willetts 1977). Susceptibility to blossom blight also depends on the variety within a species. Depending on the host species, variety, climate and infection conditions from the pre- to post-flowering period, up to three fungicide applications from growth stage 59 to 67 may be necessary to control blossom blight. Brown rot (caused by all three *Monilinia* species) is a serious problem primarily in stone fruits. In some cases, even a single fruit can be infected by different *Monilinia* species (Fig. 3; Mercier et al. 2009). Up to five sprays with effective fungicides are needed to control brown rot, depending on the crop, variety, maturing time, climate and infection conditions. Fungicides currently used for *Monilinia* control are, for instance, benzimidazoles (e.g. thiophanate methyl), sterolbiosynthesis inhibitors (e.g. myclobutanil and fenhexamide), succinate dehydrogenase inhibitors (e.g. boscalid), dicarboximides (e.g. iprodione) and quinone outer binding site inhibitors (QoI, e.g. pyraclostrobin and azoxystrobin).

The risk of fungicide resistance development is a complex interaction between the risk of the pathogen, the risk of the fungicide and the agronomic risk (Kuck and Russell 2006). The last of these refers to agronomic measures such as cropping system, varieties, fungicide spray programs, etc. These measures can be influenced by the grower and used to decrease the selection pressure of a fungicide on fungal pathogens, whereas the pathogen and fungicide risk are inherent.

Monilinia species are classified by the Fungicide Resistance Action Committee (FRAC) as pathogens with a moderate risk to develop resistance to fungicides. This estimation is based on the biology of the fungus (generations, spore dispersal and spore production), its history of fungicide resistance development, and the occurrence of the disease (frequency and control). *Monilinia* spp. developed resistance to some of the above-mentioned fungicides in some regions after several years of exposure (Elmer and Gaunt 1994; Luo et al. 2008; Ma et al. 2003; Ma et al. 2005; Penrose et al. 1985; Zehr et al. 1999).

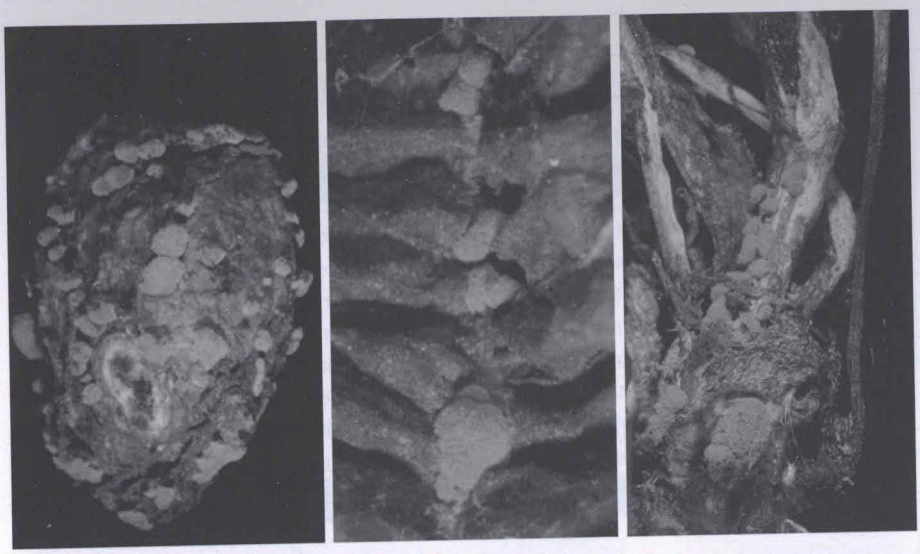

Fig. 1: Sporulation of *Monilinia laxa* on sweet cherry in early spring on plant parts infected in the previous season. Sporulation on a fruit mummy (left), the midrib of a leaf (middle), and a spur (right).

Fig. 2: Blossom infection of sweet cherry caused by *Monilinia laxa*. Early infection with diseased stigma, style and ovary (left) and more progressed infection with infected pedicel and further infection of the pedicel of the second blossom.

