Detection and Identification of Six *Monilinia* spp. Causing Brown Rot Using TaqMan Real-Time PCR from Pure Cultures and Infected Apple Fruit

Jing-ru Wang and Li-yun Guo, Department of Plant Pathology and MOA Key Lab of Pest Monitoring and Green Management, College of Plant Protection, China Agricultural University, 100193, Beijing, China; Chang-lin Xiao, United States Department of Agriculture–Agricultural Research Service, San Joaquin Valley Agricultural Sciences Center, Parlier, CA 93648; and Xiao-qiong Zhu,[†] Department of Plant Pathology and MOA Key Lab of Pest Monitoring and Green Management, College of Plant Protection, China Agricultural University

Abstract

Brown rot is a severe disease affecting stone and pome fruit. This disease was recently confirmed to be caused by the following six closely related species: *Monilinia fructicola*, *M. laxa*, *M. fructigena*, *Monilia polystroma*, *M. mumeicola*, and *M. yunnanensis*. Because of differences in geographic distributions, some of these species are important quarantine pathogens in certain countries. In this study, we developed TaqMan real-time polymerase chain reaction (PCR) assays to detect and identify the six species. Primer pairs and probes were designed for *Monilinia fructicola*, *M. fructigena*, *M. laxa*, and *Monilia polystroma* based on sequence differences

in the laccase-2 genes. Additionally, based on sequence differences in the elongation factor genes, primer pairs and probes were designed for *Monilia mumeicola* and *M. yunnanensis*. The real-time PCR assays were able to specifically identify the target pathogens, with detection limits of 10 to 100 fg of DNA, which is equivalent to one to seven conidia. The assays were also able to detect the target pathogens in a mixed DNA sample comprising all six *Monilinia* spp. and related species. The real-time PCR assays accurately detected target fungi from infected apple fruit. Furthermore, the identification results were consistent with those of traditional morphological methods.

Brown rot is one of the most economically important diseases of stone fruit, including peach and nectarine, because it can cause severe yield losses during pre- and postharvest stages. Brown rot fungi also cause diseases in pome fruit such as apple and pear and in the fruit of many other plants, including grapevine, hawthorn, guava, and tomato (Byrde and Willetts 1977). In addition to fruit diseases, brown rot fungi can also cause blossom and leaf blights (Berrie and Holb 2014; Byrde and Willetts 1977). The incidence of peach and nectarine fruit brown rot in China ranges from 20 to 100% (Zhu et al. 2008). In Europe, preharvest losses of 7 and 25% were observed in conventional and organic apple orchards, respectively (Berrie and Holb 2014).

Six closely related species of brown rot fungi have been isolated from stone and pome fruit, including *Monilinia fructicola* (G. Winter) Honey, *M. laxa* (Aderh. & Ruhland) Honey, *M. fructigena* Honey, *Monilia polystroma* G. Leeuwen, *M. yunnanensis* M. J. Hu & C. X. Luo, and *M. mumeicola* Y. Harada, Y. Sasaki & Sano (Byrde and Willetts 1977; Harada et al. 2004; Hu et al. 2011; Van Leeuwen et al. 2002). These species are differentially distributed around the world, with two or more species usually detected in a particular country or region. For example, *M. fructicola* and *M. laxa* are commonly detected in North America. Meanwhile, *M. fructigena* and *M. laxa* have been reported mostly in Europe, along with a few *M. fructicola* and *M. polystroma* isolates (Bosshard et al. 2006; De Cal et al. 2009; Duchoslavová et al. 2007; Grabke et al. 2011; Hilber-Bodmer et al. 2010; Munda and Viršček Marn 2010; Pellegrino et al. 2009; Petróczy and Palkovics 2006; Poniatowska et al. 2013; Vasic et al. 2012). Additionally, infections caused by *M. fructicola*, *M. laxa*, *M. polystroma*, and *M. mumeicola* are common in Japan, whereas *M. fructicola*, *M. yunnanensis*, *M. polystroma*, and *M. mumeicola* have often been reported in China, in addition to *M. laxa* and *M.*

fructigena, which have been detected in wild fruit tree forests in Xinjiang (Hu et al. 2011; Niu et al. 2016; Yin et al. 2015; Zhu and Guo 2010; Zhu et al. 2005, 2011, 2016).

