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ORIGINAL PAPER

Monilinia species in Hungary: morphology, culture characteristics, and molecular analysis

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Abstract *Monilinia* is a well-known pathogen of fruit trees affecting fruit production all over the world. Three species of the *Monilinia* genus are particularly important with regard to fruit trees and ornamentals, causing serious blossom and twig blight and brown rot in fruits: *Monilinia fructicola*, *Monilinia fructigena*, and *Monilinia laxa*. In this study, *Monilinia* isolates were compared and identified using classical and molecular methods. Morphological and culture characteristics were determined and pathogenicity testing performed. In addition, internal transcribed spacer regions and a genomic sequence with unknown function were analyzed and compared with sequence data from other *Monilinia* species in an international database. Four *Monilinia/Monilia* species were identified: *M. fructicola*, *Monilia polystroma*, *M. fructigena*, and *M. laxa*. *M. fructicola* was isolated from imported peach fruits. *M. polystroma* was first reported from Hungary and Europe on apple shoots and fruits. *M. fructigena* was identified on tea-rose hybrid pseudofruits, which is the first occurrence of this pathogen on this host. *M. laxa* causes brown rot of grapes, which has only been reported in New Zealand. Substitutions and insertions were detected when comparing *M. laxa*, *M. fructigena*, and *M. polystroma* sequences. In the genomic sequence with unknown function, three repetitive sequence motifs were identified in different numbers, depending on species and isolate. On the phylogram produced in this analysis, the Hungarian *M. polystroma* isolate (UFT) and *M. polystroma* reference isolates localized at a different branch than the closely related *M. fructigena* isolates and other *Monilinia* species.

Keywords *Monilinia fructigena* · *Monilinia fructicola* · *Monilinia laxa* · *Monilia polystroma* · Brown rot · ITS region

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Introduction

Monilinia is a well-known fruit tree pathogen affecting fruit production all over the world. Three species of the genus are particularly significant with regard to fruit trees and ornamentals, causing blossom and twig blight and brown rot in fruits: *Monilinia fructicola* (G. Wint.), *Monilinia fructigena* (Honey), and *Monilinia laxa* (Aderhold and Ruhland) Honey (Byrde and Willetts 1977). In 2002, a new species, *Monilia polystroma* (G. Leeuwen), named after the intense stromata formation, was distinguished from *M. fructigena* based on morphological and molecular identification (Fulton et al. 1999; van Leeuwen et al. 2002). Other species of *Monilinia* known to cause disease in fruit trees are *M. mali* (Tak.) Wetzel, which attacks apple and Japanese crab apple (Shima 1936), and *M. laxa* f. sp. *mali* (Wormald) Harrison, which causes blossom wilt in apple trees (Wormald 1954).

Identification of *Monilinia* species used to be based on the symptoms, host, morphology, and culture characteristics of the fungi (Wormald 1954; Byrde and Willetts 1977; Batra 1991). The parameters for exogenous stromata

formation on the infected areas are typical to each *Monilinia* species. According to Willetts and Bullock (1993), the stromata of *M. fructigena* are bigger in size than those of *M. fructicola* and *M. laxa*, and they often form concentric rings around the point of infection. The stromata of *M. fructigena* are yellowish or buff-colored, whereas the stromata of *M. laxa* and *M. fructicola* are grayish or hazel in color (EPPO 2003). *M. polystroma* forms a large number of yellowish stromata (van Leeuwen et al. 2002). The stromata of *M. laxa* f. sp. *mali* are similar to those of *M. laxa*, but smaller and grayish (Wormald 1954).

Colonies of *M. fructigena* are creamy/yellow, the margins are not lobed, and they do not form rosettes on potato dextrose agar (PDA). The average growth rates range from 0 to 12 mm/24 h (EPPO 2003). Colonies of *M. polystroma* are similar to those of *M. fructigena*, but black stromatal plates occur on the colonies after 10–12 days of incubation (van Leeuwen et al. 2002; EPPO 2003), and *M. polystroma* isolates grow faster than *M. fructigena* isolates under the same conditions (Holb 2004). The mean colony growth rate of *M. polystroma* isolates are 7 mm/24 h in continuous darkness, and the mean growth rate of *M. fructigena* is 3.7 mm/24 h (van Leeuwen et al. 2002). Colonies of *M. laxa* are grayish-brown or hazel, the margins are serrulate, the colonies form rosettes with black arcs on PDA, and sporulation is sparse (Byrde and Willetts 1977). *M. laxa* has lower growth rates (2–11 mm/24 h), about half of that of *M. fructicola* (EPPO 2003). *M. fructicola* forms hazel colonies with entire margins, and no rosettes develop. Sporulation is abundant and form concentric rings on PDA (Batra 1991; EPPO 2003). Reported growth rates on PDA range from 9 to 20 mm/24 h (De Cal and Melgarejo 1999), with the average around 13 mm/24 h (van Leeuwen and van Kesteren 1998).

Several molecular methods have been developed to distinguish *Monilinia* species. Fulton and Brown (1997) established a PCR-based method of targeting the group I intron in the gene for the ribosomal small subunit, but some isolates do not contain these sequences. To develop a more reliable method, Ioos and Frey (2000) designed species-specific primer pairs for the ribosomal internal transcribed spacer 1 (ITS1) region, the 5.8S rRNA gene, and the ITS2 region between the 18S and 28S rRNA genes. These primer pairs were specific for *M. laxa*, *M. fructigena*, and *M. fructicola*, but not for *M. polystroma*. According to Fulton et al. (1999), isolates of Japanese *M. fructigena* differ from European *M. fructigena* isolates by five base pairs in the ITS and formed a separate group from *M. laxa*, *M. fructigena*, and *M. fructicola*. Later, Japanese *M. fructigena* isolates were identified as a new species with the name *M. polystroma* (van Leeuwen et al. 2002). Multiplex PCR assays were developed to detect and identify *Monilinia* species based on the different lengths of the ITS fragments in different species (Cotê et al. 2004). An internal control-based universal PCR protocol was developed for *Monilinia* spp., and species-specific primers were designed using SCAR markers (Gell et al. 2007a). Finally, a real-time (TaqMan) PCR assay was developed to differentiate *M. fructicola* from other brown rot fungi of fruit crops (van Brouwershaven et al. 2010).

The aim of this study were to (1) identify *Monilinia* species occurring in Hungary using classical and molecular methods, (2) determine which species are most important in blossom and twig blight and brown rot, and (3) characterize selected isolates and compare them with other published isolates.

