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A real-time (TaqMan) PCR assay to differentiate *Monilinia fructicola* from other brown rot fungi of fruit crops

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To prevent the entry and spread of the brown rot fungus *Monilinia fructicola* in Europe, a fast and reliable method for detection of this organism is essential. In this study, an automated DNA extraction method combined with a multiplex real-time PCR based on TaqMan chemistry was developed for fast, convenient and reliable detection of both the EU quarantine organism *Monilinia fructicola* and the three other brown rot fungi *M. fructigena*, *M. laxa* and *Monilia polystroma*. Using the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA gene repeat, a *Monilinia* genus-specific primer pair and two differently labelled fluorogenic probes specific for *M. fructicola* and the group *M. fructigena/M. laxa/Monilia polystroma* were developed. The analytical specificity of the assay was assessed by testing 33 isolates of the four brown rot fungi and 13 isolates of related fungal species or other fungal species that can be present on stone and pome fruit. No cross-reactions were observed. The assay was found to have a detection limit of 0·6 pg of DNA, corresponding to 27 haploid genomes or four conidia. Comparison of a manual DNA isolation followed by a conventional PCR with an automated DNA isolation combined with the presently developed real-time PCR showed that the latter method gave improved results when tested with 72 naturally infected stone fruit samples. The detection rate increased from 65 to 97%.

Keywords: automated DNA extraction, conventional PCR, detection, duplex assay, manual DNA extraction, TaqMan probe

Introduction

Monilinia fructicola, *M. fructigena*, *M. laxa* and the anamorph species *Monilia polystroma* cause brown rot, an extremely destructive disease occurring on stone fruit trees (*Prunus* spp.) and other rosaceous fruit trees (e.g. *Malus* spp. and *Pyrus* spp.). The disease may seriously reduce or even destroy a crop by affecting blossoms and fruits, either on the tree or after harvest. *Monilinia fructigena* and *M. laxa* are established in Europe, and *Monilia polystroma,* a close relative of *M. fructigena*, is known from Japan (van Leeuwen *et al*., 2002) and has been reported to occur in Hungary (Petróczy & Palkovics, 2009). *Monilinia fructicola* is listed in Annex IV of EU Directive 2000/29 (EU, 2000) as an organism whose introduction and spread within the EU member states is prohibited. It was introduced into France in 2001 (EPPO, 2003), and is still present there (R. Ioos, LNPV, Malzéville, France, personal communication). There have been reports on *M. fructicola* from Hungary and the Czech Republic (Petróczy & Palkovics, 2006; Duchoslavová *et al*., 2007), but these findings have not been confirmed yet. Accurate and rapid identification of *Monilinia* spp. is the essential first step towards early and adequate measures to prevent introduction and further spread of *M. fructicola* within Europe.

Traditionally *Monilinia* spp. are differentiated based on morphological and cultural traits, which requires up to 10 days after initial isolation (van Leeuwen & van Kesteren, 1998; van Leeuwen *et al*., 2002). These methods generally require skilled personnel with specialized taxonomic expertise. Furthermore, visual identification is not always unambiguous due to qualitative, partly shared morphological characteristics, so that identification has to be conducted under standardized conditions and on pure cultures. Despite that, atypical isolates of *M. fructicola* may be misidentified as *M. laxa* and vice versa (van Leeuwen & van Kesteren, 1998). Consequently, classical methods alone are not adequate for phytosanitary diagnosis, as they lack speed and reliability. In the last decade, several conventional PCR-based assays were introduced for differentiation of *M. fructicola* from other *Monilinia* spp. (Fulton & Brown, 1997; Snyder & Jones, 1999; Förster & Adaskaveg, 2000; Hughes *et al*., 2000; Ioos & Frey, 2000; Côté *et al*., 2004; Gell *et al*., 2007). However, the PCR assay based on a group I intron in the 18S rDNA (SSU) (Fulton & Brown, 1997; Snyder & Jones, 1999) was not reliable, as some isolates of *M. fructicola* lack this intron (Fulton *et al*., 1999). The analytical sensitivity (detection limit) of these conventional PCR assays is sufficiently low when using pure cultures of *Monilinia* spp., but is too high for

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routine detection of *M. fructicola* on fruit samples, presumably because of the presence of PCR inhibitors in the samples. A nested PCR approach as developed by Ma *et al*. (2003) increases the analytical sensitivity, although nested PCR is prone to contamination with amplification products, because of the high number of molecules (>10^{12} per reaction) produced in an amplification reaction, and is therefore less suitable in a diagnostic setting.