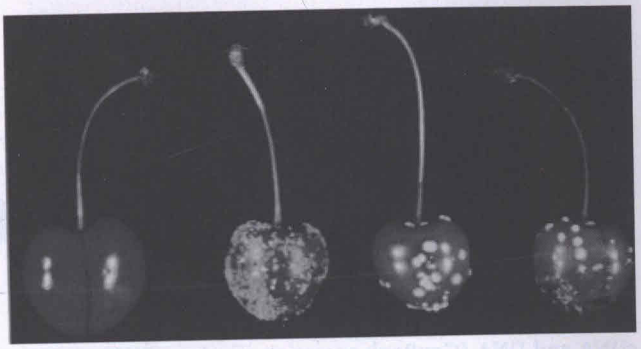

Fig. 3: Fruit infections of sweet cherries (var. Schneiders Späte). From left to right: Healthy, infected with *Monilinia laxa*, infected with *M. fructigena*, mixed infection with *M. fructigena* and *M. laxa* (arrow marks *M. laxa* sporulation).

QoIs are generally classified by the FRAC as fungicides with a high resistance risk. This is confirmed by the rapid development of QoI resistance in some "high-risk" pathogens such as powdery mildews on wheat or cucurbits shortly (i.e. 1 to 3 years) after market introduction (Kuck and Russell 2006). Resistance to QoIs is conferred by mutations in the target protein, cytochrome *b*. Most important is the G143A mutation, which leads to high resistance to all QoI fungicides. However, some plant pathogenic fungi have not developed this mutation up to now, even after high selection pressure (many years of use, high fungicidal activity on the relevant pathogens). Examples of this are *Pyrenophora teres* (Semar et al. 2007; Sierotzki et al. 2007) and *Puccinia triticina* (Koch et al. 2008). The reason has been elucidated by Grasso et al. (2006a, b), who report that an intron directly after codon 143 in the cytochrome *b* gene may prevent the occurrence of the G143A mutation. The glycine codon may play a role in the splicing of the mRNA, and changes in the 143 codon would lead to incorrect splicing and consequently to a lethal cytochrome *b* protein. Several plant pathogenic fungi have now been sequenced and introns directly after or even within codon 143 have been found in *Alternaria solani*, *Pyrenophora teres*, various *Puccinia* species, *Phakopsora pachyrhizi* and others. In most cases, these introns encode genes for maturases (enzymes encoded by self-splicing introns which catalyze excision of the intron from their own primary transcript). In *Alternaria solani* or *Pyrenophora teres* the mutations F129L and/or G137R have been reported (www.frac.info) but both mutations are of minor importance compared with the G143A because they generally lead to lower resistance factors (www.frac.info) with no or only limited impact on the field efficacy of, for example, pyraclostrobin (Semar et al. 2007).

The aim of this study was to clarify the risk of development of the G143A (F129L, G137R) mutation as a resistance mechanism to QoI in the 3 most important *Monilinia* species in stone and pome fruits. We therefore analyzed the cytochrome *b* genes of the three species for the presence and localization of intron sequences in the regions of the three relevant mutations. As a spin-off, the sequence data were used to develop a PCR-based method for rapid, sensitive, reliable identification and differentiation of the three *Monilinia* species.

2 Materials and methods

2.1 Origin of isolates

Five *M. fructigena*, four *M. laxa* and two *M. fructicola* isolates were included in the studies. Species were identified by morphological characteristics and confirmed by molecular genetic analysis as described by Cote et al. (2004). Details about the isolates are given in Table 1.

Table 1: Origin of isolates

Isolate ID	Year	Country	Origin	Crop cultivar	Species
2	2004	Germany	Limburgerhof	apple	*M. fructigena*
6	2005	France	Grenade	peach	*M. fructigena*
17	2005	Germany	Renchen-Ulm	cherry	*M. fructigena*
247	2008	France	Merville	peach	*M. fructigena*
250	2008	France	Merville	peach	*M. fructigena*
57	2005	Denmark	South-East Jutland	cherry	*M. laxa*
70	2005	Germany	Schriesheim	plum	*M. laxa*
132	2005	Italy	Ravenna	nectarine	*M. laxa*
242	2008	France	Merville	peach	*M. laxa*
252	2009	Australia	CABI Bioservices (IMI 158426)	plum	*M. fructicola*
253	2009	New Zealand	CABI Bioservices (IMI 361375)	plum	*M. fructicola*