Materials and methods

Fungal isolates, isolation, and culture characteristics

In a survey carried out from 2003 to 2009, a total of 97 *Monilinia* isolates were collected from cultivated fruits and ornamentals showing typical symptoms in different parts of Hungary. Selected isolates were chosen for deep analysis. The host, geographical origin, source of inoculum, and name of the isolates are listed in Table 1. All isolates were routinely cultured on PDA (Difco Laboratories) media to produce fungal material for molecular analysis and pathogenicity testing. Conidia from the infected host tissues were transferred to PDA by sterile needle. Plates were incubated at 24°C in darkness for 4 days. Agar plugs with mycelia from the margins of the cultures were re-plated to sterile PDA plates. Pure cultures from three repetitions were grown in 85-mm diameter plastic Petri dishes sealed with parafilm (American Can Company) in continuous darkness for 7–21 days at 24°C. Isolates were identified using classical taxonomic criteria, including culture characteristics. Colony growth rates were compared and analyzed statistically using nested design ANOVA supplemented with the Tukey HSD test.

Pathogenicity tests

Pathogenicity studies were carried out on 3–3 apple or peach fruits for each isolate, with the addition of apple shoots in the case of the UFT isolate. The fruit surface was disinfested by dipping the entire fruit in 70% ethanol for 1 min and then rinsing it with sterile distilled water. Plugs (5-mm diameter) of 10-day-old mycelia from the fungal colony margins were inserted into a wound made by puncturing a sterile needle into the fruit skin. Four inoculations of the same isolate were applied per fruit at sites 90° from each other. Potato dextrose agar plugs were used as controls. The inoculated fruits were sprayed lightly with sterile distilled water and placed in separate large glass

Table 1 Name, origin, host, source of inoculum, and identified pathogens in the isolates

Isolate	Origin	Host	Source of inoculum	Identified pathogen
M_Ch1	Budapest	*Chaenomeles speciosa* 'Simonii'	Flower	*M. laxa*
M_Ch2	Budapest	*Chaenomeles* × *superba* 'Atrococcinea Plena'	Flower	*M. laxa*
M_Ch3	Budapest	*Chaenomeles speciosa* 'Simonii'	Fruit	*M. fructigena*
M_Rosa	Budapest	*Rosa hybrid*	Pseudofruit	*M. fructigena*
UFT	Újfehértó	*Malus* × *domestica* 'Ashton bitter'	Shoot and young fruit	*M. polystroma*
M1	Budapest	*Prunus serrulata* 'Kanzan'	Fruit and shoot	*M. laxa*
M2	Budapest	*Prunus triloba*	Fruit and shoot	*M. laxa*
M3	Budapest	*Prunus tenella*	Fruit and shoot	*M. laxa*
M4	Budapest	*Malus purpurea* 'Aldenhamensis'	Fruit	*M. fructigena*
M5	Budapest	*Malus* × *scheideckeri*	Fruit	*M. fructigena*
M6	Budapest	*Chaenomeles speciosa* 'Nivalis'	Fruit	*M. fructigena*
M7	Budapest	*Chaenomeles* × *superba* 'Nicoline'	Fruit	*M. fructigena*
M8	Budapest	*Cotoneaster divaricatus*	Fruit	*M. fructigena*
M9	Budapest	*Pyrus elaeagrifolia*	Fruit	*M. fructigena*
M10	Budapest	*Pyrus pyraster*	Fruit	*M. fructigena*
M11	Budapest Fehérvári Str. fruit market	*Prunus persica*	Fruit	*M. fructicola*
M12	Budapest (Auchan) import from Spain	*Prunus persica* 'Michelini'	Fruit	*M. fructicola*
M13	Gödöllő (Tesco) import from Italy	*Prunus persica*	Fruit	*M. fructicola*
M14	Solymár	*Malus* × *domestica* 'Jonathan'	Fruit	*M. fructigena*
M15	Gödöllő	*Malus* × *domestica* 'Granny Smith'	Fruit	*M. fructigena*
M16	Felcsút	*Cydonia oblonga*	Fruit	*M. fructigena*
M17	Gödöllő	*Cydonia oblonga*	Fruit	*M. fructigena*
M18	Diósd	*Cydonia oblonga*	Fruit	*M. fructigena*
M19	Pomáz	*Pyrus domestica*	Fruit	*M. fructigena*
M20	Gödöllő	*Pyrus domestica*	Fruit	*M. fructigena*
M21	Diósd	*Prunus avium* 'Solymári gömbölyű'	Flower and shoot	*M. laxa*
M22	Gödöllő	*Prunus avium* 'Germersdorfi óriás'	Fruit	*M. laxa*
M23	Pomáz	*Prunus persica* 'Michelini'	Fruit	*M. fructigena*
M24	Pomáz	*Prunus persica* 'Cresthaven'	Fruit	*M. fructigena*
M25	Gödöllő	*Prunus domestica*	Fruit	*M. laxa*
M26	Pomáz	*Prunus domestica* 'Tuleu gras'	Fruit	*M. fructigena*
M27	Pomáz	*Prunus domestica* 'President'	Fruit	*M. laxa*
M28	Budaörs	*Prunus domestica*	Fruit	*M. laxa*
M29	Felcsút	*Prunus domestica*	Fruit	*M. laxa*
M30	Pomáz	*Prunus domestica* 'President'	Fruit	*M. fructigena*
M31	Pomáz	*Prunus domestica* 'President'	Fruit	*M. laxa*
M32	Vecsés	*Prunus domestica*	Fruit	*M. fructigena*
M33	Pomáz	*Prunus domestica* 'Bluefre'	Fruit	*M. laxa*
M34	Sárospatak	*Prunus domestica*	Fruit	*M. fructigena*
M35	Gödöllő	*Prunus domestica* 'Bluefre'	Fruit	*M. fructigena*
M36	Pomáz	*Prunus domestica* 'Cacanska lepotica'	Fruit	*M. laxa*
M37	Sárospatak	*Prunus armeniaca*	Fruit	*M. laxa*
M38	Felsőörs	*Prunus armeniaca* 'Ceglédi arany'	Flower and shoot	*M. laxa*
M39	Felsőörs	*Prunus armeniaca* 'Pannónia'	Flower and shoot	*M. laxa*
M40	Felsőörs	*Prunus armeniaca* 'Mandulakajszi'	Flower and shoot	*M. laxa*
M41	Szigetcsép	*Prunus armeniaca*	Flower and shoot	*M. laxa*
M42	Szigetcsép	*Prunus armeniaca*	Flower and shoot	*M. laxa*