Compared to conventional PCR, real-time PCR, measuring accumulation of PCR product using fluorescence, generally offers increased sensitivity, and is performed in a closed tube system, which is less prone to contamination with PCR amplicons (Hughes *et al*., 2006). Furthermore, because real-time PCR does not require electrophoresis, it is less laborious than conventional PCR, and is therefore suitable for automation and high throughput testing. Recently a SYBR green chemistry-based real-time PCR assay (Luo *et al*., 2007) has been developed. A primary disadvantage of this type of assay is that both specific and non-specific PCR products are detected, unless additional analysis is done such as melt curve analysis. Fluorogenic TaqMan probes detect only specific PCR products and eliminate the need for post-PCR processing to analyse the products. TaqMan probes can be labelled with distinguishable reporter dyes, which allow the detection of different sequences in one reaction tube.

The objective of this study was to develop a TaqMan-chemistry based duplex real-time PCR for differentiation of *M. fructicola* from the other three brown rot fungi, combined with an automated DNA isolation method enabling quick and reliable diagnosis. A primer set was developed for amplifying *Monilinia* spp. and two different TaqMan probes for detection: a FAM-labelled probe to detect *M. fructicola*, and a VIC-labelled probe for detection of *M. fructigena, M. laxa* and *Monilia polystroma* as a group. The probes have different reporter dyes that can be used together in a duplex PCR. The analytical specificity of this assay was evaluated using 33 isolates of *Monilinia* spp. and *Monilia polystroma*, and 13 isolates of related fungal species or other fungal species that can be present on stone fruit. The analytical sensitivity was assessed using DNA from *M. fructicola* and *M. fructigena* diluted in DNA from plant extract. Finally, the developed assay was compared with the assay routinely used in this laboratory: manual DNA isolation and conventional PCR, using 72 naturally infected cherries and plums showing symptoms of brown rot.

Materials and methods

Fungal isolates

Isolates used in this study are listed in Table 1. To extract fungal DNA, each isolate was grown on a plate with cherry decoction agar (Gams *et al*., 1998). Subsequently, emerging *Monilinia* spp. colonies were transferred to potato dextrose agar (Oxoid) and incubated for 5 days at 22°C.

Fruit samples

Infected fruits (25 cherries and 47 plums) with brown rot symptoms were received as part of a survey held during May–July 2005 to obtain pest state information of *M. fructicola* in the Netherlands.

DNA preparation

Genomic DNA from pure fungal cultures was extracted using the DNeasy Plant Kit (Qiagen) according to the manufacturer's instructions. The DNA was eluted in 50 μL of elution buffer. The concentration and purity of the DNA were determined using a ND-1000 Spectrophotometer (NanoDrop Technologies). The DNA suspensions were then diluted to a concentration of 2 ng μL^{-1}.

To isolate fungal DNA from infected fruit, surface growth consisting of mycelium and conidia was dissected from the fruit, removing as much plant material as possible. The dissected fungal tissue was transferred to a 1·5 mL micro centrifuge tube with a secure fitting flattop cap (Superlock tubes, BIOzym TC) containing 300 μL of extraction buffer (0·02 M PBS, 0·05% Tween T25, 2% polyvinylpyrrolidone, 0·2% bovine serum albumin) and one stainless steel bead (3·97 mm in diameter). The tube was then placed in a bead mill (Mixer Mill MM300, Retsch) for 80 s at 1800 beats min^{-1}. The mixture was centrifuged for 5 s at maximum speed in a micro centrifuge (16 100 ***g***) and 75 μL of the resulting supernatant, i.e. plant extract, was used for manual or automated DNA isolation. Manual DNA isolation was performed using the Qiagen DNeasy Plant Kit, according to the manufacturer's instructions. The DNA was eluted in 50 μL of elution buffer and was further purified using polyvinylpolypyrrolidone (PVPP, Sigma) columns. The columns were prepared by filling Multi-Spin columns (Axygen) with 0·5 cm of PVPP, and washing twice with 250 μL of DNase- and RNase-free water by centrifuging for 5 min at 4000 ***g***. The DNA suspension was applied to a PVPP column and centrifuged for 5 min at 4000 ***g***. The flow through fraction was used as input for the PCR assays.

Automated DNA isolation was performed with the KingFisher 96 magnetic particle processor (Thermo Electron Corporation) using the QuickPick Plant DNA Kit (BioNobile), following the protocol developed by the manufacturer (K. Kontu, personal communication). Briefly, 5 μL of proteinase K and 50 μL lysis buffer were added to 75 μL of plant extract. After 30 min incubation at 65°C, 5 μL of MagaZorb Magnetic Particles and 125 μL of binding buffer were added. The particle-bound DNA was washed twice with 200 μL of wash buffer and DNA was eluted in 130 μL of elution buffer. A total genomic DNA programme was used to transfer the magnetic particles through each of the wells.