Historically, *Monilinia* spp. have been differentiated based on morphological and cultural characteristics (Byrde and Willetts 1977; van Leeuwen and van Kesteren 1998). However, morphological traits vary with growth conditions and are often not reliable enough to be used for routine species-level identifications. Several polymerase chain reaction (PCR)-based methods have recently been developed to identify *Monilinia* spp. based on sequence differences in genetic markers such as simple sequence repeats (Ma et al. 2003) and random amplified polymorphic DNA sequences (Côté et al. 2004; Gell et al. 2007). Other PCR-based *Monilinia* identification procedures have focused on specific genetic regions, including internal transcribed spacer (ITS) regions 1 and 2 (Gell et al. 2007; Ios and Frey 2000) and the β -tubulin (Fan et al. 2014; Hu et al. 2011; Ma et al. 2003) and *cyt b 1* genes (Hily et al. 2011; Miessner and Stammler 2010).

These molecular methods involved a conventional PCR (Ios and Frey 2000; Ma et al. 2003), multiplex PCR (Côté et al. 2004; Gell et al. 2007; Hily et al. 2011; Hu et al. 2011; Miessner and Stammler 2010), or real-time PCR (Fan et al. 2014; Garcia-Benitez et al. 2017; Papavasileiou et al. 2016; Van Brouwershaven et al. 2010) to separate up to four *Monilinia* spp. at one time. However, subsequent attempts by other researchers to use the same methods to identify some isolates were unsuccessful (Fan et al. 2007; Hu et al. 2011; Zhu et al. 2011). Comparisons of *Monilinia* sequences revealed that differences are restricted to small genetic regions. Additionally, the primers designed for these PCR-based methods are unable to amplify the sequences of recently discovered species such as *M. polystroma*, *M. mumeicola*, and *M. yunnanensis*. Thus, assays used to identify *Monilinia* spp. must be able to distinguish between all six aforementioned related *Monilinia* spp. consistently. In a previous study, we designed primers specific for the six *Monilinia* spp. based on the laccase gene, and a universal PCR method was developed that could identify five of the six *Monilinia* spp. (Zhu et al. 2016). A conventional PCR may not be sensitive enough to identify *M. fructigena*. Real-time PCR may represent a better option than conventional PCR because of its greater specificity and sensitivity.

A reliable method for accurately and rapidly identifying *Monilinia* spp. is essential for phytosanitary inspections and for ensuring that imported and exported fruit (e.g., peach, apple, and pear) comply with plant quarantine regulations. Therefore, the objectives of this study were to (i) design primers and probes specific for the laccase

[†]Corresponding author: X.-q. Zhu; E-mail: mycolozhu@cau.edu.cn

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gene and elongation factor of *Monilinia* spp. and (ii) develop Taq-Man real-time PCR assays for differentiating among the six *Monilinia* spp. from pure cultures and in infected apple fruit.

Materials and Methods

Fungal isolate collection. In total, 54 isolates of six species of *Monilinia* or *Monilia* causing brown rot of stone and pome fruit were analyzed. These isolates were collected from 35 geographic

populations from nine countries: China, the United States, France, Japan, The Netherlands, Poland, Spain, England, and Ireland (Table 1). All isolates were identified based on morphological and PCR using species-specific primers or sequence comparison of ITS, β -tubulin, and laccase genes in the previous studies (Zhu et al. 2011, 2016). The following nontarget fungal pathogens known to cause diseases in stone and pome fruit were also included in this study: *Botrytis cinerea*, *Botryosphaeria dothidea*, *Alternaria* sp., *Penicillium* sp., and *Colletotrichum fructicola* (Table 1).