Table 1 continued

Isolate	Origin	Host	Source of inoculum	Identified pathogen
M43	Szigetcsép	*Prunus armeniaca*	Flower and shoot	*M. laxa*
M44	Szigetcsép	*Prunus armeniaca*	Flower and shoot	*M. laxa*
M45	Szigetcsép	*Prunus armeniaca*	Flower and shoot	*M. laxa*
M46	Szigetcsép	*Prunus armeniaca*	Flower and shoot	*M. laxa*
M47	Szigetcsép	*Prunus armeniaca*	Flower and shoot	*M. laxa*
M48	Soroksár	*Prunus armeniaca*	Flower and shoot	*M. laxa*
M49	Soroksár	*Prunus armeniaca*	Flower	*M. laxa*
M50	Soroksár	*Prunus armeniaca*	Flower and shoot	*M. laxa*
M51	Soroksár	*Prunus armeniaca*	Flower and shoot	*M. laxa*
M52	Soroksár	*Prunus armeniaca*	Flower and shoot	*M. laxa*
M53	Soroksár	*Prunus armeniaca*	Flower	*M. laxa*
M54	Soroksár	*Prunus armeniaca*	Flower and shoot	*M. laxa*
M55	Soroksár	*Prunus armeniaca*	Flower and shoot	*M. laxa*
M56	Soroksár	*Prunus armeniaca*	Flower and shoot	*M. laxa*
M57	Soroksár	*Prunus armeniaca*	Flower and shoot	*M. laxa*
M58	Soroksár	*Prunus armeniaca*	Flower	*M. laxa*
M59	Soroksár	*Prunus armeniaca*	Flower and shoot	*M. laxa*
M60	Soroksár	*Prunus armeniaca*	Flower and shoot	*M. laxa*
M61	Soroksár	*Prunus armeniaca*	Flower and shoot	*M. laxa*
M62	Alsóörs	*Prunus cerasus* 'Kántorjánosi'	Flower and shoot	*M. laxa*
M63	Alsóörs	*Prunus cerasus* 'Újfehértói fürtös'	Flower and shoot	*M. laxa*
M64	Ócsa	*Malus* × *domestica* 'Golden Delicious'	Fruit	*M. fructigena*
M65	Ócsa	*Malus* × *domestica* 'Starking'	Fruit	*M. fructigena*
M66	Budaörs	*Malus* × *domestica*	Fruit	*M. fructigena*
M67	Zsámbék	*Cydonia oblonga*	Fruit	*M. fructigena*
M68	Budakeszi	*Cydonia oblonga*	Fruit	*M. fructigena*
M69	Pálköve	*Cydonia oblonga*	Fruit	*M. fructigena*
M70	Balatonalmádi	*Cydonia oblonga*	Fruit	*M. fructigena*
M71	Diósjenő	*Cydonia oblonga*	Fruit	*M. fructigena*
M72	Pesterzsébet	*Pyrus domestica*	Fruit	*M. fructigena*
M73	Budaörs	*Pyrus domestica*	Fruit	*M. fructigena*
M74	Nagykanizsa	*Pyrus domestica*	Fruit	*M. fructigena*
M75	Érd	*Prunus persica*	Fruit	*M. laxa*
M76	Felcsút	*Prunus persica*	Fruit	*M. fructigena*
M77	Sóskút	*Prunus persica* 'Suncrest'	Fruit	*M. laxa*
M78	Diósjenő	*Prunus persica*	Fruit	*M. laxa*
M79	Tiszacsécse	*Prunus domestica*	Fruit	*M. laxa*
M80	Budaörs	*Prunus domestica*	Fruit	*M. fructigena*
M81	Budaörs	*Prunus domestica*	Fruit	*M. laxa*
M82	Rábakecöl	*Prunus domestica*	Fruit	*M. laxa*
M83	Sóskút	*Prunus domestica* 'Bluefre'	Fruit	*M. laxa*
M84	Sóskút	*Prunus domestica* 'Stanley'	Fruit	*M. laxa*
M85	Diósjenő	*Prunus domestica*	Fruit	*M. laxa*
M86	Nagykanizsa	*Prunus domestica*	Fruit	*M. laxa*
M87	Révfülöp	*Vitis vinifera* 'Zala gyöngye'	Fruit	*M. laxa*
M88	Budaörs	*Vitis vinifera*	Fruit	*M. fructigena*
M89	Budapest	*Chaenomeles speciosa*	Fruit	*M. laxa*
M90	Budapest	*Prunus serrulata* 'Ichiyo'	Flower and shoot	*M. laxa*

Table 1 continued

Isolate	Origin	Host	Source of inoculum	Identified pathogen
M91	Budapest	*Prunus cerasifera* 'Nigra'	Fruit	*M. fructigena*
M92	Budapest	*Malus* 'Hopa'	Fruit	*M. fructigena*

vessels containing wet sheets of filter paper at the bottom and incubated at 24°C. The wet sheets of filter paper were replaced after 5 days of incubation. Fruits were examined 14 days after inoculation.

Apple shoots (cv. Mutsu and Granny Smith) were inoculated with UFT isolate based on Koch's postulates, demonstrating that pure culture of the fungus is able to cause shoot blight and necrosis on apple fruits. Shoots for inoculation were disinfested with 70% ethanol, rinsed with sterile water, and blotted dry. A V-shaped incision was made with a scalpel at a 45° angle to expose the tissue under the bark. An agar plug (3-mm diameter) containing young hyphae from 7-day-old culture of the UFT isolate was immediately placed in the incision and wrapped with parafilm. Control shoots were wounded in the same way and inoculated with sterile PDA plugs. The shoots were placed into water-containing vessels, enclosed in plastic bags, and incubated at 24°C. Disease development was evaluated after 14 days by observing necrosis, shoot blight, and the appearance of stromata.

DNA isolation

DNA extraction was based on a modification of the method in Dellaporte et al. (1983). All isolates were grown on PDA plates. The mycelium was removed from the media using a sterile toothpick, ground in cetyl trimethyl ammonium bromide (CTAB) buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris–HCl pH 8.0, 0.2% 2-mercapto-ethanol) using a mortar and pestle, and incubated for 20 min at 60°C. Proteins were removed with chloroform:isoamyl alcohol (24:1), and DNA was precipitated in isopropanol (Maniatis et al. 1989). The pellet was re-suspended in 50 µl of TE buffer containing 10 µg/ml RNase. The final concentration of DNA was approximately 100 ng/µl.

PCR amplification

Three species-specific reverse primers (ITS4Mfcl, ITS4Mfgn, ITS4MLx) (Ioos and Frey 2000) and ITS-Monilia forward primer (Table 2) based on sequence data from the NCBI database (synthesized by Biomi Kft. Gödöllő, Hungary) were used to amplify the ITS regions of the ribosomal DNA (ITS1-5.8S-ITS2) for identification. The length of the amplified product is 374 bp for all species.