Table 1 Isolates used for assessment of analytical specificity of the brown fruit rot fungi real-time assay

Species	Isolate code[a]	Geographic origin	Host or substrate
Monilinia spp. and *Monilia polystroma*			
M. fructicola	CBS 166·24, PD 0603202070[b,c]	Unknown	*Prunus triflora*
M. fructicola	CBS 167·24[d]	New Zealand	*Pr. persica*
M. fructicola	CBS 203·25	USA	*Malus sylvestris*
M. fructicola	CBS 204·25	USA	*Pr. persica*
M. fructicola	CBS 205·25	USA	*Pr. domestica*
M. fructicola	CBS 301·31	Australia	*Pr. domestica*
M. fructicola	CBS 329·35	USA	*M. sylvestris*
M. fructicola	CBS 350·49	USA	*Pr. avium*
M. fructicola	CBS 101511, dar 27029	Australia	*Pr. persica*
M. fructicola	LNPV 08-0445, PD 083485779	France	*Pr. avium*
M. fructicola	PD 0803485584	Unknown	*Pr. avium*
M. fructicola	PD 0803485592	Unknown	*Pr. persica*
M. fructigena	CBS 231·57	the Netherlands	*Pr. domestica*
M. fructigena	CBS 348·72	the Netherlands	*M. sylvestris*
M. fructigena	CBS 493·50	the Netherlands	*M. sylvestris*
M. fructigena	CBS 494·50	the Netherlands	*Pr. cerasus*
M. fructigena	CBS 495·50	the Netherlands	*Pyrus communis*
M. fructigena	CBS 578·77	the Netherlands	*Py. communis*
M. fructigena	CBS 101499, es-48	Spain	*Pr. domestica*
M. fructigena	CBS 101500, cc 752	Poland	*Pr. domestica*
M. fructigena	CBS 101502, PD 0603202062[b]	the Netherlands	*M. pumila*
M. fructigena	PD 0703468039	Czech Republic	*Pr. persica*
M. laxa	CBS 132·21	UK	*Py. malus*
M. laxa	CBS 298·31	Ireland	*M. sylvestris*
M. laxa	CBS 299·31	UK	*Pr. domestica*
M. laxa	CBS 333·47	the Netherlands	*Pr. cerasus*
M. laxa	CBS 488·50	the Netherlands	*Pr. domestica*
M. laxa	Jap 2466, PD 0603202054[b]	Japan	*Pr. mume*
M. polystroma	CBS 101504, Jap 2317	Japan	*M. pumila*
M. polystroma	CBS 102686, Jap 1815	Japan	*M. pumila*
M. polystroma	CBS 102687, Jap 2314	Japan	*M. pumila*
M. polystroma	CBS 102688, Jap 2316,	Japan	*M. pumila*
M. polystroma	Jap 2315, PD 0603202046[b]	Japan	*M. pumila*
Other species			
Alternaria mali	CBS 106·24	USA	*M. sylvestris*
Botrytis cinerea	PD 89/1757	the Netherlands	Unknown
Cladosporium spp.	PD 97/4384	the Netherlands	Lobelia *sp.*
Colletotrichum acutatum	PD 79/407	the Netherlands	*Allium porrum*
Coniothyrium fuckelii	PD 95/567	the Netherlands	*Rosa* sp.
Cylindrocarpon obtusiusculum	CBS 101069, PD 98/8/1415	the Netherlands	*Ribes* sp.
Penicillium expansum	IPO 1324	Unknown	Unknown
Pestalotia funerea	PD 83/728	the Netherlands	*Thuja plicata*
Phomopsis viticola	CBS 267·80, PD 05/01586691	Italy	*Vitis vinifera*
Peacilomyces variotii	IPO 1063	Unknown	Unknown
Sclerotinia sclerotiorum	PD 93/879	the Netherlands	*Brassica oleracea*
Stigmina carpophila	PD 89/566	the Netherlands	*Prunus* sp.
Trichoderma viride	IPO 1260	Unknown	Unknown

[a]Isolate codes. CBS = Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; cc = Central Science Laboratory (Food and Environment Research Agency, since 1 April 2009), York, UK; es = Department of Plant Protection, CIT-INIA, Madrid, Spain; IPO = Plant Research International, Wageningen, the Netherlands; Jap = Faculty of Agriculture and Life Science, Hirosaki University, Japan; LNPV = Laboratoire Nationale de la Protection des Végétaux, Malzéville, France; PD = Plant Protection Service, Wageningen, the Netherlands.
[b]Isolates used for determination of analytical sensitivity (detection limit) and used as reference strains.
[c]Until July 2007 preserved as *M. laxa.*
[d]Until May 2008 preserved as *M. laxa.*

Each series of DNA extractions included multiple controls: a negative control (DNase- and RNase-free water, one for every five samples) to monitor false positives caused by cross-contamination during DNA isolation, and a positive control to check efficiency of the DNA isolation. The positive controls were aliquots of a batch of extract from known *M. fructigena*-infected fruit tissue, prepared in the same manner as the samples.