Table 1. Isolates analyzed in this study

Isolate name	Species	Location	Host
ABC15	<i>Monilia yunnanensis</i>	Changping, Beijing, China	<i>Malus domestica</i>
ABC3	<i>M. yunnanensis</i>	Changping, Beijing, China	<i>M. domestica</i>
ABH19	<i>M. yunnanensis</i>	Huairou, Beijing, China	<i>M. domestica</i>
AK7-1	<i>M. yunnanensis</i>	Akesu, Xinjiang, China	<i>M. domestica</i>
AK8-1	<i>M. yunnanensis</i>	Akesu, Xinjiang, China	<i>M. domestica</i>
AK9-1	<i>M. yunnanensis</i>	Akesu, Xinjiang, China	<i>M. domestica</i>
AL1-2	<i>M. yunnanensis</i>	Alaer, Xinjiang, China	<i>M. domestica</i>
AL2-1	<i>M. yunnanensis</i>	Alaer, Xinjiang, China	<i>M. domestica</i>
LBD4	<i>M. yunnanensis</i>	Daxing, Beijing, China	<i>Pyrus</i> sp.
323031	<i>Monilinia fructicola</i>	Pinggu, Beijing, China	<i>Prunus persica</i>
324051	<i>M. fructicola</i>	Pinggu, Beijing, China	<i>P. persica</i>
C67	<i>M. fructicola</i>	Changping, Beijing, China	<i>P. persica</i>
H111	<i>M. fructicola</i>	Pinggu, Beijing, China	<i>P. persica</i>
H605	<i>M. fructicola</i>	Pinggu, Beijing, China	<i>P. persica</i>
KAC3-4	<i>M. fructicola</i>	United States	Unknown
NE18	<i>M. fructicola</i>	New Zealand	Unknown
NE20	<i>M. fructicola</i>	New Zealand	Unknown
PLY6	<i>M. fructicola</i>	Yantai, Shandong, China	<i>P. persica</i>
PLY9	<i>M. fructicola</i>	Yantai, Shandong, China	<i>P. persica</i>
RI37	<i>M. fructicola</i>	United States	Unknown
2319	<i>Monilia polystroma</i>	Japan	Unknown
CBS102686	<i>M. polystroma</i>	Japan	Unknown
LHX12	<i>M. polystroma</i>	Xinji, Hebei, China	<i>Pyrus</i> sp.
LHX6	<i>M. polystroma</i>	Xinji, Hebei, China	<i>Pyrus</i> sp.
LS13	<i>M. polystroma</i>	Laiyang, Shandong, China	<i>Pyrus</i> sp.
LS25	<i>M. polystroma</i>	Laiyang, Shandong, China	<i>Pyrus</i> sp.
LS4	<i>M. polystroma</i>	Laiyang, Shandong, China	<i>M. domestica</i>
LS5	<i>M. polystroma</i>	Laiyang, Shandong, China	<i>M. domestica</i>
SS3	<i>M. polystroma</i>	Laiyang, Shandong, China	<i>M. domestica</i>
CBS101499	<i>Monilinia fructigena</i>	Spain	Unknown
CBS101500	<i>M. fructigena</i>	Poland	Unknown
CBS101502	<i>M. fructigena</i>	England	Unknown
EA1	<i>M. fructigena</i>	England	Unknown
EA2	<i>M. fructigena</i>	England	Unknown
EA3	<i>M. fructigena</i>	England	Unknown
GD8-1	<i>M. fructigena</i>	Yili, Xinjiang, China	<i>M. sieversii</i>
GM7-1	<i>M. fructigena</i>	Yili, Xinjiang, China	<i>P. armeniaca</i>
GM8-2	<i>M. fructigena</i>	Yili, Xinjiang, China	Unknown
HX14-1	<i>M. fructigena</i>	Yili, Xinjiang, China	<i>P. cerasifera</i>
HX17-1	<i>M. fructigena</i>	Yili, Xinjiang, China	<i>M. sieversii</i>
474	<i>M. laxa</i>	United States	Unknown
BC11	<i>M. laxa</i>	United States	Unknown
CBS489.50	<i>M. laxa</i>	The Netherlands	Unknown
CBS298.31	<i>M. laxa</i>	Ireland	Unknown
P3	<i>M. laxa</i>	France	Unknown
HD17-1	<i>M. laxa</i>	Yili, Xinjiang, China	<i>P. cerasifera</i>
TL5-2	<i>M. laxa</i>	Tuoli, Tacheng, China	<i>M. sieversii</i>
HX8-1	<i>M. laxa</i>	Yili, Xinjiang, China	<i>P. cerasifera</i>
HX9-1	<i>M. laxa</i>	Yili, Xinjiang, China	<i>P. cerasifera</i>
MDA12-1	<i>M. laxa</i>	United States	Unknown
3231	<i>Monilia mumecola</i>	Japan	<i>P. mume</i>
HWL10-4b	<i>M. mumecola</i>	Wuhan, Hubei, China	<i>P. persica</i>
HWL10-20a	<i>M. mumecola</i>	Wuhan, Hubei, China	<i>P. persica</i>
BJ6	<i>Botrytis cinerea</i>	Unknown	Unknown
C38	<i>Colletotrichum fructicola</i>	Changping, Beijing, China	<i>Fragaria ananassa</i>
F4	<i>Alternaria</i> sp.	Xinjiang, China	<i>Ziziphus jujube</i>
Pe	<i>Penicillium</i> sp.	Beijing, China	Unknown
ZY7	<i>Botryosphaeria dothidea</i>	Zhaoyuan, Shandong, China	<i>M. domestica</i>