To recover a "genomic sequence with unknown function", UniMon_Rev and UniMon_Forw primers were designed (Table 2) similar to those used by Cotê et al. (2004). UniMon_Rev and UniMon_Forw primers are universal for *M. fructicola*, *M. fructigena*, *M. laxa*, and *M. polystroma* species, and they are suitable for identifying species by the length of PCR product. The amplified region is 397 bp for *M. laxa*, 417 bp for *M. fructigena*, 433 bp for *M. polystroma*, and 594 bp for *M. fructicola*.

Approximately, 100 ng of DNA was amplified in 1× PCR reaction buffer (Fermentas) containing 250 µM of each dNTP, 7.5 mM $MgCl_2$, 20 pM primers, and 2.5 units *Taq* DNA polymerase (Fermentas) in a total volume of 50 µl. In all cases, the PCR reactions were carried out in a Perkin-Elmer 9700 thermocycler (Applied Biosystems) with initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 55°C for 1 min, and elongation at 72°C for 1.5 min, and final elongation at 72°C for 10 min. Amplified products were separated by electrophoresis in 1% agarose gels in 1× TBE buffer, stained with Gel Red (Biotium), visualized under UV light, and photographed using a BioDoc-It Imaging System (UVP).

Cloning and sequencing of PCR products

Amplified DNA products were purified using the High Pure PCR Product Purification Kit (Bio-Rad) and ligated into pGEM-T Easy (Promega) following the manufacturer's instructions. The vector was transformed into *Escherichia coli* DH5α competent cells, white colonies were selected, and recombinant plasmids purified. Digestion with *Eco*RI (Fermentas) confirmed the presence of an insert of the expected size. The purified plasmids were sent to Biomi Kft. for sequencing using M13 reverse and M13 forward primers, the ABI PRISM BigDye Terminator Cycle Sequencing kit, and the ABI PRISM 310 instrument (Applied Biosystems).

Phylogenetic analysis

Nucleic acid sequence analysis and comparisons were performed using the Clustal X (Thompson et al. 1997), MEGA 3.1 (Kumar et al. 2004), and GAP programs of the Genetic Computer Group (GCG) sequence analysis software package, version 10.0 (Devereux et al. 1984), using

Table 2 Primers used for PCR amplification

Primer	Sequence	Specific to
ITS4Mfcl (rev)	5′-TGGGTTTTGGCAGAAGCACACT-3′	*M. fructicola*
ITS4Mfgn (rev)	5′-CACGCTCGCCAGAGAATAACC-3′	*M. fructigena*, *M. polystroma*
ITS4MLx (rev)	5′-TGGGTTTTGGCAGAAGCACACC-3′	*M. laxa*
ITSMonilia (forw)	5′-GGTAGACCTCCCACCCTTGTGTA-3′	*M. fructicola*, *M. fructigena*, *M. laxa*, *M. polystroma*
UniMon_Rev	5′-GAGCAAGGTGTCAAAACTTCCAT-3′	*M. fructicola*, *M. fructigena*, *M. laxa*, *M. polystroma*
UniMon_Forw	5′-ATCGGCTTGGGAGCGG-3′	*M. fructicola*, *M. fructigena*, *M. laxa*, *M. polystroma*

default parameters. Multiple alignments were used as input data to construct phylogenetic trees with the UPGMA distance method implemented in Clustal X. Bootstrap analysis was performed with 10,000 replicates. Trees were visualized using TreeExplorer in the MEGA 3.1 program.

Results

A total of 97 isolates were identified based on culture characteristics and molecular identification (Table 1). We determined that three isolates were *M. fructicola*, one was *M. polystroma*, 39 were *M. fructigena*, and 54 were *M. laxa*.

Hosts and culture characteristics of the isolates

M11, M12, and M13 isolates originating from imported peaches (Italy and Spain) were identified as *M. fructicola* (Table 1). Peach fruits showing brown rot covered with conidial tufts were bought in early October 2005 at a vegetable market and two supermarkets. Colonies were hazel, and sporulation showing concentric rings was abundant. The colony was not rosetted, and the margin was even. The average growth rate of *M. fructicola* isolates was 10.87 mm/24 h ± 0.097 SEM ($P < 0.001$, Fig. 1a).

UFT isolate from 'Ashton Bitter' apple trees (in an orchard at Újfehértó) was the only isolate identified as *M. polystroma*. In April 2006, brownish dieback was present on the leaf petioles and laminas and on the small fruits and fruit pedicels. Infected areas were covered with yellowish exogenous stromata. Colonies of the UFT isolate grown on PDA were yellowish in color, and irregular black stromatal crusts occurred on the edges of the colonies after 10–12 days of incubation. The margins of the colonies were slightly undulate. The mean growth rate was 7.4 mm/24 h.

Thirty-nine isolates were identified as *M. fructigena*, causing brown rot of fruit crops (*Cydonia oblonga*, *Malus* × *domestica*, *Prunus persica*, *Prunus domestica*, *Pyrus communis*, and *Vitis vinifera*), and ornamentals (*Malus* hybrid 'Hopa', *Malus purpurea* 'Aldenhamensis',