Table 2 Oligonucleotides used as primers and probes in this study

Oligonucleotide	Sequence (5' to 3')	Orientation	Target	Reference
ITS1Mfc1	TATGCTCGCCAGAGGATAATT	Forward	*Monilinia fructicola*	Ioos & Frey, 2000
ITS4Mfc1	TGGGTTTTGGCAGAAGCACAC T	Reverse	*M. fructicola*	Ioos &Frey, 2000
ITS1Mfgn1	CACGCTCGCCAGAGAATAACC	Forward	*M. fructigena*	Ioos & Frey, 2000
ITS4Mfgn1	GGTGTTTTGCCAGAAGCACACT	Reverse	*M. fructigena*	Ioos & Frey, 2000
ITS1Mlx1	TATGCTCGCCAGAGAATAATC	Forward	*M. laxa*	Ioos & Frey, 2000
ITS4Mlx1	TGGGTTTTGGCAGAAGCACACC	Reverse	*M. laxa*	Ioos & Frey, 2000
Mon139F	CACCCTTGTGTATYATTACTTTGTTGCTT[a]	Forward	*Monilinia* spp.	This study
Mon139R	CAAGAGATCCGTTGTTGAAAGTTTTAA	Reverse	*Monilinia* spp.	This study
P_fc	FAM-TATGCTCGCCAGAGGATAATT-MGBNFQ		*M. fructicola*	This study
P2_fgn/lx/ps	VIC-AGTTTGRTTATTCTCTGGCGA[b]-MGBNFQ		*M. fructigena*, *M. laxa*, *Monilia polystroma*	This study

[a]Y = C or T
[b]R = A or G

Real-time PCR

For real-time (TaqMan) PCR of *M. fructicola* and *M. fructigena/M. laxa/Monilia polystroma,* a genus-specific primer pair and two minor groove-binding (MGB) probes with 3' non-fluorescent quencher (NFQ) were designed with the Primer Express software (Applied Biosystems). Primers were obtained from Isogen Life Science, and the MGB-NFQ probes were obtained from Applied Biosystems. The TaqMan assays were based on sequences in the internal transcribed spacer (ITS) region of the nuclear multi-copy ribosomal RNA gene (Table 2). Based on the differences between the sequence of *M. fructicola* and related *Monilinia* species (Fig. 1), the potentially best discriminating combination was selected: the primers Mon139F and Mon139R, amplifying a 140 bp fragment, were chosen together with 6-carboxyfluorescein (FAM)-MGB probe P_fc for detection of *M. fructicola*, and VIC-MGB probe P2_fgn/lx/ps for detection of the group consisting of *M. fructigena, M. laxa* and *Monilia polystroma* (Table 2). Five microlitres of genomic DNA were amplified in 25 μL volumes in 0·2 mL optical grade plates with optical adhesive covers (Applied Biosystems). The 25 μL reaction mixture contained: 1 × TaqMan Universal PCR Master Mix (Applied Biosystems), 200 nM of each of the primers Mon139F and Mon139R, 200 nM of probe P_fc and 200 nM of probe P2_fgn/lx/ps (Table 2). Real-time PCR was performed in an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems), using the following conditions: 95°C for 10 min, followed by 40 to 55 cycles at 95°C for 15 s and 60°C for 1 min. The emission was measured at the annealing-extension step. The threshold value was set at a fluorescence (ΔRn) of 0·1. A cycle threshold (Ct) value below 40 was scored as a positive result. For the assessment of analytical sensitivity and analytical specificity, 55 PCR cycles were performed to monitor signals above the cut-off Ct of 40.

Similar to each series of DNA extractions, each series of amplifications included controls: a negative control (DNase- and RNase-free water) to test for contamination with DNA as well as controls to monitor the performance of the PCR for amplification of each of the four brown rot fungi. The controls comprised DNA (100 pg, 10 pg and 1 pg) from the reference strains *M. fructicola* CBS 166·24, *M. fructigena* CBS 101502, *M. laxa* Jap 2466 and *Monilia polystroma* Jap 2315.