DNA extraction. The mycelia of all isolates maintained on potato dextrose agar (PDA) slants at 4°C or in 15% glycerol at –80°C (Zhu and Xiao 2015) were revived on PDA and transferred to pea broth. The mycelia were grown in Petri dishes containing pea broth (with no agitation) at 22°C in darkness for 5 days. The mycelial DNA was extracted as described by Zhu et al. (2011), and the DNA concentration was quantified with a NanoDrop 2000 (Thermo Scientific, Wilmington, DE).

TaqMan probe and real-time PCR primer design. The laccase and elongation factor gene sequences of the six *Monilinia* spp. and two closely related nontarget fungi (*Botrytis cinerea* and *Sclerotinia sclerotiorum*) were obtained in a previous study (Zhu et al. 2016), and were aligned with DNAMAN, version 6.0. TaqMan probes and real-time PCR primers specific for *M. fructicola*, *M. laxa*, *M. fructigena*, *M. polystroma*, *M. yunnanensis*, and *M. mumeicola* were designed based on target gene sequence differences. The TaqMan probes needed to satisfy the following criteria: (i) 13 to 30 bp long for black-hole quencher (BHQ) and 13 to 25 bp long for minor groove binder (MGB), (ii) 30 to 80% GC content, (iii) no G bases at the 5' end and no G as the second base if tagged with 6-carboxyfluorescein (FAM) without six sequential A bases, (iv) fewer than two G and C bases in the five bases at the 3' end, (v) probe should be as close as possible to the primer, and (vi) no complementary secondary structures.

The criteria used to design the real-time PCR primers were as follows: (i) 18 to 30 bp long, (ii) 40 to 60% GC content, (iii) no A bases at the 3' end, (iv) 50- to 150-bp amplicon, (v) no distinct hairpin structure, (vi) species specific, and (vii) optimal annealing temperature. All primers were synthesized by Shanghai Genecore Biotech (Shanghai, China).

Assessment of the primer specificity for *Monilinia* spp. in conventional PCR. To determine the ideal annealing temperature for each primer pair, a temperature-gradient PCR was conducted with temperatures of 50 to 66°C. Three isolates of each *Monilinia* sp. were used for this initial PCR. For each primer pair, the PCR was completed in a 25-μl reaction volume containing 2.5 μl of 10× PCR buffer (TaKaRa Bio, Dalian, China), 200 μM each dNTP, 0.4 μM forward and reverse primers, 0.5 U of Ex Taq DNA polymerase, and 20 to 30 ng of genomic DNA. The PCR conditions were as

follows: 94°C for 3 min; 30 cycles of 94°C for 30 s, annealing temperature for 30 s, and 72°C for 40 s; and 72°C for 4 min. The PCR was completed in an ABI 9902 96-well PCR amplification system (Applied Biosystems, Carlsbad, CA). The resulting PCR products were separated by electrophoresis in 2% agarose gels using a Tris-acetate EDTA buffer. The amplified products stained with ethidium bromide were visualized and photographed with a MultiImage Light Cabinet (Alpha Innotech Corporation, U.S.A.).

Primer specificity was assessed in a conventional PCR using DNA from 20 isolates of the six target pathogens and nontarget fungi (Table 1). The PCR was completed as described above, using optimized annealing temperatures.