2.2 *Analysis of cDNA and DNA sequences*

RNA and DNA were isolated from approximately 20 mg fresh mycelium from pure cultures using the NucleoSpin RNA Kit and NucleoSpin Plant Kit respectively (both from Machery and Nagel, Düren, Germany). RT-PCR was carried out using the Verso cDNA kit (Thermo, Ulm, Germany). For *M. fructigena*, the primer pair KES 194 5'-AAAGCAAAGAATCTGTTTA-AGGTTGC and KES 223 5'-CGGATCATATAGAGCACCTAGAA-CATTAG resulted in 237 bp and 4960 bp PCR products with cDNA and DNA (GenBank accession number: GU933644) as templates respectively. For *M. laxa*, the same primer resulted in 237 bp (cDNA) and 5944 bp (DNA, GU933642) products. PCR was carried out with 2x HotStart-IT FideliTaq Mastermix (USB, Staufen, Germany) and the following conditions: an initial heating step for 2 min at 95°C was followed by 35 cycles for 15 s at 95°C, 30 s at 50°C, 10 min at 68°C and a final amplification step for 5 min at 68°C. For *M. fructicola*, 493 bp (cDNA) and 7435 bp (DNA, GU933643) fragments were amplified with the primer pair KES 115 5'-GAGTTTGCATTGGATTAGCCA and KES 126 5'-GAGGTTTATATTACGGATCATATAGAG using the PCR conditions described above. PCR products were sequenced by the Sanger method and primer walking sequencing.

2.3 *Identification of* Monilinia *species*

DNA from the 3 *Monilinia* species (*M. fructigena*, *M. laxa* and *M. fructicola*) was isolated from approximately 20 mg fresh mycelium from pure cultures using the NucleoSpin Plant Kit (Macherey and Nagel, Düren, Germany), and in the final step the DNA was eluted with 100 µl elution buffer. With the sequence information from the study above, a primer pair (KES 1238 5'-AGCTTTCCTGGGTTTGTCAAA and KES 1261 5'-TCCAATTCATGGTAYAGCACTCATA [Y = 50% T and 50% C]) was selected which annealed to the cytochrome *b* gene of all three species but generated amplification products of different, species-specific length. PCR was carried out with 2.5 µl DNA solution, 12.5 µl HotStart-IT FideliTaq Mastermix (USB), 0.5 µl KES 1238 (10 pmol/µl) and 1.0 µl KES 1261 (10 pmol/µl) in a total volume of 25 µl per reaction and the following conditions: an initial heating step for 2 min at 95°C, 35 cycles for 15 s at 95°C, 30 s at 55°C, 10 min at 68°C followed by a final amplification step for 5 min at 68°C. Amplified products were separated in 1.5% agarose gel in 1x TAE buffer, stained with ethidium bromide and visualized under UV light.

3 Results

3.1 *PCR amplification*

With the primer pairs described, PCR products could be amplified with DNA and cDNA as template material. The PCR products of cDNA and DNA differed in length for all 3 *Monilinia* species, i.e. the cDNA sequences were significantly shorter than the corresponding DNA sequences. The differences between these PCR products indicated the presence of intron sequences which are removed during mRNA maturation. DNA-cDNA alignments revealed large intron sequences with several thousand base pairs in all three *Monilinia* species (Figs. 4–6).

3.2 *Cytochrome b sequence and impact on QoI resistance risk*

Analysis of the cytochrome *b* gene showed that the three *Monilinia* species differed in their sequence. While mRNA sequences of *M. laxa* and *M. fructigena* were nearly identical in the fragment analyzed, both species differed in the presence and positioning of introns in the gene. The sequence of the cDNA fragment of *M. fructicola* differed more from those of *M. laxa* and *M. fructigena*. Regarding the localization of the intron sequences, *M. laxa* and *M. fructicola* showed an intron sequence directly after codon 143 (Fig. 6) of the cytochrome *b* gene. *M. fructigena* also had an intron in the cytochrome *b* gene but not in the direct vicinity of codon 143 (Fig. 6). No introns were identified directly before or after codons 129 and 137 in any of the three species (Figs. 4, 5).