Conventional PCR

The conventional PCR assays were performed in 25 μL reactions containing 1 × PCR buffer with 1·5 mM $MgCl_2$ (Roche), 1·25 Units of *Taq* DNA polymerase (Roche), 200 μM of each dNTP, 0·2 μM of each primer ITS1Mfc1 and ITS4Mfc1, ITS1Mfgn1 and ITS4Mfgn, or ITS1Mlx1 and ITS4Mlx1 (Table 2) (Ioos & Frey, 2000), and 5 μL of template DNA. The PCR was performed in a 96-well Peltier-type thermocycler (PTC-200, MJ-Research). After amplification, 10 μL of the PCR

CACCCTTGTGTATTATTACTTTGTTGCTTTGGCGAGCTGCCTTCGGGCCTTGTATGCTCGCCAGAGGATAATTAAACTCTTTTTATTAATGTCGTCTGAGTACTATATAATAGTTAAAACTTTCAACAACGGATCTCTTG *M. fructicola* (Z73777)
CACCCTTGTGTATTATTACTTTGTTGCTTTGGCGAGCTGCCTTCGGGCCTTGTATGCTCGCCAGAGGATAATTAAACTCTTTTTATTAATGTCGTCTGAGTACTATATAATAGTTAAAACTTTCAACAACGGATCTCTTG *M. fructicola* (Z73778)
CACCCTTGTGTAT**C**ATTACTTTGTTGCTTTGGCGAGCTGCCTTCGGGCCTTG**CAC**GCTCGCCAGAG**A**ATAA**CC**AAACTCTTTTTATCAATGTCGTCTGAGTACTATACAATAGTTAAAACTTTCAACAACGGATCTCTTG *M. fructigena* (AF150677)
CACCCTTGTGTAT**C**ATTACTTTGTTGCTTTGGCGAGCTGCCTTCGGGCCTTG**CAC**GCTCGCCAGAG**A**ATAA**CC**AAACTCTTTTTATCAATGTCGTCTGAGTACTATACAATAGTTAAAACTTTCAACAACGGATCTCTTG *M. fructigena* (AF150680)
CACCCTTGTGTAT**C**ATTACTTTGTTGCTTTGGCGAGCTGCCTTCGGGCCTTG**CAC**GCTCGCCAGAG**A**ATAA**CC**AAACTCTTTTTATCAATGTCGTCTGAGTACTATACAATAGTTAAAACTTTCAACAACGGATCTCTTG *M. fructigena* (Z73779)
CACCCTTGTGTATTATTACTTTGTTGCTTTGGCGAGCTGCCTTCGGGCCTTGTATGCTCGCCAGAG**A**ATAAT**C**AAACTCTTTTTATTAATGTCGTCTGAGTACTATATAATAGTTAAAACTTTCAACAACGGATCTCTTG *M. laxa* (Z73784)
CACCCTTGTGTATTATTACTTTGTTGCTTTGGCGAGCTGCCTTCGGGCCTTGTATGCTCGCCAGAG**A**ATAAT**C**AAACTCTTTTTATTAATGTCGTCTGAGTACTATATAATAGTTAAAACTTTCAACAACGGATCTCTTG *M. laxa* (Z73785)
CACCCTTGTGTATTATTACTTTGTTGCTTTGGCGAGCTGCCTTCGGGCCTTGTATGCTCGCCAGAG**A**ATAAT**C**AAACTCTTTTTATTAATGTCGTCTGAGTACTATATAATAGTTAAAACTTTCAACAACGGATCTCTTG *M. laxa* (Z73786)
CACCCTTGTGTAT**C**ATTACTTTGTTGCTTTGGCGAGCTGCCTTCGGGCCTTGT**GC**GCTCGCCAGAG**A**ATAAT**C**AAACTCTTTTTATCAATGTCGTCTGAGTACTATATAATAGTTAAAACTTTCAACAACGGATCTCTTG *M. polystroma* (AM937114)

Figure 1 Partial sequence alignment of the ITS region showing species-specific base substitutions in bold. The regions chosen for the design of primers and the species-specific probes are underlined. The respective forward and reverse primers and the probes P_fc for *Monilinia fructicola* and P2_fgn/lx/ps for *M. fructigena/M. laxa and Monilia polystroma* are indicated in Table 2.

product was electrophoresed on a 1·5% agarose gel according to standard methods (Sambrook *et al*., 1989) along with a 100 bp DNA ladder (GeneRuler 100 bp DNA Ladder, Fermentas GmbH) to size fragments. The PCR products were viewed and photographed under UV light. Each series of amplifications contained DNA (10 ng, 1 ng, 100 pg) from the reference strains as used for the TaqMan assays.

Interpretation of PCR results

The results of each sample in a PCR series (conventional or real-time PCR) were considered to be reliable if all controls in the series gave the expected results. In case the results of the controls were not as expected, the following procedures were followed. If one of the positive amplification controls gave a negative result, a technical failure had occurred and for all samples with a negative result the corresponding PCR was repeated. If the positive DNA isolation control gave a negative result, there was a failure in the DNA extraction procedure or inhibitors of the PCR were present in the DNA and the PCR assay was repeated with undiluted, 10- and 100-fold diluted DNA extract. If one of the negative controls was positive a contamination had occurred and the assay was repeated with DNA extract from the duplicate sample. An extensive review regarding the use of positive and negative controls and their interpretation has been published previously (Kox *et al*., 2005, 2007).