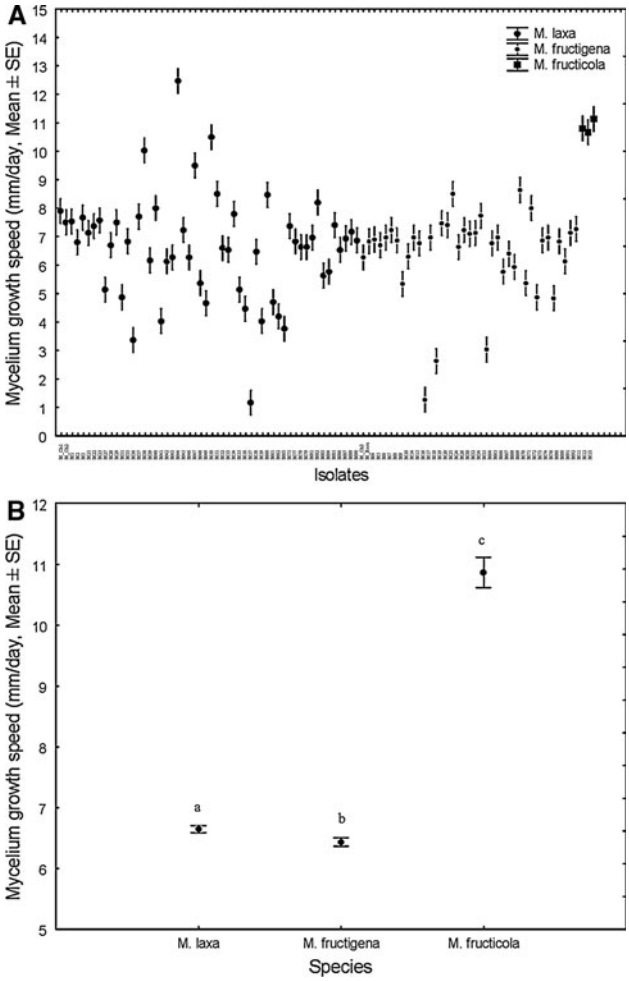

Fig. 1 **a** Statistical analysis of isolate growth rates. The *dots* show the mean of growth speed (mm/24 h) of each *Monilinia* isolate and was performed with their standard errors. **b** Mycelial growth rate of among *Monilinia* species. *Vertical bars* denote the mean of mycelium growth rate (mm/24 h) of the different *Monilinia* species with standard errors. Significant differences among groups are represented by *different letters* above the bar graphs. The model had the statistics $F = 70.38$, $df = 95.192$, $R^2 = 0.985$, $P < 0.001$

Malus × *scheideckeri*, *Chaenomeles speciosa* 'Nivalis' and 'Simonii', *Chaenomeles superba* 'Nicoline', *Cotoneaster divaricatus*, *Pyrus elaeagrifolia*, *Pyrus pyraster* 'Veszprémi', *Prunus cerasifera* 'Nigra', *Rosa* sp.). Most of

the isolates were yellowish in color on PDA, but some of them were rather brownish or gray (typical to *M. laxa*). The margins of the colonies were usually even or slightly undulate, but serrulated (like *M. laxa*) on some occasions. Sporulation was not observed. The average growth rate of *M. fructigena* isolates was 6.44 mm/24 h ± 0.137 SEM ($P < 0.001$). Three isolates showed a lower growth rate than other isolates: M16 (1.3 mm/24 h), M18 (2.6 mm/24 h), and M35 (3.0 mm/24 h) (Fig. 1a).

Fifty-four isolates were identified as *M. laxa*, causing blossom and twig blight and brown rot of fruit trees (*Prunus armeniaca*, *Prunus dulcis*, *Prunus domestica*, *Prunus cerasus*, *Prunus avium*, *Prunus persica*, *Vitis vinifera*) and ornamentals (*Chaenomeles speciosa* 'Simonii', *Chaenomeles* × *superba* 'Atrococcinea Plena', *Prunus tenella*, *Prunus triloba*, *Prunus serrulata* 'Kanzan' and 'Ichiyo'). Typical colonies of the isolates were grayish white to dark gray and brownish gray, with serrulated margins, but a few isolates were yellowish in color with undulated margins (like *M. fructigena*). Sporulation in darkness was observed only with the M33 isolate. The average growth rate of *M. laxa* isolates was 6.65 mm/24 h ± 0.147 SEM ($P < 0.001$). Two isolates showed lower or higher growth rate compared to other isolates: M57 (1.2 mm/24 h) and M44 (12.5 mm/24 h) (Fig. 1a).

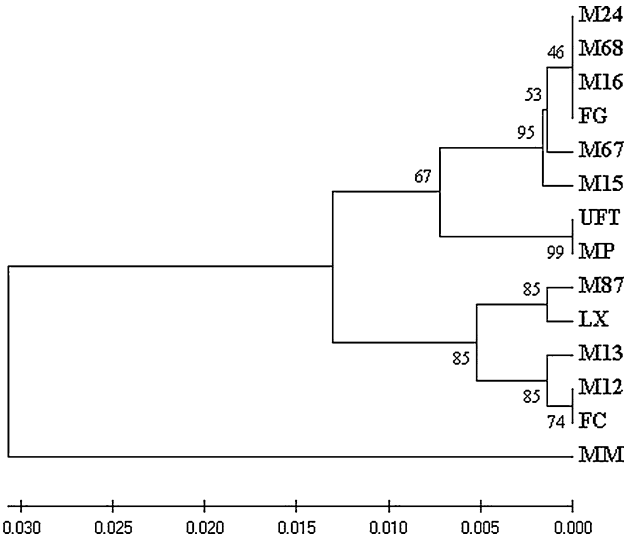

Fig. 2 Phylogenetic tree of the ITS region. *Horizontal lines* show the genetic distances between the different *Monilinia* isolates based on the analyzed sequence data, and *vertical lines* show the genetic identity until the branches. *Numbers* at nodes indicate the percent occurrence of nodes in 10,000 bootstrap re-sampling. Isolates (in *bold*) and GeneBank Accession numbers for reference isolates found in the database: *M. Fructicola*, **FC**, FM994935; *M. Laxa*. **LX**, AB125618; *M. Fructigena*, **FG**, Z73779; *M. Polystroma*, **MP**, Y17876; *M. Mali*, **MM**, AB1256619; and our own isolates: **M12**, FM994935; **M13**, FM994934; **M15**, AM937111; **M16**, AM937109; **M24**, AM937110; **M67**, AM937112; **M68**, AM937113; **UFT**, AM937114

Pathogenicity of the isolates

The pathogenicity of *M. fructicola* and *M. laxa* isolates was tested on peach fruits, whereas *M. polystroma* and *M. fructigena* isolates were tested on apple fruits. The pathogenicity of *M. polystroma* was also confirmed on apple shoots.

Surface-sterilized fruits inoculated with each isolate developed typical brown rot symptoms, and control fruits remained healthy. The first symptoms occurred 2–5 days after inoculation. Pathogens were re-isolated from the inoculated fruits. Pathogenicity tests of the UFT isolate were also successful on apple shoots, and the fungus was re-isolated.

Analysis of the ITS region

PCR analysis of the ITS region identified the M11-13 isolates as *M. fructicola*. Sequences of the M12 and M13 isolates were analyzed. The ITS region showed one base difference in the M12 isolate from the M13 and *M. fructicola* reference isolates (Figs. 2, 3). Thymine was present in the 5.8S ribosomal gene at position 281 instead of cytosine, which resulted in a change from serine (TCG) to leucine (TTG).

In the nucleotide sequence of the ITS region in the UFT isolate, five nucleotides were found that could distinguish *M. polystroma* from *M. fructigena* (Fig. 3) (Fulton et al. 1999; van Leeuwen et al. 2002). The only difference detected between the UFT isolate and the published sequence of *M. polystroma* was at nucleotide 371, where the UFT sequence was identical to the three other species, but the published *M. polystroma* sequence contained an extra 'T' (Fig. 3).