3.3 *Cytochrome b sequence and species identification of* M. laxa, M. fructigena *and* M. fructicola

The selected primer pair resulted in amplification products of different length for each *Monilinia* species: 1412 bp for *M. fructigena*, 3469 bp for *M. laxa* and 2577 bp for *M. fructicola*. The differences in length could easily be differentiated by agarose gel electrophoresis and the species therefore identified. Fig. 7 shows the amplification products for *M. fructigena*, *M. laxa* and *M. fructicola*.

No cross-reaction was found with other fungal pathogens, such as *Venturia inaequalis*, *Gloeosporium album*, *Botrytis cinerea*, *Alternaria alternata* and *Septoria tritici* (data not shown).

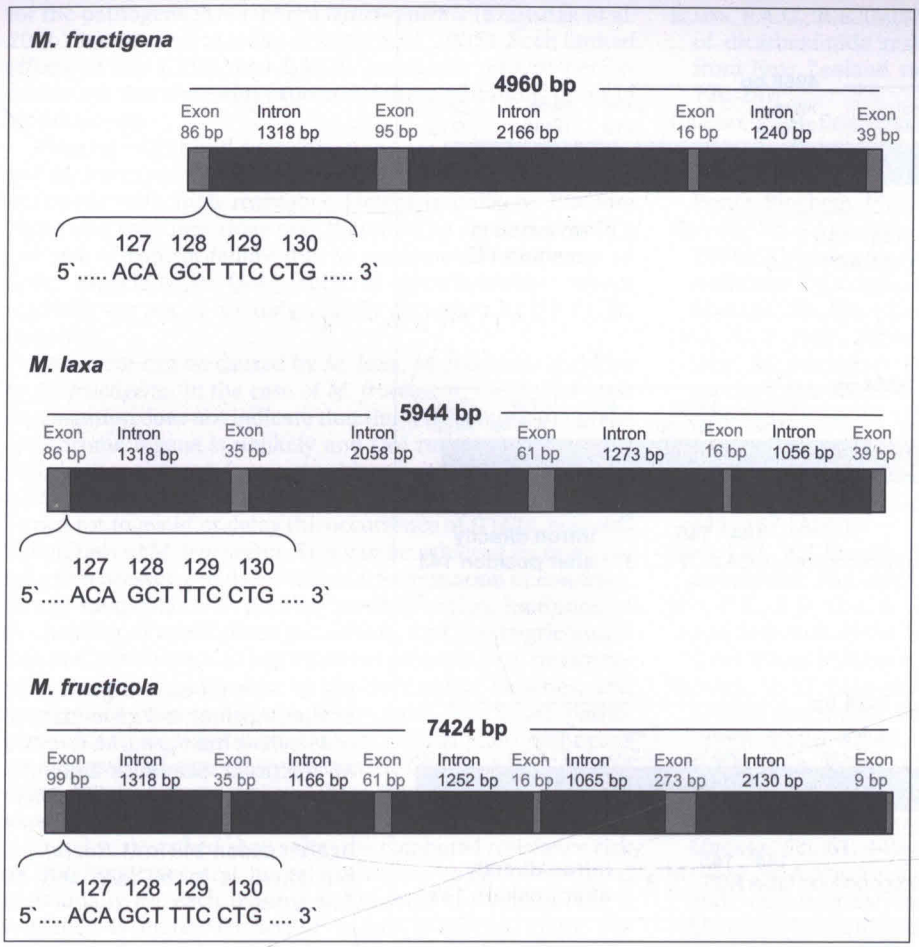

Fig. 4: Intron-exon organization of the cytochrome *b* gene (partial) of *Monilinia fructigena*, *M. laxa* and *M. fructicola*. Sequence adjoining codon 129 is given in detail. No intron is present directly before or after codon 129.