Data analysis

The data obtained from standard curves, plotting the Ct of each reaction against the logarithmic values of DNA concentration were analysed by linear regression. The slope of the curves (the regression coefficient k) was used to determine the average amplification efficiency $E = 10^{-1/k}$, with $E = 2$ corresponding to 100% efficiency (Rasmussen, 2001).

The diagnostic utility of each test was quantified by calculating the detection rate with naturally infected fruit samples. The detection rates of each method were expressed as proportions and compared with Fisher's exact test (Kendall & Stuart, 1979). Differences in Ct values were tested using paired Student's t-tests (Altman, 1991). Confidence intervals (CI) for means were calculated using standard methods (Altman, 1991).

Results

Selection of primers and probes for real-time PCR

Based on the alignment of sequences available from the National Center of Biotechnology Information (NCBI) DNA database (GenBank), generic primers for *M. fructicola, M. fructigena, M. laxa* and *Monilia polystroma* were selected and two specific probes, one FAM-labelled (P_fc) to detect *M. fructicola* and one VIC-labelled (P2_fgn/lx/ps) to detect the combination of *M. laxa, M. fructigena* and *Monilia polystroma* (Fig. 1, Table 2). The probes have different reporter dyes to be used in a duplex PCR. To minimize the likelihood of non-specific detection, the probe sequences were compared with sequences in GenBank using the BLASTN database search program (Altschul *et al*., 1997). Probes P_fc and P2_fgn/lx/ps showed only sequence homologies with *M. fructicola* and *M. fructigena/M. laxa/Monilia polystroma*, respectively.

Performance characteristics of duplex real-time PCR

The analytical specificity of the real-time PCR assays was tested by performing reactions using DNA (10 ng) from *M. fructicola* (12 isolates), *M. fructigena* (10 isolates), *M. laxa* (six isolates), *Monilia polystroma* (five isolates), the closely related *Botrytis cinerea* and *Sclerotinia sclerotiorum* and 11 isolates of other fungi occurring on stone and pome fruit (Table 1). FAM fluorescence of probe P_fc could only be measured when the assay contained DNA of *M. fructicola*, and VIC fluorescence of probe P2_fgn/lx/ps could only be measured when the assay contained DNA of *M. fructigena, M. laxa* or *Monilia polystroma*. Amplification of duplicate DNA samples spiked with 10 pg of *M. fructigena* DNA (isolate CBS 101502) were used to verify that negative results were not due to inhibition. All reactions were positive showing that no inhibition occurred.

The analytical sensitivity was assessed by testing a dilution series starting with 10 ng of DNA from *M. fructicola* (isolate CBS 166·24) and *M. fructigena* (isolate CBS 101502). To mimic the presence of fruit tissue in a mycelium sample, the dilutions contained 20 ng DNA from healthy plum. The preparation of each series of dilutions was replicated eight times. The detection limit of the assay (mean of lowest detectable amount [0·4 pg] + three standard deviations [0·2 pg]) was 0·6 pg of *Monilinia* spp. DNA (Table 3). Standard curves plotting the Ct of each reaction against the logarithmic values of DNA concentration of *M. fructicola* (Fig. 2) and *M. fructigena* were calculated. The assay showed a linear response from 10 ng down to 0·4 pg with amplification efficiencies of 1·96 and 1·93 for *M. fructicola* and *M. fructigena*, respectively (Table 3).

To demonstrate that the multiplex PCR method is able to detect *M. fructicola* in fruit that is co-infected with one of the other brown rot fungi, DNA from *M. fructicola* (isolate CBS 166·24) and *M. fructigena* (isolate CBS 101502) mixed in different ratios (ranging from 1:10 000 to 10 000:1) were amplified (data not shown). When both *Monilinia* spp. were present in equal amounts, both targets were amplified equally well. Excess of either target had a negative effect on the amplification of the other target, although it only abolished amplification at 1000-fold excess.

Comparison of DNA extraction methods

To determine whether the DNA isolation method influences the results of the amplifications, the automated

Table 3 Performance characteristics of the duplex real-time PCR assay for *Monilinia fructicola* and *M. fructigena*

Target DNA[a]	Detection limit[b,c] (pg DNA)	Dynamic range (pg DNA)		Linear regression[b,d]		
		From	To	*k*	R^2	E^e
M. fructicola	0·6	10·000	0·4	3·42	1·00	1·96
M. fructigena	0·6	10·000	0·4	3·49	1·00	1·93

[a]*M. fructicola* CBS 166·24 and *M. fructigena* CBS 101502 in the presence of 20 ng DNA from healthy plum.
[b]Calculated from eight separately prepared series of dilutions.
[c]Detection limit = mean of lowest detectable amount + three standard deviations.
[d]Linear regression analysis: k = slope of linear regression between logarithmic values of DNA quantity and Ct values; R^2 = average squared regression coefficient; E = efficiency of amplification.
[e]$E = 10^{-1/k}$, with $E = 2$ corresponding to 100% efficiency (Rasmussen, 2001).

