Identification and Differentiation of *Monilinia* Species Causing Brown Rot of Pome and Stone Fruit using High-Resolution Melting (HRM) Analysis

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ABSTRACT

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Brown rot is a devastating disease of stone fruit caused by *Monilinia* spp. Among these species, *Monilinia fructicola* is a quarantine pathogen in Europe but has recently been detected in several European countries. Identification of brown rot agents relies on morphological differences or use of molecular methods requiring fungal isolation. The current study was initiated to develop and validate a high-resolution melting (HRM) method for the identification of the *Monilinia* spp. and for the detection of *M. fructicola* among other brown rot pathogens. Based on the sequence of the *cytb* intron from *M. laxa*, *M. fructicola*, *M. fructigena*, *M. mumeicola*, *M. linhartiana*, and *M. yunnanensis* isolates originating from several

countries, a pair of universal primers for species identification and a pair of primers specific to *M. fructicola* were designed. The specificity of the primers was verified to ensure against cross-reaction with other fungal species. The melting curve analysis using the universal primers generated six different HRM curve profiles, each one specific for each species. The HRM analysis primers specific to *M. fructicola* amplified a 120-bp region with a distinct melt profile corresponding to the presence of *M. fructicola*, regardless of the presence of other species. HRM analysis can be a useful tool for rapid identification and differentiation of the six *Monilinia* spp. using a single primer pair. This novel assay has the potential for simultaneous identification and differentiation of the closely related *Monilinia* spp. as well as for the differentiation of *M. fructicola* from other common pathogens or saprophytes that may occur on the diseased fruit.

Additional keyword: peach.

Brown rot is one of the most important and damaging diseases of stone and pome fruit worldwide. The disease appears initially on blossoms and spreads to the twigs and leaves, causing blight (Holb et al. 2013). The fruit are also affected at any stage of their development; however, they exhibit the highest susceptibility at the stage of ripening, resulting in substantial yield losses due to pre- and postharvest fruit rots (Hong et al. 1997; Villarino et al. 2012). Brown rot is mainly caused by *Monilinia fructicola*, *M. laxa*, and *M. fructigena* which have a global geographical distribution (EFSA Panel on Plant Health 2011), and in some countries are characterized as quarantine pathogens. *M. fructicola* (G. Winter) Honey (anamorph *Monilia fructicola* L. R. Batra) is widely distributed in America, Australia, China, and Japan. *Monilinia laxa* (Aderh. & Ruhland) Honey (anamorph *Monilia laxa* (Ehrens.) Sacc. & Voglino) is the most common agent of brown rot in Europe and is widely distributed around the world (EFSA Panel on Plant Health 2011; Riccioni and Valente 2015). *Monilinia fructigena* Honey (anamorph *Monilia fructigena* ((Pers.) Pers.) occurs in Europe, Asia, and North Africa and is considered the main cause of brown rot in pome fruit (EFSA Panel on Plant Health 2011). In recent years, four newly discovered *Monilia* spp. have been reported as causal agents of brown rot but are of less importance and limited geographical distribution. *M. polystroma*, an asexual species, has been reported in Japan, Hungary, Poland, and China (Petróczy et al. 2012; Poniatowska et al. 2013; Van Leeuwen et al. 2002; Zhu and Guo 2010). *M. mumeicola* has been reported on mume in Japan and on peach in China (Hu et al. 2011); *M. yunnanensis* has been reported on peach in China (Hu et al. 2011); and *M. linhartiana* has been reported on quince in Spain (Moral et al. 2011).

Although *Monilinia fructicola* is a quarantine pest in the European Union (EPPO 2015), it has recently entered several southern and central European countries (Bosshard et al. 2006; De Cal et al. 2009; Duchoslavová et al. 2007; Papavasileiou et al. 2015b; Pellegrino et al. 2009; Poniatowska et al. 2013). Furthermore, recent studies have shown a considerable increase in the *M. fructicola* population in apricot orchards in Switzerland (Jänsch et al. 2012) and peach orchards in Spain (Villarino et al. 2013), and a high occurrence of *M. fructicola* in Greece (Papavasileiou et al. 2015b). A considerable increase in the frequency of *M. fructicola* has also been observed in China after its first report (Zhu et al. 2011), while *M. fructicola* almost entirely replaced indigenous populations of *M. laxa* in California (Michailides et al. 1987). The possible shift in the *Monilinia* spp. population in favor of *M. fructicola* in Europe, combined with the fact that *M. fructicola* grows faster, sporulates more abundantly, has a better dissemination capacity, and is more prone to fungicide resistance development, makes the need for a better surveillance of borders and a continuous monitoring of *Monilinia* spp. population structure imperative. Thus, to detect the possible changes in the *Monilinia* spp. populations, an accurate, fast, reliable, and low-cost identification technique is needed.

