CHARACTERIZATION OF *MONILINIA LINHARTIANA* ISOLATES

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SUMMARY

Monilinia linhartiana is a significant fungal pathogen of quince causing leaf blotch, shoot blight and mummification of developing young fruits. In our study, five isolates originating from infected quince shoots were characterized. Morphological characteristics were determined before performing molecular assays. Three genomic regions were sequenced and analysed: the ITS region, a polymorphic region and the β-tubulin gene. The ITS region (ITS1-5.8 rDNA-ITS2) sequences of different *Monilinia* species showed high similarity, thus *M. linhartiana* isolates could not be safely separated from *M. aucupariae* isolates. By converse, based on the 28S rDNA sequences, *M. linhartiana* isolates could be distinguished from the closely related species, offering prospects for the unambiguous identification of *Monilinia* species. The 28S rDNA and β-tubulin genes of *M. linhartiana* were sequenced for the first time.

Keywords: beta-tubulin, disjunctors, ITS, *Monilinia linhartiana*, quince, 28S rDNA.

INTRODUCTION

Quinces (*Cydonia oblonga*) have been grown for centuries in Europe, where summers are sufficiently hot for the fruit to fully ripen, Turkey being the world's leading producer (Topcu *et al*., 2015). Shoot blight and fruit rot caused by *Monilinia* spp. are significant diseases of quince, which requires chemical treatments every year. Brown rot symptoms caused by *Monilinia fructigena* or *Monilinia fructicola*, occasionally by *Monilinia laxa* or *Monilinia polystroma,* appear on mature fruits (Chalkley, 2015). *Monilinia linhartiana* causes leaf blotch, shoot blight and mummification of young fruits. However, the disease caused by *M. linhartiana* is one of the most destructive diseases of quince worldwide (Batra, 1991). Yield losses could reach 90-95%, as reported from the Mediterranean region (Altinyay, 1972; Moral *et al*., 2008; Nevado *et al*., 2011).

M. linhartiana (Prill. and Delacr.) N.F. Buchw. (anamorph: *Monilia cydoniae* Schellenb.) was first described by the Hungarian botanist and mycologist György Linhart, and was named after him (Wormald, 1926). In Hungary, the disease has been observed since the 1930s (Glits, 2000), although the pathogen has rarely appeared in recent decades. Nowadays the total area of quince orchards is steadily increasing in Hungary, thus the pathogen showed up again causing infection more frequently. In Turkey, the fungus was studied in detail in the 1970s (Altinyay, 1972) and it is still present nowadays (Ünlü and Boyraz, 2010), but no further studies have been conducted. Recent studies were only carried out in Spain by Moral *et al.* (2011) who examined the pathogen by molecular methods, and reconsidered the existing plant protection strategies.

M. linhartiana colonises leaves, shoots and young fruits of quince (Naidenov, 1915), overwintering in pseudosclerotial or mycelial form in twigs (Altinyay, 1972). Two infection cycles can be distinguished during the vegetation period. Ascospores as primary inoculum infect the young leaves and the mycelium spreads along the shoots. The secondary inoculum develops on the surface of primary infected leaves, and infects the flowers or young fruits.

In early spring, apothecia with ascospores develop on stromatized fruit mummies (pseudosclerotia). The life cycle of *M. linhartiana* is closely related to the host plant phenology (Moral *et al*., 2011), as the first apothecia appear at the bud break stage of quince. Ascospores infect the young leaves, causing foliar necrosis along the primary veins starting from the base of the leaf. Wormald (1926) observed that the infection spreads rapidly; the fungus penetrates through the petioles into the axis of the shoots and blights them. Conidial coating appears on the necrotised tissues (Sumstine, 1913). Conidia induce the secondary infection and attack the young fruits. The infected fruits shrivel on the shoots or fall to the ground, but conidia never develop on their surfaces (Batra, 1991). These infected fruits turn into pseudosclerotial structures.

M. linhartiana belongs to the section *Disjunctoriae* of the genus *Monilinia*. Conidial chains contain small modified conidia called disjunctors, which can be found between macroconidia (Honey, 1936). The species of the section are monotrophic, inhabiting a single host, or are oligotrophic for they attack several species of one host genus

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(Batra, 1991). In the section *Junctoriae*, the macroconidia are joined directly, like the conidial chains of *M. laxa* or *M. fructicola*. These pathogens are polytrophics, infecting several genera of a family. The identification and classification of the species is feasible based on host specificity, morphology of stromata and conidial chains, shape and size of the conidia, and cultural characteristics on cultivation media (Batra, 1991). However, closely related species cannot be differentiated in all cases by morphology and cultural characteristics (van Leeuwen and van Kesteren, 1998), whereas reliable identification can be obtained by PCR-based methods (De Cal and Melgarejo, 1999).