Assessment of the real-time PCR probe concentrations, and specificity and sensitivity of primers and probes. Optimal probe concentrations were based on real-time PCR results for seven dilutions (0.01 to 1.3 μM) for each probe. The PCR was conducted in 20-μl reaction volumes containing 10 μl of 2× TaqMan universal PCR master mix (Shanghai Genecore Biotech), 0.5 μM forward and reverse primers, and 10 to 15 ng of genomic DNA. The PCR conditions were as follows: 94°C for 3 min; 30 cycles of 94°C for 30 s, optimized annealing temperature for 30 s, and 72°C for 40 s; and 72°C for 4 min. The PCR was completed in an ABI 7500 96-well PCR instrument (Applied Biosystems, U.S.A.).

To improve the efficiency of the PCR, the primer and probe specificities were also tested using the annealing temperatures mentioned above. Similar to the conventional PCR, primer specificity was also tested for real-time PCR assays using DNA extracted from the target pathogens and nontarget fungi (Table 1). The PCR assays were conducted as described above to optimize the probe concentration. Additionally, primer specificity was tested using a mixture consisting of DNA from the six *Monilinia* spp. and five reference species (i.e., *Botryosphaeria dothidea*, *Alternaria* sp., *Botrytis cinerea*, *C. fructicola*, and *Penicillium* sp.). Each PCR included a 60-ng genomic DNA mixture as the template.

The sensitivity of the real-time PCR method for the six *Monilinia* spp. was determined with a series of samples with decreasing DNA concentrations. Seven 10-fold dilutions of DNA extracted from pure fungal cultures (10 ng μl⁻¹ to 10 fg μl⁻¹) were assessed using three

Table 2. Polymerase chain reaction primers and probes used in this study

Target species	Primer, probe	Sequence (5'–3') ^a	Temp (°C) ^b	Product (bp)	Probe (μM)	Equation	r ²	Efficiency (%)
<i>Monilinia fructicola</i>	MfcF	TGCTCACTCAAGTAAGTTGATCTGC	59	144	0.2	$y = -3.4215x + 22.51$	0.9911	96
	MfcR	TCCATCGCCGTATTGAAGT	59	...	...	...	...	...
	MfcP	FAM-TACCTCCATCAAGTGCCCTATCGCT-BHQ	...	...	...	...	...	...
<i>Monilinia fructigena</i>	MfgF	TCAATCGATACCAACTGGTACGAT	62	123	0.5	$y = -3.2673x + 25.577$	0.9912	102
	MfgR	TTCTCAACTGAAAGCCAATATTCTTTAG	62	...	...	...	...	...
	MfgP	FAM-CACTAGAGGTAAGTGATATGACAT-MGB	...	...	...	...	...	...
<i>Monilinia laxa</i>	MlaF	AATTGATACCAACTGGTACGATGTG	59	111	0.8	$y = -3.452x + 24.56$	0.9878	95
	MlaR	AAAGCCAATATTCTTGATATCAAGTTAGTG	59	...	...	...	...	...
	MlaP	FAM-AGGTAAGTGATAAAACATCCTTT-MGB	...	...	...	...	...	...
<i>Monilia polystroma</i>	MpoF	ATACCAACTGGTACGATGTTACTCCTAC	61	105	1.0	$y = -3.12x + 24.236$	0.9862	109
	MpoR	AAGCCAATATTCTTTATATAAAGTTAGCGC	61	...	...	...	...	...
	MpoP	FAM-CTAGAGGTAAGTGATATGGCA-MGB	...	...	...	...	...	...
<i>Monilia mumeicola</i>	MmuF	AAGTTGCATCCCTTCGCTGT	62	67	1.0	$y = -3.158x + 27.326$	0.9934	107
	MmuR	ATGACCAGGGGCATCTGTAATT	62	...	...	...	...	...
	MmuP	FAM-ACGTTTCAGGATAATCTAA-MGB	...	...	...	...	...	...
<i>Monilia yunnanensis</i>	MyuF	TCATTGGTAAGTTGCATCCCC	59	76	0.8	$y = -3.11x + 30.823$	0.9952	109
	MyuR	GATGACCAGGGGCATCTATAATTATT	59	...	...	...	...	...
	MyuP	FAM-CATTACGTTGTTACAGATAA-MGB	...	...	...	...	...	...