Identification of *Monilinia* spp. was originally based on both quantitative and qualitative characteristics of the fungal cultures (Lane 2002; Van Leeuwen and van Kesteren 1998; Van Leeuwen et al. 2002). However, some of these characteristics often coexisted in strains of different species, making the development of new, rapid, and more reliable molecular identification techniques necessary for accurate identification of *Monilinia* spp. The first molecular techniques were based on the presence of a 418-bp group-I intron located in the small-subunit ribosomal DNA gene of *M. fructicola* that was absent in *M. laxa* and *M. fructigena* (Fulton and Brown 1997). However, this method is no longer reliable because the intron was found to be absent in some *M. fructicola*

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isolates (Côté et al. 2004; Förster and Adaskaveg 2000; Fulton et al. 1999; Hughes et al. 2000). Another method makes use of the internal transcribed spacer (ITS) between the 18S and the 28S ribosomal RNA genes (Ioos and Frey 2000) yet it was found that the variation in the ITS region was not enough to separate between *M. fructigena* and *M. polystroma* isolates (Van Leeuwen et al. 2002). Later, a multiplex polymerase chain reaction (PCR) was developed based on a polymorphic sequence of *M. fructigena*, allowing the discrimination of *M. fructigena*, *M. fructicola*, and *M. laxa*, while the latest diagnostic assay was based on a highly conserved region within a *cytb* gene intron located at codon 143 of the gene (Hily et al. 2011; Miessner and Stammeler 2010). A multiplex real-time PCR was also developed for differentiating *M. fructicola* from *M. fructigena*, *M. laxa*, and *M. polystroma* (Van Brouwershaven et al. 2010).

Over recent years, a new alternative and powerful molecular technique called high-resolution melting (HRM) analysis has been introduced and utilized for DNA fingerprinting. HRM analysis is a closed-tube, post-PCR method for identifying genetic variation in DNA sequences by measuring changes in fluorescence level of the melting DNA amplicon (Madesis et al. 2014; Sakaridis et al. 2014). Prior to HRM analysis, the region of interest is amplified using PCR in the presence of a double-stranded (ds)DNA binding dye that does not interfere with the reaction and fluoresces brightly when bound to DNA or fluoresces at a low level in the unbound state (Ganopoulos et al. 2012; 2013; Reed and Wittwer 2004). After the PCR, the DNA amplicon is gradually heated until the dsDNA denatures into two single strands, causing the release of the binding dye and a drop in the fluorescent level. The whole process is monitored by the HRM instrument's software, resulting in a characteristic melting profile of

the amplicon (Ganopoulos et al. 2012; Zambounis et al. 2015). HRM analysis has already started to be used for the identification and discrimination of plant pathogens such as viruses (Bester et al. 2012) and bacteria (Gori et al. 2012; Ricchi et al. 2011); however, when it comes to phytopathogenic fungi, this method is still in its infancy (Zambounis et al. 2015) and has only been used for discriminating the *Fusarium oxysporum* formae speciales complex (Ganopoulos et al. 2012) and *Phyllosticta* spp. on banana (Wong et al. 2013).

This study was conducted to investigate whether an HRM assay can be developed for the simultaneous detection and identification of six different, closely related *Monilinia* spp. associated with brown rot disease of pome and stone fruit, and whether it is possible to be used with fungal DNA isolated directly from infected peach fruit tissues.