Five *M. fructigena* isolate sequences were sent to the international database and analyzed. Two nucleotide changes were found in the ITS region (Fig. 3). In the M15 isolate, "A" was found at position 191, but other isolates contained "G" at the same position. Similarly, all isolates contained "T" at position 348, except M67, which contained "A". Nucleotide variation in the 5.8S rDNA gene at position 191 caused an amino acid change from glutamic acid to lysine.

One sequence of *M. laxa* (M87) was sent to the international database and analyzed. In the ITS region, only one nucleotide change was found between M87 and the reference isolate (Fig. 3). In position 212, "C" changed to "G" resulted in no change at the amino acid level.

Analysis of the genomic region with unknown function

A 594-bp fragment was amplified from *M. fructicola* using universal primer pairs targeting the genomic region with unknown function. These primer pairs only amplify 397

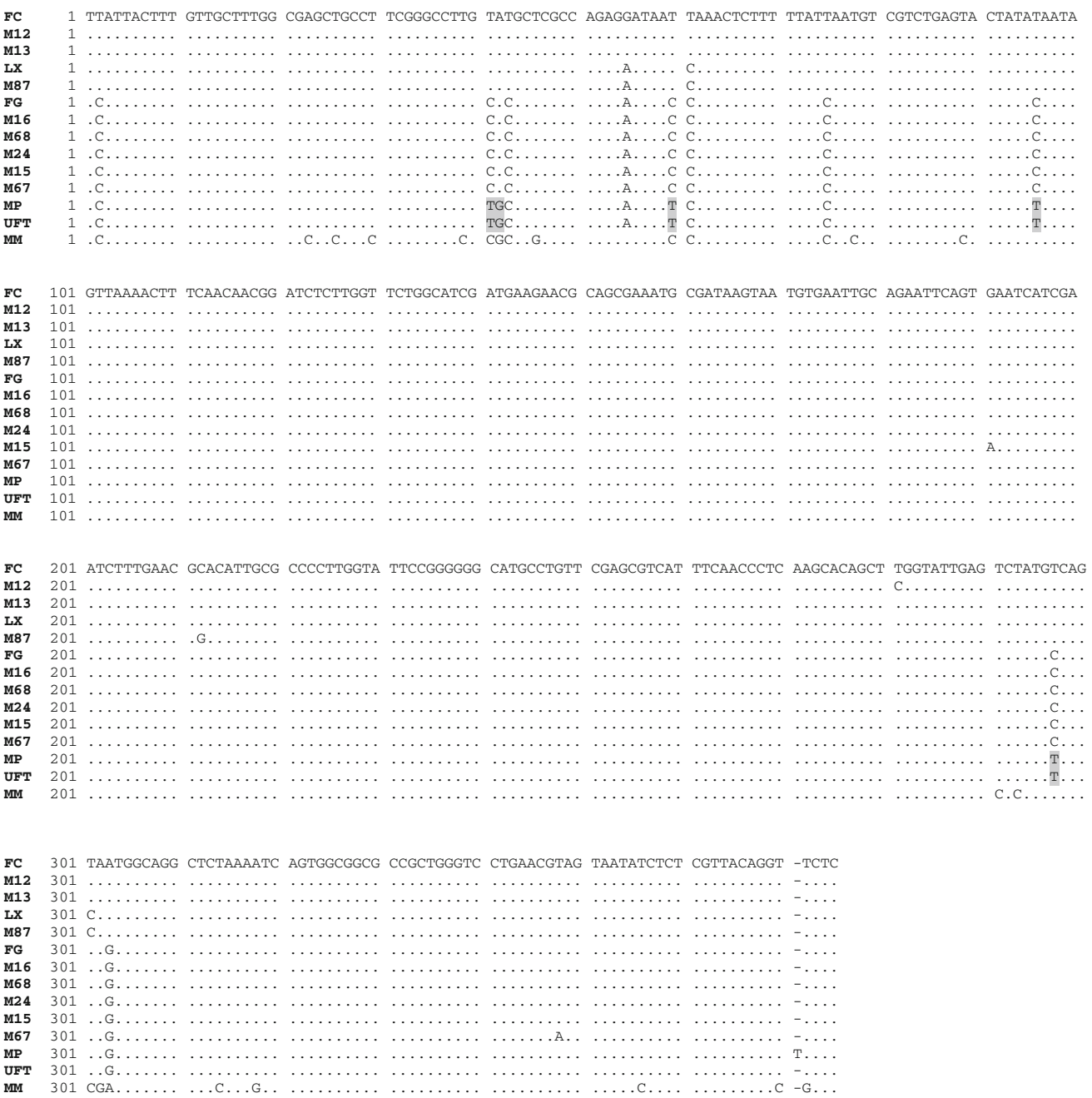

Fig. 3 Sequence alignment of the ITS region of *Monilinia* species and selected isolates. Identical nucleotides are represented by *dots*; absent nucleotides are indicated by *hyphens*. Five nucleotides that distinguish *M. polystroma* from *M. fructigena* are *shaded*. Isolates and GeneBank Accession numbers are given in Fig. 2

and 417-bp fragments from *M. laxa* and *M. fructigena*, respectively. PCR identified the M11-13 isolates as *M. fructicola* (Fig. 4). A 16 base substitution and eight base pair deletion was found on comparing the M12 and M13 isolates with the reference sequence (Fig. 5).

On comparing the *M. fructigena* and *M. polystroma* sequences with the genomic sequence with unknown function revealed insertions and substitutions in the *M. polystroma* sequences (Fig. 6). The Hungarian UFT and published *M. polystroma* sequences were almost identical. Three repetitive sequence motifs (CAT, CCT, TAGTCCA or TAGTCCC) were identified. The CAT and CCT motifs occurred twice in all *M. fructigena* isolates and three times in *M. polystroma* and UFT isolates, whereas the TAG TCCA or TAGTCCC motif occurred three times in all *M. fructigena* isolates, five times in *M. polystroma*, and four times in the UFT isolate (Fig. 6). Regarding this region, the Hungarian *M. fructigena* and *M. laxa* isolates