^a FAM = 6-carboxyfluorescein, BHQ = black-hole quencher, and MGB = minor groove binder.

^b Annealing temperature.

Fig. 1. (Continued from previous page)

B	<i>M. fructicola</i>	ATTGCTCTCTGGAAGTTCGAGACACCAAAGTACTATGTTA	40
	<i>M. laxa</i>	-----c-----	40
	<i>M. mumecola</i>	-----c-----	40
	<i>M. fructigena</i>	-----c-----	40
	<i>M. yunnanensis</i>	-----c-----	40
	<i>M. polystroma</i>	-----c-----	40
	<i>M. fructicola</i>	CCGTCATTGGTAAGTGGTTCCCTTCACAGTATAT	80
	<i>M. laxa</i>	-----t-cgt--a--g-----tgt-c-cc	80
	<i>M. mumecola</i>	-t-----t-cat-----g-g-gt-c...	77
	<i>M. fructigena</i>	-----t-ca-t-----g--t--g-gg-tc	80
	<i>M. yunnanensis</i>	-----t-cat-----g-a-t-gt-g-tc	80
	<i>M. polystroma</i>	-----t-ca-t-----g--t--g-gg-tc	80
	<i>M. fructicola</i>	ACAATATCCTAACAATAATCCTAGATGCCCTGGACATCG	120
	<i>M. laxa</i>	-tg-ccat-----ta-----	120
	<i>M. mumecola</i>	gg--at-----tac-----t-----	117
	<i>M. fructigena</i>	g-g-gat-----cta-----t-----	120
	<i>M. yunnanensis</i>	--g--at-----ta-----t-----	120
	<i>M. polystroma</i>	--g-gat-----cta-----t-----	120
	<i>M. fructicola</i>	TGATTTTCATCAAGAACATGATCACTGGTACTTCCCAAGCT	160
	<i>M. laxa</i>	-----	160
	<i>M. mumecola</i>	-----	157
	<i>M. fructigena</i>	-----	160
	<i>M. yunnanensis</i>	-----	160
	<i>M. polystroma</i>	-----	160

Fig. 2. Specificity of the TaqMan probe and primer sets for targeted *Monilinia* spp. TaqMan probe and primers for **A**, five *Monilinia fructicola* isolates (1 to 5); **B**, five *M. laxa* isolates (1 to 5); **C**, three *Monilinia mumecola* isolates (1 to 3); **D**, five *Monilinia fructigena* isolates (1 to 5); **E**, five *Monilinia yunnanensis* isolates (1 to 5); and **F**, five *M. polystroma* isolates (1 to 5). Isolates 6 to 26 and 4 to 24 refer to the other *Monilinia* spp. and nontarget fungi.

fungi was used for the real-time PCR. At the same annealing temperature of 59°C, the primer sets for *M. fructicola*, *M. fructigena*, *M. laxa*, *M. mumecola*, and *M. yunnanensis* specifically amplified the respective target target sequences with a Ct value less than 30.

The detection limits for the real-time PCR analyses of DNA from pure cultures including mycelia and conidia were 10 to 100 fg, with the lowest Ct value of 20 (Table 2). The limit of detection for all six species was equivalent to one to seven conidia (Fig. 3). Additionally,

the limit of detection for the real-time PCR assays was 10 to 100 fg for five of the six examined fungi when DNA from apple tissues was added. The exception was *M. fructigena*, whose primer and probe had a detection limit of 1 pg. There was no amplification of DNA for the real-time PCR assays conducted using genomic DNA extracted from healthy apple tissue. The efficiencies of the real-time PCR assays calculated using purified fungal DNA in water or the same DNA spiked with DNA from apple fruit was 90 to 110% for all six species. For a particular primer set, there was no cross-reaction with the DNA from any of the other five target pathogens and no signals were obtained in reactions using DNA extracted from any of the non-target fungi or from apple tissue.