MATERIALS AND METHODS

Fungal cultures. In total, 8 *M. laxa*, 11 *M. fructicola*, 5 *M. fructigena*, 2 *M. linhartiana*, 2 *M. mumecola*, and 2 *M. yunnanensis* representative isolates from Greece, Spain, China, and the United States were included in the study (Table 1). Isolates originating from Greece were collected for the requirements of previous studies (Papavasileiou et al. 2015a, b), while isolates originating from the United States, Spain, and China were kindly offered to us by Dr. Guido Schnabel (Clemson University, South Carolina), Dr. Juan Moral (University of Cordoba, Spain), and Prof. Chao-Xi Luo (Huazhong Agricultural University, China), respectively. Isolates originating from China were received as DNA samples.

DNA extraction and sequencing. For DNA extraction the isolates were initially grown in potato dextrose broth (Sigma-Aldrich) for 2 days at 22°C. Subsequently, the mycelium was harvested by filtration, dried, lyophilized, ground to a fine powder using micropestles (Eppendorf International), and stored at -20°C until use. DNA was extracted from 50 to 100 mg of air-dried frozen mycelium using Puregene Core kit A (Qiagen GmbH), according to the manufacturer's protocol, and quantified with the a P330 nanophotometer (Implen GmbH). DNA was stored at -20°C until use. The primers P450intron6-2-fwd (AGGTGAGTAGGAAATACAGATAAATG) and P450intron6-2-rev (AGTTCAACTCAGATCTAAAGATACCTC), designed by Hily et al. (2011), were used to amplify a part of an intron, located in the *cytb* gene. The PCR reagents and amplification protocol were performed as described by Hily et al. (2011), in a total volume of 50 µl of the reaction mixture.

To verify the presence of the PCR products, after the end of each reaction, aliquots of the amplicons (5 µl) were separated by electrophoresis on a 1.5% agarose gel in Tris-acetate EDTA buffer, stained with ethidium bromide (0.1 µg ml⁻¹), and viewed under UV light. The QIAquick PCR Purification Kit (Qiagen GmbH) was used for the purification of the PCR products of each isolate, according to the manufacturer's recommendations. The purified products were sequenced in both directions using the P450intron6-2-fwd and P450intron6-2-rev primers. The sequencing was carried out by Cellular and Molecular Immunological Applications (CEMIA) S.A.

Sequence analysis and primer design. The obtained sequences from *M. laxa*, *M. fructicola*, *M. fructigena*, *M. linhartiana*, *M. mumecola*, and *M. yunnanensis* isolates were aligned using the CLUSTALW algorithm available in the software package MEGA v. 4 (Tamura et al. 2007) and Bioedit (Hall 1999). Sequences of *M. fructicola*, *M. laxa*, *M. fructigena* and *M. mumecola* deposited in GenBank (GenBank accession numbers GU952815.1, GU952817.1, HM149254.1, and JN204425.1, respectively) were also included in the study.

The targeted intron in the *cytb* gene location was found to be highly effective for the separation of the *Monilinia* spp. included in this study. Based on the sequences, a pair of primers (MoUni-fwd and MoUni-rev) was designed for the discrimination of *M. laxa*, *M. fructicola*, *M. fructigena*, *M. linhartiana*, *M. mumecola*, and *M. yunnanensis* by HRM analysis. A second set of primers (Mfcola-fwd and Mfcola-rev)

TABLE 1. Host, region, and country of origin of the *Monilinia* spp. isolates used in the study