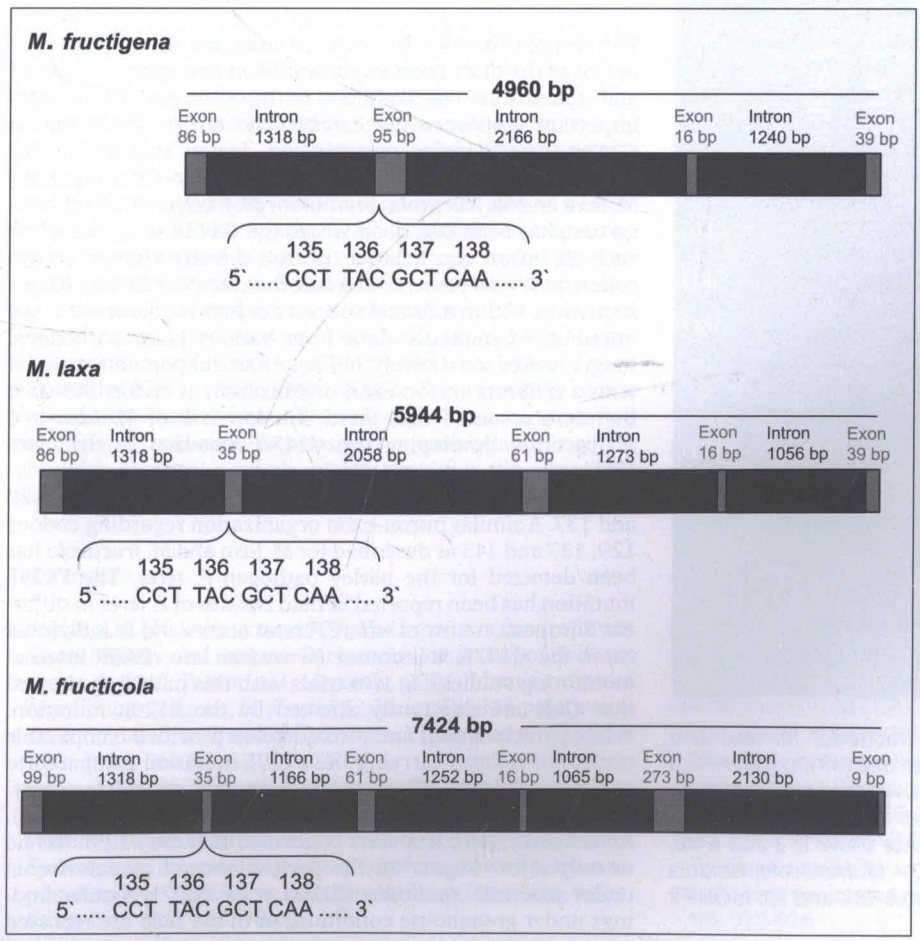

Fig. 5: Intron-exon organization of the cytochrome *b* gene (partial) of *Monilinia fructigena*, *M. laxa* and *M. fructicola*. Sequence adjoining codon 137 is given in detail. No intron is present directly before or after codon 137.