Detection of *Monilinia* spp. from infected apple fruit. The real-time PCR assays using DNA extracted from apple tissue inoculated with specific *Monilinia* spp. revealed that amplification with a Ct value less than 30 occurred only with DNA extracted from apple tissue with visible brown rot symptoms. Additionally, the primers and probes were specific for the target fungal pathogen, enabling the identification of the pathogen responsible for the symptoms. No amplification was observed with a Ct value less than 35 when

DNA from healthy apple fruit tissue was used. The respective fungi were reisolated from each of the infected apple fruit using a tissue-isolation method, and the results were consistent with that of the real-time PCR.

Discussion

Real-time PCR primers and probes specific for *M. fructicola*, *M. laxa*, *M. fructigena*, *M. mumecola*, *M. yunnanensis*, and *M. polystroma* were designed based on the sequence differences in the laccase or elongation factor genes. The TaqMan real-time PCR method developed in this study accurately identified the six *Monilinia* spp. using DNA from pure cultures, a mixture of target and nontarget fungal DNA, or DNA from infected apple fruit.

This is the first report describing the rapid and accurate identification of the six closely related *Monilinia* spp. using a TaqMan real-time PCR method based on laccase and elongation factor genes. Previously reported methods were only able to identify up to four *Monilinia* spp. at one time, and were based on universal PCR (Ioos and Frey 2000; Ma et al. 2003), multiplex PCR (Côté et al. 2004; Gell et al. 2007; Hily et al. 2011; Hu et al. 2011; Miessner and

Fig. 3. Sensitivity of the TaqMan probe and primer sets using DNA from conidia. TaqMan probe and primers for **A**, *M. fructicola*; **B**, *M. laxa*; **C**, *Monilia mumecola*; **D**, *Monilinia fructigena*; **E**, *Monilia yunnanensis*; and **F**, *M. polystroma*. The threshold cycle (Ct) value refers to the cycle number during which the generated fluorescence for a reaction crosses the fluorescence threshold (i.e., significantly above the background fluorescence).

Stammler 2010), or real-time PCR (Fan et al. 2014; Garcia-Benitez et al. 2017; Papavasileiou et al. 2016; Van Brouwershaven et al. 2010).

The primers used in the previous PCR-based assays to identify *Monilinia* spp. were designed mainly based on single-nucleotide polymorphisms. In contrast, there were more differences in the laccase gene sequence among the *Monilinia* spp. which were used to develop the universal PCR for identifying five of the six *Monilinia* spp. (Zhu et al. 2016). In this study, we attempted to design real-time PCR primers and probes for all six species based on the laccase gene sequence differences. However, the differences were not sufficient for designing primers and probes for distinguishing *M. mumeicola* and *M. yunnanensis*. Thus, the elongation factor gene, in which polymorphisms occur primarily in one region (Niu et al. 2016), was selected for designing primers and probes for the two species.

The designed primers and probes were species specific, as indicated by lack of amplification of nontarget PCR products. Additionally, the primers and probes were species specific and sensitive for samples consisting of DNA from target and nontarget species, or for DNA from the target fungus and apple fruit. Moreover, the *Monilinia* isolates examined in this study were from China, the United States, Japan, France, Italy, and England, which are countries affected by brown rot disease. The efficiencies of the real-time PCR assays calculated using both purified fungal DNA in water and the same DNA spiked with DNA from apple fruit were 90 to 110%. The detection limit of the real-time PCR assays from both purified fungal DNA in water and the same DNA spiked with DNA from apple fruit was 10 to 100 fg and no amplification was observed with a Ct value less than 35 when DNA from healthy apple fruit tissue was used. These results suggested that the method developed in this study is species specific and DNA of apple does not interfere the real-time PCR.

In the present study, the developed real-time PCR method was validated only with DNA from infected apple fruit exhibiting brown rot symptoms and DNA from healthy apple fruit tissue. We did not use infected apple fruit without brown rot symptoms (latent infections). Because the limit of detection for all six species was equivalent to one to seven conidia, and the limit of detection was 10 to 100 fg for five of the six examined fungi when DNA from apple tissues was added, the real-time PCR assay has the potential to detect latent infections in apple fruit by *Monilinia* spp. Thus, it may be applicable for testing imported or exported apple fruit for the presence of these plant quarantine pathogens.

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