<i>Monilinia</i> spp.	Isolate code	Origin ^a	Region	Host
<i>Monilinia laxa</i>	Mlx4	Greece	Thessaly	Peach
<i>M. laxa</i>	Mlx31	Greece	Thessaly	Peach
<i>M. laxa</i>	Mlx22	Greece	Macedonia	Peach
<i>M. laxa</i>	Mlx36	Greece	Macedonia	Cherry
<i>M. laxa</i>	Mlx15	Greece	Macedonia	Apricot
<i>M. laxa</i>	Mlx11	Spain	Las Angastara	Peach
<i>M. laxa</i>	Mlx12	Spain	Las Angastara	Peach
<i>M. laxa</i>	GEARI6A3C	United States	Middleton, RI	Cherry
<i>M. fructicola</i>	Mfl2	Greece	Thessaly	Cherry
<i>M. fructicola</i>	Mfl5	Greece	Thessaly	Peach
<i>M. fructicola</i>	Mfl23	Greece	Macedonia	Plum
<i>M. fructicola</i>	Mfl26	Greece	Macedonia	Cherry
<i>M. fructicola</i>	Mfl28	Greece	Macedonia	Apricot
<i>M. fructicola</i>	SCMD145	United States	Spartanburg, SC	Peach
<i>M. fructicola</i>	SCMD136	United States	Spartanburg, SC	Peach
<i>M. fructicola</i>	MDbbp4	United States	Kent, MD	Peach
<i>M. fructicola</i>	MDbbp5	United States	Kent, MD	Peach
<i>M. fructicola</i>	Mfl11	China	Zhejiang	Peach
<i>M. fructicola</i>	Mfl12	China	Hubei	Peach
<i>M. fructigena</i>	Mfg26	Greece	Macedonia	Quince
<i>M. fructigena</i>	Mfg28	Greece	Macedonia	Quince
<i>M. fructigena</i>	Mfg36	Greece	Macedonia	Apple
<i>M. fructigena</i>	Mfg21	Spain	Unknown	Peach
<i>M. fructigena</i>	Mfg11	Spain	Mansano La Camna	Peach
<i>M. linhartiana</i>	Mlh11	Spain	Las Palomas	Quince
<i>M. linhartiana</i>	Mlh12	Spain	Las Palomas	Quince
<i>M. mumecola</i>	Mmc11	China	Hubei	Peach
<i>M. mumecola</i>	Mmc12	China	Hubei	Peach
<i>M. yunnanensis</i>	Mmc11	China	Yunnan	Peach
<i>M. yunnanensis</i>	Mmc12	China	Yunnan	Peach

^a Isolates originating from Greece were collected for the requirements of a previous study (Papavasileiou et al. 2015b), while isolates originated from the United States, Spain, and China were kindly offered to us by Dr. Guido Schnabel (Clemson University, South Carolina), Dr. Juan Moral (University of Cordoba, Spain), and Prof. Chao-Xi Luo (Huazhong Agricultural University, China), respectively.

specific to *M. fructicola* was designed for the detection of the quarantine pathogen, again via the use of HRM. Primers were designed for robust performance and specificity of the following HRM analysis. The sequences of the primers are listed in Table 2.

HRM analysis. PCR amplification, DNA melting, and end-point fluorescence level acquiring PCR amplifications were performed in a 20-ml volume containing 20 ng of genomic DNA, 4 μ M MoUni-fwd and MoUni-rev primers, 0.2 mM each dNTP, 2.5 mM MgCl₂, 1.5 mM

SYTO 9 Green fluorescent nucleic acid stain (Life Technologies Corp.), and 1 U of Kapa Taq DNA polymerase (Kapa Biosystems). The PCR amplifications were performed in a Rotor-Gene 6000 real-time 5P HRM PCR thermocycler (Corbett Research) using the following thermal parameters: an initial preheat for 10 min at 95°C; followed by 35 cycles at 95°C for 20 s, 55°C for 20 s, and 72°C for 15 s; and terminated with a final extension at 72°C for 10 min. After the PCR amplification, HRM was performed from 68 to 90°C, rising by 0.1°C in each step, with a 2-s hold time for each step and with 90 s of premelt conditioning on the first step. The HRM data were analyzed with the Rotor-Gene Q series software (version 2.3.1; Qiagen). The melting curves were normalized in order to limit the temperature range between the pre- and postmelt regions and eliminate the fluorescence variance. In the difference graph, *M. linhartiana* was used as a reference baseline curve. All experiments were repeated in triplicate.

Identification of *Monilinia* spp. on artificially inoculated peach. To determine whether this method can discriminate *Monilinia* spp. without the need of culturing the isolates, an HRM analysis was performed using DNA directly extracted from artificially infected fruit. One isolate from each of the available *M. fructicola*, *M. laxa*, *M. linhartiana*, and *M. fructigena* cultures was randomly selected and used for the artificial inoculations. The selected isolates were cultured on potato dextrose agar medium under continuous light at 25°C for 6 days. Peach fruit ('Andross') which had received no fungicide applications during the growing season were collected just before harvest from AUTH (Aristotle University of Thessaloniki), School of Agriculture experimental orchards. Fruit were free of infection during the collection and storage. The surface of the fruit was disinfested by submerging them in a 10% solution of commercial sodium hypochlorite for 1 min, and then rinsed twice