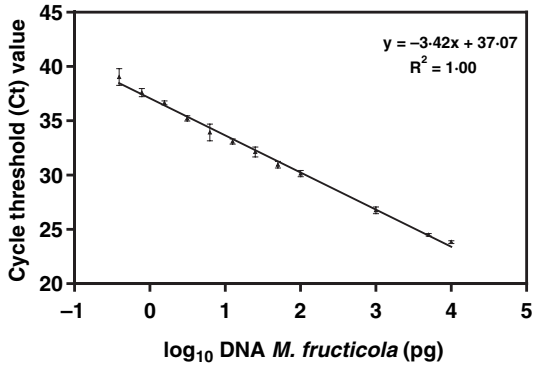

Figure 2 Standard curve of cycle threshold (Ct) values calculated from amplifications of serial dilutions of DNA from *Monilinia fructicola* isolate CBS 166·24 using the duplex real-time PCR assay. The preparation of each series of dilutions was replicated eight times. Ct values shown are mean values of eight reactions; error bars represent standard deviations.

DNA extraction method on the KingFisher 96 magnetic particle processor using the QuickPick Plant DNA kit was compared with the manual isolation using the Qiagen DNeasy Plant Kit followed by PVPP purification. The DNA isolation methods were tested on 50 fruit samples infected with *M. fructigena* using the real-time PCR for the three other brown rot species in this study. Results obtained after isolation with both methods gave similar results (P = 0·142, two-tailed paired Student's *t*-test). The mean Ct-values were 19·1 (95% CI 18·3–21·3) for the manual method and 20·0 (95% CI 18·7–21·3) for the automated method.

Comparison of conventional and real-time PCR assays using infected fruit samples

Gel-based conventional PCR assays, using the primers ITS1Mfc1 and ITS4Mfc1 for *M. fructicola*, ITS1Mfgn1 and ITS4Mfgn1 for *M. fructigena* and ITS1Mlx1 and ITS4Mlx1 for *M. laxa* (Ioos & Frey, 2000) was used in a comparison with the real-time TaqMan assay developed in this study (Table 4). The conventional PCR assays included a manual DNA isolation and the TaqMan assays included automatic DNA isolation using the KingFisher 96 magnetic particle processor. Both assays, with different but equivalent DNA isolation methods, as demonstrated in the previous paragraph, were tested on mycelium/conidia dissected from 72 naturally infected samples of *Prunus* spp. (plums and cherries). For 49 samples the results were consistent in both assays. This results in 68% concordance between both assays. In two of these samples no brown rot fungi could be detected. Because it was suspected that inhibition was the cause of these negative results, duplicate samples spiked with *M. fructigena* DNA were tested (Table 4). Both reactions were negative showing that the samples contained inhibitors of the PCR. Twenty-three samples were positive only in the real-time PCR; the difference was due to inhibition in the conventional PCR as verified by negative results of spiked duplicate samples. Also, dilution of the DNA did not improve the result. The detection rates of the conventional and real-time assays were 65 and 97%, respectively ($P < 0·001$, two-tailed Fisher's exact test).

Table 4 Results of conventional PCRs and duplex real-time PCR on 72 naturally brown rot affected stone fruits

Number of samples	Result	
	Conventional PCR assay	TaqMan PCR assay
24	*Monilinia fructigena*	*M. fructigena* and/or *M. laxa*
14	*M. laxa*	*M. fructigena* and/or *M. laxa*
9	*M. fructigena* and *M. laxa*	*M. fructigena* and/or *M. laxa*
23	Inhibition[a]	*M. fructigena* and/or *M. laxa*
2	Inhibition[a]	Inhibition[b]

[a]Inhibition tested by spiking a duplicate DNA sample with 1 ng of DNA of *M. fructigena* CBS 101502.
[b]Inhibition tested by spiking a duplicate DNA sample with 10 pg of DNA of *M. fructigena* CBS 101502.

Discussion

In this study an automated duplex real-time TaqMan PCR assay has been developed and evaluated for rapid and specific detection of the brown rot pathogens *M. fructicola*, *M. fructigena*, *M. laxa* and *Monilia polystroma* on

pome and stone fruit with clear symptoms (mycelium/conidia) of brown rot. The assay is a PCR using two differently labelled TaqMan probes enabling differentiation of the EU quarantine fungus *M. fructicola* from the other three brown rot fungi. One generic specific primer pair is used for amplification of part of the ITS region of all four brown rot fungi. Even though the TaqMan assay was developed for the identification of fungal structures directly on the fruit, it can also be used to identify the cultures obtained from the fruit, circumventing the need for pure cultures for morphological/cultural identification under standardized conditions.