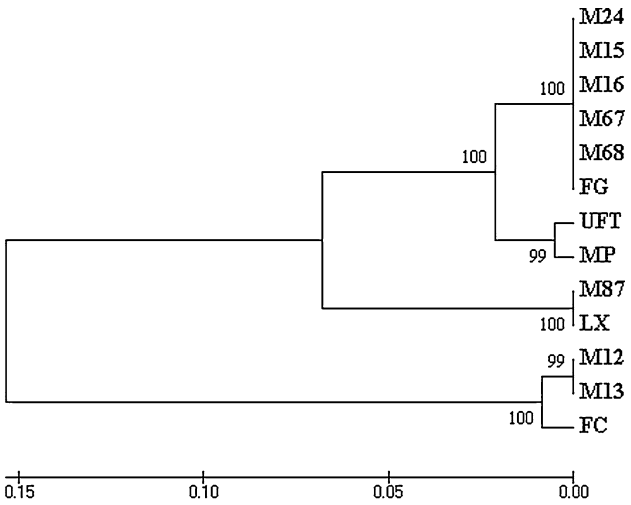

Fig. 4 Phylogenetic tree of the genomic region with unknown function. *Horizontal lines* show the genetic distances between the different *Monilinia* isolates based on the analyzed sequence data, and *vertical lines* show the genetic identity until the branches. *Numbers* at nodes indicate the percent occurrence of nodes in 10,000 bootstrap resampling. Isolates and GeneBank Accession numbers for reference isolates found in the database: *M. Fructicola*, **FC**, AF506700; *M. Laxa*, **LX**, AF506702; *M. Fructigena*, **FG**, AF506701; *M. Polystroma*, **MP**, AY456197; and our own isolates: **M12**, FM994904; **M13**, FM994903; **M15**, AM937116; **M16**, AM937115; **M24**, AM937117; **M67**, AM937118; **M68**, AM937119; **UFT**, AM937120

were identical with the sequence of the published isolates (Figs. 4, 6).

Discussion

The intent of this study was to establish which *Monilinia* species occur in Hungary and cause blossom wilt, twig blight, and brown rot. Isolates were identified by morphological characterization and molecular identification. Three species were identified from field and one species from imported fruits. Regarding classical mycological identification methods, they are simple and inexpensive, but not reliable in every case. Molecular identification is required for unerring identification.

M. fructicola spread in different countries, including Europe, from shipments exported with the pathogen (Bosshard et al. 2006; Ondejková et al. 2010). Rotten imported fruits might be thrown into the communal waste by consumers. The pathogen could overwinter in the mummies and, in spring, among favorable weather conditions, produce conidia on the surface of the mummies, which may cause infection in the orchards.

After reporting the occurrence of the quarantine pathogen on imported peaches from Spain (Petróczy and Palkovics 2006), the Spanish Plant Protection Authority (NPPO) confirmed the presence of *M. fructicola* in Ivars de Noguera (Lleida, Catalonia) and Castillonroy (Huesca, Aragon). Orchards within a radius of 5 km were quarantined, and another zone (10-km radius) surrounding the orchards was also delimited and phytosanitary measures applied in packing stations and nurseries to prevent any further spread of the disease (EPPO 2006; Gell et al. 2007b).

M. fructicola was not found in Hungarian orchards during the survey, though the Hungarian Plant Protection Authority identified the pathogen in some cases from different areas of the country. *M. fructicola* might be an important pathogen in the future, affecting fruit production, but *M. laxa* is currently the most responsible for blossom blight and brown rot in stone fruits. According to new Chinese research, the distribution of brown rot fungi is

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M12     1 TGAAAAAGCT GCCTCATCTA ATAGCAAAAG GAGTGTAAAT AATAAACCTT TTAACTTCTT AGCCGCTCCA TAGCTCTTCT CTCCCCTTTC TTTACCTACC
M13     1 .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
FC      1 .......... .C....G... .......... .......... .......... .......... .......... .......... .......... ..........

M12   101 TAGACACCCG TAGACCTCTC TAGTACTTTC CATTATCCTT TCACGACATT CGTGATCTAC CTCCCTAGTA CCTAGTAGCC ATTACCGCGT CGTTTAGTTC
M13   101 .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
FC    101 .G........ .......... .G........ .......... .......... .......... .......... .......... .......... ..........

M12   201 GCTAGCCATC TTACCATTAT TGTTATTGTT ATTGTTCTTA TTATTATTTT TATTTTTATT ATCATCATTA TCTTTATTTT TATCATCTTA CTGTCTACGG
M13   201 .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
FC    201 .......... .......... .......... C..A..A... ..T....... ....A.C..C ..T..TT... .T......A. .......... ..........

M12   301 TACTTGCTGT ACT------- -ACGGAGCAA ACACGAAATA GATACTGTAC CAAGCGTGTA CTCTGTCCCT ATTTATCATT TGCTCAAGCA AAAAGTACTA
M13   301 .......... ...------- -.......... .......... .......... .......... .......... .......... .......... ..........
FC    301 .......... ...ACCCGTG G......... .......... .......... .......... .......... .......... .......... ..........

M12   393 CTGTACAAAC GGACTCTATT ACGGAATACC TAGGTACATA CCTAGGTACA TCCAGACCCA TCAATAGCCA AAAATGTAAG TGGGGGGG
M13   393 .......... .......... .......... .......... .......... .......... .......... .......... ........
FC    401 .......... .......... .......... .......... .......... .......... .......... .......... ........
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Fig. 5 Sequence alignment of the genomic region with unknown function of *Monilinia fructicola* isolates. Identical nucleotides are represented by *dots*; absent nucleotides are indicated by *hyphens*. Isolates and GeneBank Accession numbers are given in Fig. 4