TABLE 2. List of primers designed for the requirements of the study

Primer	Sequence (5' to 3')
MoUni-fwd	GATATAGTCAAGCCCCATTYGAAAG
MoUni-rev	CTTTCAGTCTTTAAATAGTATAGAAAGCAAT
Mfcola-fwd	GGCTTTTTTATTCGAGAATAAAATGGCTTAAG
Mfcola-rev	GAGAAAAGAAGGGCGTCGGTTAAACC

TABLE 3. Isolates of different fungal species used in the *Monilinia fructicola*-specific diagnostic test^a

Fungal species	Isolate code	Host of origin	Region
<i>M. fructicola</i>	MFM5	Peach	Skidra
<i>M. laxa</i>	MLM82	Peach	Larissa
<i>M. fructigena</i>	MFR5	Apple	Kastoria
<i>Botrytis cinerea</i>	BCP12	Strawberry	Pieria
<i>Alternaria alternata</i>	AAM26	Pomegranate	Xanthi
<i>Aspergillus niger</i>	ANP36	Pomegranate	Drama
<i>Penicillium expansum</i>	PEP16	Apple	Skidra

^a Isolates belonging in the isolate bank of the Laboratory of Plant Pathology of AUTH, obtained and characterized for the requirements of previous studies (Kanetis et al. 2015; Konstantinou et al. 2011, 2015; Papavasileiou et al. 2015a).

Fig. 1. Multiple nucleotide sequences of *Monilinia fructicola*, *M. laxa*, *M. fructigena*, *M. linhartiana*, *M. mumecola*, and *M. yunnanensis* corresponding to the targeted intron of the *cytb* gene using the primers P450intron6-2-fwd and P450intron6-2-rev (Hily et al. 2011). Universal primers MoUni-fwd and MoUni-rev, designed for the discrimination of *M. fructicola*, *M. laxa*, *M. fructigena*, *M. linhartiana*, *M. mumecola*, and *M. yunnanensis*, are enclosed in the left-most boxes and *M. fructicola*-specific primers Mfcola-fwd and Mfcola-rev are enclosed in the right-most boxes.

with sterile water. Fruit were placed in sterilized plastic containers lined with wet paper towels. With the help of a sterile lancet, a U-shaped incision (30 mm in diameter) was made on the surface of the fruit to a depth of 20 mm. Inoculation of fruit was done after lifting the skin within the incision and placing a 5-mm² mycelial plug of each isolate culture inside the fruit tissue. In total, three replicate fruit were inoculated with each isolate. The containers with the fruit were then covered with lids to ensure high relative humidity and incubated at 22°C for 6 days. At the end of the incubation period, DNA was extracted directly from the skin and flesh of the fruit lesions using the Nucleospin Plant II extraction kit (Macherey-Nagel GmbH & Co.), according to the manufacturer's protocol.

Development of a *M. fructicola*-specific diagnostic test.

In order to verify the specificity of the *M. fructicola*-specific primers against other pathogens and to test the method's ability to detect *M. fructicola* in mixed inoculations, an HRM analysis was performed using DNA of the most common *Monilinia* spp. and

other fungal pathogens or saprophytes that can be found on stone fruit and *M. fructicola* (Table 3). The analysis was performed on individual samples and, at the same time, on a 1:1 mixture with DNA extracted from a representative isolate of *M. fructicola*.

The PCR amplification and HRM analysis were conducted in a Rotor-Gene 6000 real-time 5P HRM PCR thermocycler (Corbett Research). The reaction reagents and thermal program of the PCR were the same as described above. Upon completion of the PCR, HRM was performed at a temperature ramp from 73 to 85°C, with increments of 0.1°C every 2 s. Rotor-Gene Q series software (version 2.3.1; Qiagen) was used to analyze the data while the melting profiles were subjected to normalization and a difference plot was obtained using *M. fructicola* as the baseline.