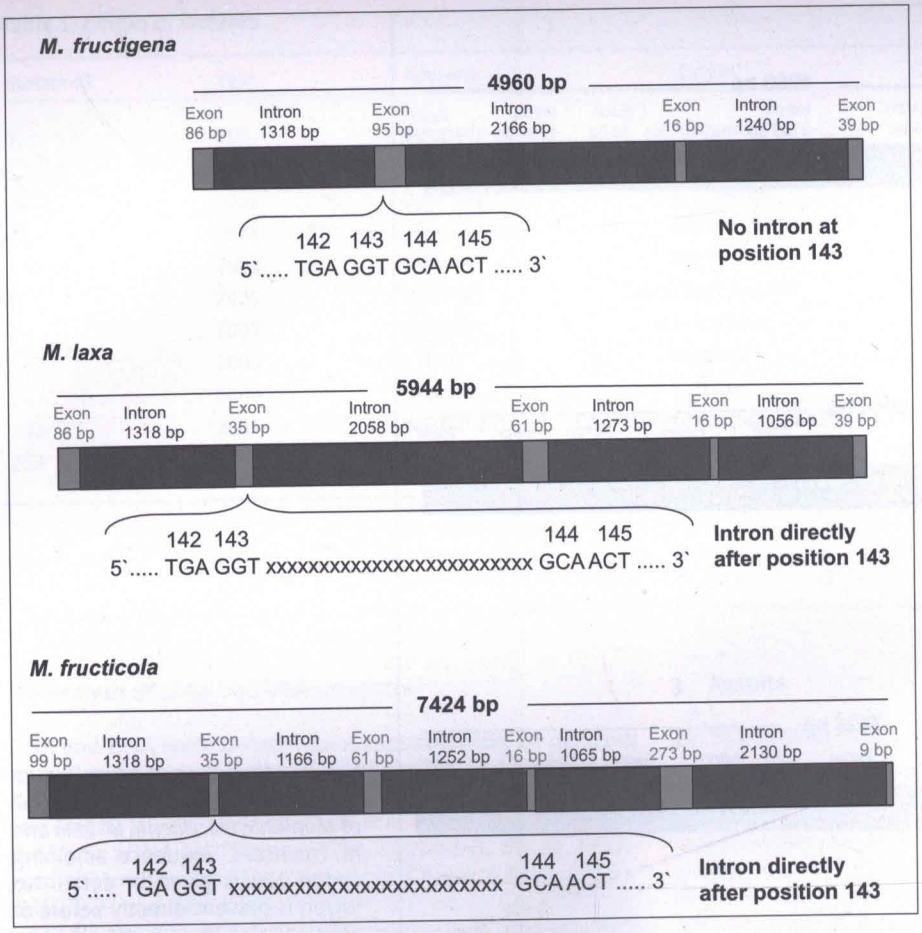

Fig. 6: Intron-exon organization of the cytochrome *b* gene (partial) of *Monilinia fructigena*, *M. laxa* and *M. fructicola*. Sequence adjoining codon 143 is given in detail. There is no intron present directly before or after codon 143 in *M. fructigena*, but there is in *M. laxa* and *M. fructicola*.

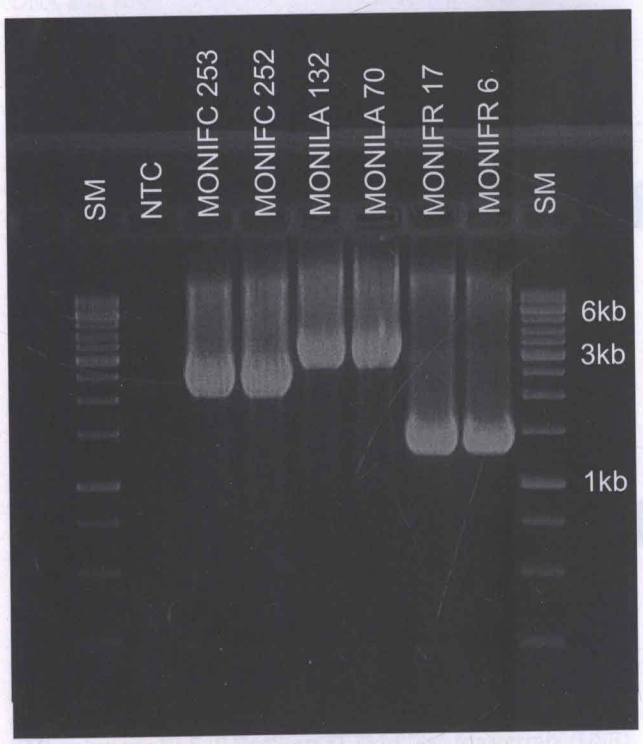

Fig. 7: Identification of *Monilinia fructicola*, *M. laxa* and *M. fructigena* by PCR amplification with one primer pair, which results in different sizes of amplified DNA products. SM = Size marker (GeneRuler 1 kb DNA Ladder from Fermentas, St. Leon-Rot, Germany), brighter fragments show 1, 3 and 6 kb. NTC = non-template control. MONIFC = *M. fructicola*, isolates 253 and 252. MONILA = *M. laxa*, isolates 132 and 70. MONIFR = *M. fructigena*, isolates 17 and 6.