The analytical specificity of the assay was excellent; moreover, it proved useful for a revised identification of two isolates (CBS 166·24 and CBS 167·24, deposited by E.E. Honey in 1924) that were preserved as *M. laxa* at the Centraalbureau voor Schimmelcultures (CBS), Fungal Biodiversity Centre. Both isolates gave a positive signal for the *M. fructicola* probe P_fc. To verify this finding the ITS regions were sequenced and found to be identical to those of *M. fructicola*. This outcome demonstrates the analytical specificity of the real-time TaqMan PCR assay.

In the case of a fruit co-infected with *M. fructicola* and one of the other three brown rot fungi, it is possible that *M. fructicola* will not be detected because of competition of both targets for PCR reagents, primers in particular, as this PCR assay uses generic primers. This is a common phenomenon, as observed for other PCR assays using generic primers (Kox *et al*., 2005). Experiments with mixtures of DNA from *M. fructicola* and *M. fructigena* show that this competition only results in false negatives if one of the targets is in more than 1000-fold excess of the other. The assay should therefore be able to detect *M. fructicola* in the presence of large quantities of the other brown rot fungi.

The detection limit of the assay is 0·6 pg of DNA established in the presence of purified plant DNA. With a mean haploid genome size of 0·022 pg for the two *Monilinia* spp. listed in the Fungal Genome Size Database (Kullman *et al*., 2005; Gregory *et al*., 2007), 0·6 pg of DNA corresponds to 27 fungal genomes. As a conidium of the brown rot fungi contains on average 6·6 nuclei (with a range from 4 to 10) (Hall, 1963; Hoffman, 1972), it is estimated that the detection limit of the assay is four conidia. Compared to the conventional assays designed by Ioos & Frey (2000) that have been implemented in this laboratory with detection limits of 10 pg of DNA (or 240 conidia), this is a more than a 10-fold improvement of analytical sensitivity. The higher detection limit of the conventional PCR should not be a problem for detection when using this assay on fruit with visible surface growth, which was the scope of this assay, because sufficient fungal DNA should be extracted to obtain a positive PCR result. Therefore similar detection rates are expected for conventional and real-time PCR. However, when testing 72 infected fruits with the conventional PCR, 25 (35%) gave false-negative results, while the TaqMan assay only resulted in two (3%) false-negatives. Similar inhibition rates have been reported in other studies comparing conventional and TaqMan assays (Baric *et al*., 2006; van Gent-Pelzer *et al*., 2007). Spiking duplicate DNA samples with *M. fructigena* DNA showed that inhibitors were associated with the DNA isolated from the fruit surface. Both PCRs were preceded by different DNA isolation methods, but the study showed that there were no significant differences in mean Ct-values between the manual and automated DNA isolation methods used. Therefore, the observed difference in detection rates for both assays, 65 and 97% for conventional and real-time PCR, respectively, cannot be explained by difference in effectiveness of both DNA isolation methods to remove inhibitors. A likely cause for the lower detection limit of the TaqMan PCR compared to the conventional PCR is that in real-time PCR smaller amplicons are produced (139 vs 365 bp), that are known to be amplified more efficiently. In conclusion, the real-time PCR has a lower detection limit and is less susceptible to inhibition than conventional PCR, and therefore is now the method of choice in this laboratory.

Real-time TaqMan PCR has many advantages compared to conventional PCR, but there is one disadvantage: the high costs for reagents and consumables. Using list prices valid in the Netherlands, these costs are calculated as four times higher than that of conventional PCR. However, these material costs can be (partly) compensated by the lower labour costs. Baric *et al*. (2006) calculated that the total costs (material and labour) of real-time PCR is 1·5 times higher than that of conventional PCR. Automation of DNA extraction will reduce the costs further.

Manual and automated DNA extraction methods have been shown to be equally fit for amplification. The automated DNA extraction enables high-throughput testing in the laboratory, while manual extraction is the method of choice for on-site detection on a portable real-time PCR platform. Real-time PCR has proven to be a suitable method for testing samples for *Phytophthora ramorum*, the causal agent of sudden oak death, at the point of sampling (Tomlinson *et al.,* 2005). A short-time diagnosis for detecting *Monilinia* spp. on fruits is essential and therefore application of this new real-time PCR on a portable real-time platform is preferred in cases where the time of transport of samples is unacceptably long.

Since 2006, the TaqMan assay has been used as a tool in the annual surveys that are held to obtain information about the pest status of *M. fructicola* in the Netherlands. The samples taken for the surveys held in 2006, 2007 and 2008 were cherries and plums with visible symptoms of brown rot. The DNA was isolated from the dissected fungal tissue using the automated method. None of the samples was positive for *M. fructicola*. The possible application of the assay for detection of quiescent infections or airborne spores needs further study.

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