Assay sensitivity. To determine the detection limits of the *M. fructicola*-specific primers, an HRM analysis was performed using known DNA concentrations of *M. fructicola*. Concentrations of 0.001, 0.01, 0.1, 1, and 10 ng per reaction tube were obtained

Fig. 2. High-resolution melting (HRM) analysis using the MoUni-fwd and MoUni-rev primers for the discrimination of *Monilinia fructicola*, *M. laxa*, *M. fructigena*, *M. linhartiana*, *M. mumecola*, and *M. yunnanensis*. **A**, Normalized HRM curves of *Monilinia* spp. **B**, Difference graph of HRM curves using *M. fructicola* as the reference baseline curve.

from serial dilutions of the *M. fructicola* isolate Mf15. The PCR amplification and HRM analysis conditions were the same as described above.

RESULTS

HRM analysis for the identification of *Monilinia* spp.

Primers MoUni-fwd and MoUni-rev, designed for the discrimination of *M. fructicola*, *M. laxa*, *M. fructigena*, *M. linhartiana*, *M. mumecola*, and *M. yunnanensis* amplified a fragment of 250, 150, 432, 148, 249, and 150 bp for each species, respectively. Sequences were identical within species for all the available isolates tested; however, they were different between species (Fig. 1). Analysis of the normalized HRM curves produced with the tested primers generated six distinct melting profiles, allowing the differentiation of all *Monilinia* spp. (Fig. 2A). Specifically, the difference graph generated using the mean *M. fructicola* curve as a baseline revealed part of the curve

sitting outside the 80% confidence interval (CI) curve, indicating that *Monilinia* spp. through the HRM curves were, indeed, different (Fig. 2B).

HRM analysis for the detection of *Monilinia fructicola*, *M. laxa*, *M. fructigena*, and *M. linhartiana* in planta. When the same HRM analysis using the MoUni-fwd and MoUni-rev primers was applied with DNA extracted directly from inoculated fruit, it allowed the detection and discrimination of *M. fructicola*, *M. laxa*, *M. fructigena*, and *M. linhartiana* genotypes. As depicted in the normalized data (Fig. 3A), HRM curves of *Monilinia* spp. were clustered together into four groups, one for each species, clearly distinguishable among them. This was also confirmed by the examination of the HRM difference plots, in which species had different plots distinct from each other (Fig. 3B).

Specific detection of *M. fructicola* from mixed DNA samples. *M. fructicola*-specific primers Mfccla-fwd and Mfccla-rev, designed for the detection of *M. fructicola* in HRM analysis,

Fig. 3. High-resolution melting (HRM) analysis using the MoUni-fwd and MoUni-rev primers for the detection of *Monilinia fructicola*, *M. laxa*, *M. fructigena*, and *M. linhartiana* extracted from artificially inoculated fruit along with reference strains of *M. fructicola*, *M. laxa*, *M. fructigena*, and *M. linhartiana*. **A**, Normalized data. **B**, Difference graph of HRM curves using *M. fructicola* as the standard genotype.

amplified a 120-bp product, part of the *cytb* gene. Analysis of the normalized HRM curves showed that all mixtures containing DNA of *M. fruticola* were clustered together and were easily distinguishable from the curves produced by *M. laxa*, *M. fructigena*, *Botrytis cinerea*, *Alternaria alternata*, *Aspergillus niger*, and *Penicillium expansum* isolates (Figs. 4A and 5A).

Furthermore, analysis of the differentiation curves, with the mean *M. fruticola* curve as reference, revealed part of the curve sitting outside the 80% CI curve, suggesting that the curves representing strains other than *M. fruticola* were in fact all different from

M. fruticola (Figs. 4B and 5A). The assay was highly sensitive because the *M. fruticola*-specific primers Mfcola-fwd and Mfcola-rev successfully detected down to 0.01 ng of genomic *M. fruticola* DNA, as shown in the difference graph generated using 0.001 ng of *M. fruticola* as the reference baseline curve (Fig. 6).