4 Discussion

The aim of this analysis was to estimate the risk of resistance to QoI of the three most important *Monilinia* species on pome and stone fruits. The likelihood of the occurrence of the most important resistance mechanism, the target site mutation G143A, was therefore investigated. Intron sequences after codon 143 in the cytochrome *b* gene were identified for *M. laxa* and *M. fructicola*, but not for *M. fructigena*. Up to now, no case has been described where the G143A occurred when such an intron constellation (intron directly after or within codon 143) was present. The fact that the cytochrome *b* DNA sequences within a fungal species are highly conserved (own unpublished sequence data from various plant pathogenic fungi) makes it relatively unlikely that subpopulations exist with a different intron-exon organization at codon 143. It is therefore assumed that there is a low risk of *M. laxa* and *M. fructicola* developing the G143A mutation in the cytochrome *b* gene.

However, no introns are located directly near codons 129 and 137. A similar intron-exon organization regarding codons 129, 137 and 143 as described for *M. laxa* and *M. fructicola* has been detected for the barley pathogen *P. teres*. The F129L mutation has been reported in field isolates of *P. teres* in different European countries where *P. teres* occurs and in individual cases the G137R was found (www.frac.info; BASF internal monitoring studies). *In vivo* trials with this pathogen showed that QoIs are differently affected by the F129L mutation. While pyraclostrobin and picoxystrobin provided comparable control of isolates carrying the F129L mutation compared to wild-type isolates, efficacy of azoxystrobin on isolates carrying the F129L mutation was reduced (Drobny et al. 2008). Additionally, field trial data confirmed that the F129L has no or only a low impact on the performance of pyraclostrobin under practical conditions (Semar et al. 2007). Similar findings under greenhouse conditions or in the field are reported

for the pathogens *Pyrenophora tritici-repentis* (STAMMLER et al. 2006) and *Alternaria solani* (PASCHE et al. 2005). Such limited effects of the F129L and G137R mutations on QoI performance are therefore also expected if these mutations occur in *Monilinia* spp.

Blossom blight and twig infections are caused by *M. laxa* and *M. fructicola* and for both pathogens development of QoI resistance with high resistance factors is unlikely. Blossom blight and twig infections may therefore be controlled with a low risk of control failure due to resistance development to QoIs, especially to QoIs, such as pyraclostrobin, whose activities are not or are only slightly decreased by the F129L mutation.

Brown rot can be caused by *M. laxa*, *M. fructicola* and also by *M. fructigena*. In the case of *M. fructigena*, the intron-exon organization does not indicate that the G143A mutation in the cytochrome *b* gene is unlikely and QoI resistance may occur with high resistance factors in this species. Resistance management strategies are therefore recommended in controlling brown rot to avoid or delay the occurrence of G143A-mutated individuals of *M. fructigena*. This can be achieved by reducing selection pressure, tools for which are alternation or combination of fungicides with another mode of action, limitation of the number of applications per season, and good agricultural practice, which leads to less infection pressure (e.g. phytosanitary measures, cultivation of less susceptible varieties, and appropriate crop cultivation unfavorable for target pathogens). Since all monitoring data on various plant pathogens are summarized on a yearly basis in the QoI Working Group of FRAC, it is recommended to follow the current management strategies published by this group at www.frac.info.

The case described shows that the combined resistance risk of QoI fungicides and fungal pathogens must be analyzed individually for each pathogen. Although the cytochrome *b* sequence is highly conserved within a species, there are differences between the species of a genus, especially in the intron-exon organization, and "small" differences may have dramatic effects on the risk of resistance to QoI fungicides.

A simple and sensitive method for identification of the *Monilinia* species was developed based on differences in the intron-exon organization in the cytochrome *b* gene. The species could be differentiated in a single reaction. Since only one primer pair is used, no multiplexing of different primer pairs, with all the possible concomitant disadvantages of sensitivity and specificity, was necessary. The use of a mitochondrial gene as the target, which is present in numerous copies (up to several hundreds) in each cell, makes the test robust and very sensitive. Several molecular diagnostic methods have been developed for identification of *M. fructicola* (e.g. COTE et al. 2004; IOOS and FREY 2000; MA and MICHAELIDIS 2003; VAN BROUWERSHAVEN et al. 2009) and were recently summarized by EPPO (OEPP/EPPO 2009). The advantage of our method over those is that all three species can be identified and differentiated simultaneously from each other in a single tube with identical primers, which simplifies the procedure and provides information on the species in the sample.

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