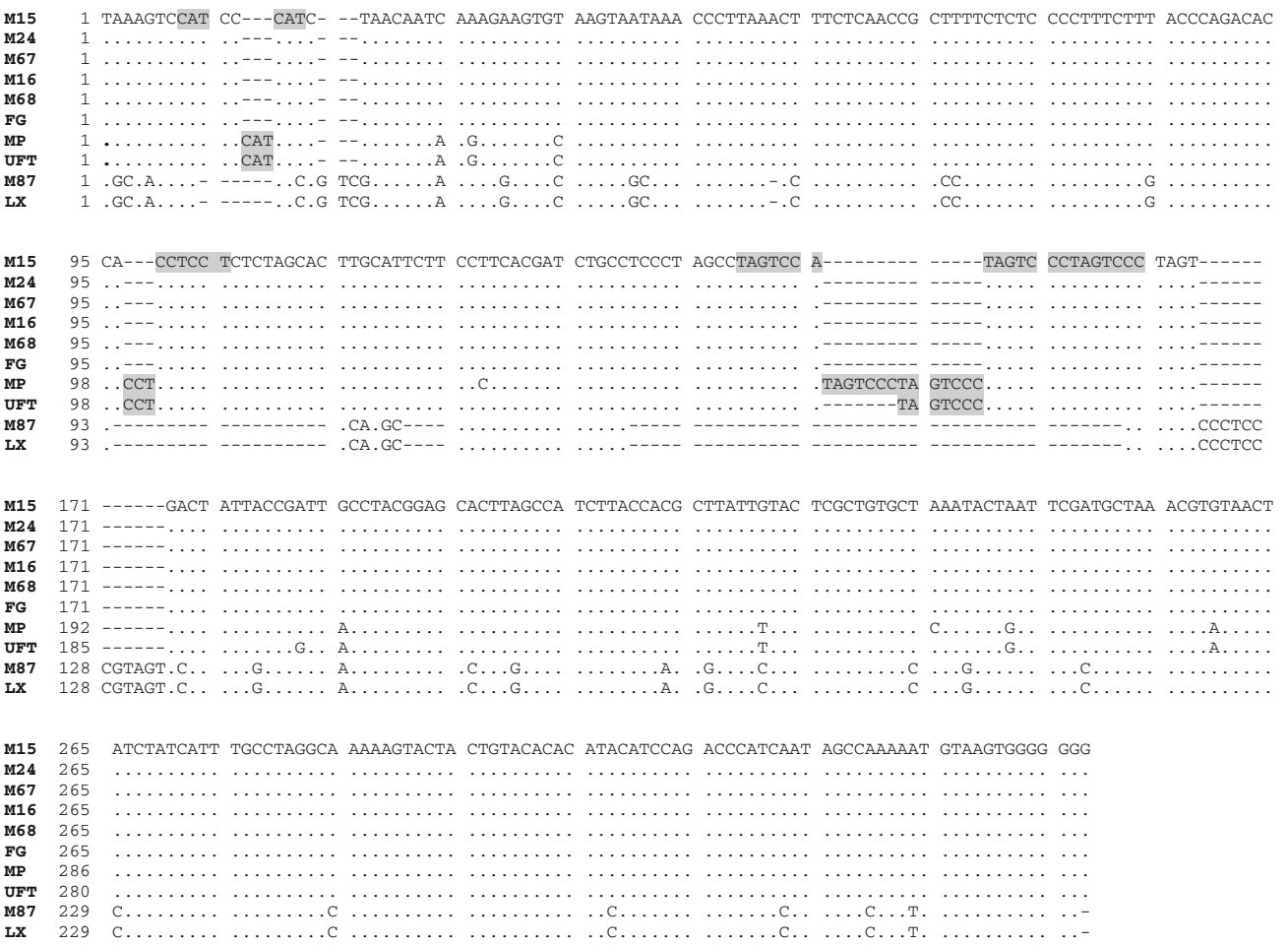

Fig. 6 Sequence alignment of the genomic region with unknown function of *Monilinia* species and selected isolates. Identical nucleotides are represented by *dots*; absent nucleotides are indicated by *hyphens*. Repetitive sequence motives are *shaded* (CAT, CCT, TAGTCCA, or TAGTCCC). Isolates and GeneBank Accession numbers are given in Fig. 4

different; *M. fructicola* is the most prevalent (93%), with *M. laxa* making up only 2% (Zhu et al. 2011).

M. polystroma caused shoot blight and dieback on the leaf petioles, laminas, and small fruit pedicels of apples. Symptoms were previously only published for apple fruits (Fulton et al. 1999). The pathogen is currently not included in the EPPO lists of quarantine pathogens. As a result of the report and existence of this new pathogen in Europe, EPPO might plan to perform a pest risk analysis to determine whether to place *M. polystroma* on one of the lists. The North American Plant Protection Organization has drawn attention to this pathogen in an early warning (Anonymous 2009) as a consequence of our report (Petróczy and Palkovics 2009). Later, the pathogen was reported in plum fruits in China (Zhu and Guo 2010).

M. fructigena and *M. laxa* are common pathogens in Hungary. A new *M. fructigena* host is reported here, tea-rose hybrid pseudofruits (M_Rosa isolate), confirmed by traditional and molecular identification. Brown rot in grapes caused by *M. laxa* (M87 isolate) is the first data about this host–pathogen interaction in Hungary and Europe, which was previously reported only in New Zealand (Pennycook 1989).

In the case of *M. fructigena* and *M. laxa*, no differences were observed in the literature with regard to the symptoms, size, color, and berth of stromata. Culture characteristics of the pathogens were mostly similar to those in the literature, but, in some cases, difficulties occurred during the identification based on culture morphology as mentioned by Muñoz et al. (2008). According to EPPO (2003), the colonies of *M. laxa* are gray/hazel, whereas those of *M. fructigena* are yellowish or creamy. In the case of M26, M88, and M91 *M. fructigena* isolates, the color of the colonies were hazel, which is typical of *M. laxa* or *M. fructicola*, but not *M. fructigena*. The M83 *M. laxa* isolate culture was creamy on PDA, which is characteristic of *M. fructigena*. Colonies of *M. laxa* are rosetted and zones develop. The culture of M88 *M. fructigena* isolate

formed rosettes with arcs on PDA, which is typical of *M. laxa*. Statistical analysis (nested design ANOVA with Tukey HSD test) of the colony growth rate of different isolates showed significant differences among species (Fig. 1b). *M. fructigena* and *M. laxa* had lower growth rates, about half that of *M. fructicola*, which was similar to that found by EPPO (2003). *M. polystroma* was not included in the statistical probe because only one isolate was available. We found that the growth rate of *M. polystroma* isolate (UFT, 7.4 mm/24 h) was higher than the mean for *M. fructigena* isolates (6.44 mm/24 h), which was similar to that reported by van Leeuwen et al. (2002).

Species-specific primers designed by Ioos and Frey (2000) for the ITS1 region, 5.8S rRNA gene, and ITS2 region of *M. fructigena*, *M. laxa*, and *M. fructicola* did not work specifically under our conditions, causing cross reactions between species at the annealing temperature given by the authors (55°C). Raising the temperature to 70°C yielded specific PCR products, but the quantity of PCR products decreased significantly. A sequence analysis of isolates belonging to the same species showed only slight variability at the nucleic acid level, and it proved to be a reliable method for identifying *Monilinia* species.

In addition to the ITS region, a genomic region with unknown function was chosen for molecular analysis because it was the only available sequence data for *M. polystroma*, and this region was more diverse among the different *Monilinia* species than the ITS region. Regarding Hungarian *Monilinia* isolates, multiplex PCR, which was developed by Cotê et al. (2004) for this region, was a suitable method for separating *Monilinia* species by different fragment lengths. Molecular verification is required in every case to complete classical identification because the latter is not reliable in all samples, as observed by Sonoda et al. (1982). Nucleic acid sequence analysis of this region is suitable for identification of *Monilinia* species, and this region showed remarkable variability in *M. fructicola* and *M. polystroma* isolates. The genomic region with unknown function is primarily suitable and important for reliable diagnosis, but analysis of other genomic regions may contribute to better understanding of the evolution of *Monilinia* species.

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