DISCUSSION

Monilinia spp. can be disseminated through several natural means but also with importation or exportation of plant propagative

Fig. 4. High-resolution melting (HRM) analysis using the *Monilinia fruticola*-specific primers Mfcola-fwd and Mfcola-rev. *M. fruticola* DNA was mixed in a 1:1 ratio with genomic DNA from *M. laxa* or *M. fructigena* and in a 1:1:1 ratio with both *M. laxa* and *M. fructigena*. Individual isolates of *M. fruticola*, *M. laxa*, and *M. fructigena* were also used in the HRM analysis as reference isolates. **A**, Normalized data. **B**, Difference graph of HRM curves generated using *M. fruticola* as the reference genotype.

material and fresh or dried fruit (EFSA Panel on Plant Health 2011). Therefore, availability of modern detection and identification methods of plant pathogens on infected material is of high importance for phytosanitary agencies. Difficulties in robust identification of members of the genus *Monilinia* at the species level using morphological characteristics has led to the development of several molecular techniques (Côté et al. 2004; Gell et al. 2007; Hu et al. 2011; Hughes et al. 2000; Ioos and Frey 2000; Luo et al. 2007; Van Brouwershaven et al. 2010). However, most of the developed protocols distinguish only the three main *Monilinia* spp. associated with brown rot of stone fruit (*M. fructicola*, *M. fructigena*, and *M. laxa*) and most of them necessitate the use of pure fungal cultures.

A few recent studies have shown that HRM can be effective in providing consistent shape and profile in melting curves that correspond to several bacterial or fungal species causing diseases on plants and, thus, enabling their accurate identification (Ganopoulos et al. 2012; Gori et al. 2012; Wong et al. 2013). The aim of this study

was to develop and apply an HRM analysis for the detection and discrimination of *Monilinia* spp. The design of the universal primers MoUni-fwd and MoUni-rev was based on the sequences within the sixth intron in *M. fructicola* and *M. laxa* and seventh intron in *M. fructigena*, located in the *cytb* gene. This region was selected based on the findings of two previous studies suggesting that *cytb* sequences in *Monilinia* spp. are highly conserved and phylogenetically informative and have been used for discrimination of *M. fructicola*, *M. laxa*, and *M. fructigena* (Hily et al. 2011; Hu et al. 2011). The assay was validated with fungal isolates originating from several hosts and continents to cover, as much as possible, natural variations within the main *Monilinia* spp. which are agents of brown rot. Our results confirmed that the sequences amplified with MoUni-fwd and MoUni-rev primers possessed variations allowing, through a very clear and reproducible HRM curve, analysis the differentiation of the six *Monilinia* spp. tested: *M. fructicola*, *M. laxa*, *M. fructigena*, *M. linhartiana*, *M. mumecola*, and *M. yunnanensis*.

Fig. 5. High-resolution melting (HRM) analysis using the *Monilinia fructicola*-specific primers Mfcola-fwd and Mfcola-rev. *M. fructicola* DNA was mixed in a 1:1 ratio with genomic DNA from *Botrytis cinerea*, *Alternaria alternata*, *Aspergillus niger*, and *Penicillium expansum*. An individual isolate of *M. fructicola* was used in the HRM analysis as a reference isolate. **A**, Normalized data. **B**, Difference graph of HRM curves generated using *M. fructicola* as the reference genotype.

Furthermore, this method was capable of discriminating *M. fructicola*, *M. laxa*, *M. fructigena*, and *M. linhartiana* directly from infected peach fruit tissues without the need of isolating and culturing the pathogens before DNA extraction. This is particularly important not only because of the rapid nature of the test but also because it is well known that different *Monilinia* spp. may occur on the same infected fruit (Miessner and Stammeler 2010). Thus, application of this method offers a simultaneous detection of different *Monilinia* spp. that co-occur on infected fruit samples and can be utilized as a rapid diagnostic test contributing to a faster and more accurate monitoring of imports and exports of stone fruit, to eventually limit the spread of brown rot pathogens.

The HRM analysis primers specific to *M. fructicola* (Mfcola-fwd and Mfcola-rev) amplified a 120-bp region with a distinct melt profile corresponding to the presence of *M. fructicola* regardless of the presence of other species. The amplicon was kept short to ensure good sensitivity of species detection. Compared with other methods, HRM-based genotyping and species identification possesses higher resolving power. New reverse-transcription PCR machines combined with HRM analysis require minimal time, and the costs of consumables are typically less than those for standard methods. Furthermore, HRM methods is easy to perform and presents high sensitivity, high specification, precision, and accuracy and is closed tube, and could be used as a quantitative or semiquantitative method. These properties would render HRM analysis the method of choice in the field of phytopathogenic fungal species identification. HRM has been proven extremely sensitive because it could discriminate even formae speciales of *F. oxysporum*, not to mention mutations in the *cytb* gene, or even detect adulterations in food as low as 1% (wt/wt) (Chatzidimopoulos et al. 2014; Madesis et al. 2014; Zambounis et al. 2015). Thus, this technique could be utilized as a new fast,

reliable, and inexpensive tool for better border surveillance against the quarantine pathogen *M. fructicola*.

The HRM analysis developed in this study has many advantages compared with the other PCR-based molecular techniques used for the identification of *Monilinia* spp. The developed assay expands the range of *Monilinia* spp. that can be identified in a single test, whereas previously developed assays focused only on the four main *Monilinia* spp. such as *M. laxa*, *M. fructicola*, *M. fructigena*, and *M. polystroma* (Côté et al. 2004, Van Brouwershaven et al. 2010). In our study, *M. polystroma* was not included despite the fact that it's a species that has recently been identified as a brown rot agent in some European and Asian countries. This was because, despite our efforts, we were unable to find any *M. polystroma* isolate either from laboratories that had isolated it in the past or any fungal collection bank. HRM analysis does not require gel electrophoresis of the PCR products, allowing the analysis of a large number of samples simultaneously, saving time, avoiding hazardous chemicals such as ethidium bromide, and eliminating the chance of cross-contaminating the samples. Furthermore, after the HRM analysis, samples can be grouped together based on their normalized profile and limiting the cost of sequencing to only some representative samples of each group (Sakaridis et al. 2014). On the downside, the increased sensitivity of the HRM at the nucleotide level requires the use of homogenous samples (single DNA extraction protocol and the same amount of DNA) in order to prevent variation and for the results to be reproducible (Chatzidimopoulos et al. 2014).

The HRM method has several advantages compared with other methods developed earlier, as described previously; however, in addition, there are two important disadvantages. There is a necessity for a specific HRM thermocycler that currently is of relatively high

Fig. 6. High-resolution melting (HRM) analysis using the *Monilinia fructicola*-specific primers Mfcola-fwd and Mfcola-rev. Difference curves were generated using 0.1, 1, 5, or 10 ng of genomic DNA from *M. fructicola* while 0.001 ng of genomic DNA from *M. fructicola* was used as the reference genotype.

cost and can be found only in well-equipped laboratories and, in addition, there is a necessity for the availability of positive controls in the lab that performs the tests.

Currently, diagnostic protocols applied for the identification of *Monilinia* spp. include the end-point PCR proposed by Ioos and Frey (2000) and the real-time PCR assay developed by Van Brouwershaven et al. (2010). Recently, one more assay, the multiplex PCR test that had been developed by Côté et al. (2004), was validated and found useful for routine diagnosis applications because it was fulfilling the basic performance requirements (Riccioni and Valente 2015). The data presented in the current study suggest that, in addition to previously developed tests, HRM analysis can be a powerful tool for the identification of the main agents of brown rot of stone fruit belonging to *Monilinia* spp. and, in particular, of *M. fructicola*, a quarantine pathogen in European countries. This is particularly important when taking into account the fact that brown rot of stone and pome fruit is caused by several closely related species of *Monilinia* that cause similar symptoms on their hosts. In addition, the method has shown several advantages that have been reviewed elsewhere (Iacumin et al. 2015; Reed et al. 2007; Vossen et al. 2009). It is considered to be a rapid, simple, and inexpensive method with no need for post-PCR processing of the PCR products using gels and gel electrophoresis. In consequence, this novel method is expected to be an attractive alternative contributing to more rapid and accurate phytosanitary control programs implemented throughout Europe, aiming to identify *Monilinia* spp. on stone fruit. In the current study, the method was evaluated using DNA that had been extracted from either pure fungal cultures or artificially inoculated fruit showing decay and fungal sporulation on the decayed tissues. Investigating possible applications of this novel method for pathogen detection in quiescent infections or airborne inoculum could contribute not only toward the rapid diagnosis of the pathogens but also toward a better understanding of the epidemiology of brown rot on stone fruit and the plant–microbe interactions.

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