Molecular studies of the internal transcribed spacer (ITS) region are generally applied for the identification of *Ascomycota* fungi, and are also appropriate for phylogenetic and evolutionary studies (Capote *et al*., 2012). Carbone and Kohn (1993) examined the ITS region of *Sclerotiniaceae*, separating the sections *Junctoriae* and *Disjunctoriae* of the genus *Monilinia*. Several brown rot species have been characterized by the ITS1-5.8S rDNA-ITS2 region (Holst-Jensen *et al*., 1997; Fulton *et al*., 1999; Ioos and Frey, 2000). Holst-Jensen *et al.* (1997) found cospeciation between host and pathogen. Species of the section *Junctoriae* infect rosaceous hosts with stone or pome fruits. Four evolutionary lineages were found in the section *Disjunctoriae*: one group of species on dry stone fruits of rosaceous hosts, one group of species on capsular fruits of ericaceous hosts, one group of species on sweet berry fruits of ericaceous hosts, and one parasite species of *Crataegus* sp. Moral *et al.* (2011) studied the ITS region of *M. linhartiana*, and the phylogenetic analyses demonstrated that the pathogen was closely related to *Monilinia mali* and *Monilinia amelanchieris* and other *Monilinia* species infecting dry stone fruits of rosaceous hosts.

MATERIALS AND METHODS

Sample collection and isolation. Infected shoots showing typical symptoms were collected in different locations across Hungary in 2013 and 2014, and in Turkey in 2015 from orchards and gardens (Table 1). Conidia from infected leaves were transferred onto Petri dishes containing potato dextrose agar (PDA, BioLab Zrt., Hungary), and individual conidia were separated and placed onto culture medium under an inverted microscope. Petri dishes were incubated in the dark at 24°C for 10 days.

Morphological and cultural characteristics. The symptoms visible on the infected parts and the appearance of conidial chains were observed, and the length and width of 100 conidia and 20 disjunctors per isolate were measured under a light microscope (Nikon Eclipse, Japan). Colonial morphology was observed on PDA, malt extract agar (MEA, BioLab Zrt., Hungary) and leonian malt agar (LMA, 1.0 g KH_2PO_4, 1.0 g $MgSO_4$, 10.0 g sucrose, 5.0 g peptone, 15.0 g agar powder, 10.0 g malt extract/litre) media. Mycelial plugs (5 mm in diameter) from single-spore cultures were transferred to the center of the agar plates, and incubated in the dark at 24°C. Colour, structure, growth rate, pigmentation, conidia production and growth rate (mm/24 h) of the isolates were determined.

Pathogenicity tests. The pathogenicity of *M. linhartiana* isolates was tested on young leaves, young fruits (approx. 3-4 cm in diameter) and fruits of *Cydonia oblonga*. Before inoculation, the surface of leaves and fruits was disinfected with 70% ethanol for 1 min, then washed in sterile water. The petioles of the leaves and the fruit peels were pricked by a sterile needle, then mycelial plugs (3 mm in diameter) were placed into the punctures. For control, sterile PDA plugs were used. Five pieces of each organ (shoots and fruits) per isolate were inoculated. On each shoot two leaves were inoculated, while in the case of young and mature fruits only one mycelial plug was inserted per fruit. Inoculated materials were placed into large sterile glass vessels on glass beads and incubated under natural light at 22-25°C for 3 weeks in 95 ± 2 RH.

Molecular investigations. DNA was extracted using the cetyl-trimethyl-ammonium-bromide (CTAB) method (Maniatis *et al*., 1989), followed by phenol-chloroform extraction and isoamyl alcohol (24:1) precipitation carried out according to a protocol described by Gell *et al.* (2007b).

Three genomic regions were examined by PCR: the ITS region and the 28S rDNA gene (primers ITS5 and NL4), the β-tubulin gene (primers BT-forw and BT-rev), and a polymorphic region (primers UniMon-F and UniMon-R) (Table 2). The BT primer pair was designed using the data of McKay *et al.* (1998). Each PCR reaction mixture contained 100 ng of fungal DNA, 1 mM of both primers, and Dream Taq Green PCR Master Mix 2× (Thermo Scientific, Lithuania) diluted to a final volume of 50 µl. During PCR reactions, the initial denaturation step (94°C, 3 min) was followed by 35 cycles as shown in Table 2. Final elongation was 10 min at 72°C. PCR products were separated in 1% GelRed (Biotium Inc. USA) stained agarose gel, and purified using the High Pure PCR Product Purification Kit (Roche, Germany) according to the manufacturer's protocol. Final DNA concentrations were verified by spectrophotometry (NanoDrop 2000). Fragments were sequenced in an ABI Prism automatic sequencer (BaseClear B.V., Leiden, The Netherlands) in three replicates. Nucleotide sequence identities and protein sequence similarities were determined by BLAST analyses (Altschul *et al*., 1999). Sequences were aligned by L-INS-i method using the MAFFT 7 program (Katoh *et al.,* 2002, 2005). Phylogenetic studies were set up in BEAST v2.3.2 (Bayesian Evolutionary Analysis Sampling Trees) software (Drummond *et al.,* 2012). For the analyses, the HKY85 substitution model was applied (Halpern and Bruno, 1998), from

Table 1. *Monilinia* isolates used in this study with the accession numbers of the gene sequences.

Isolate	Species	Host plant	Location	Date of collection	Accession number		
					ITS region	BT gene	Polymorphic region
MLH1	*Monilinia linhartiana*	*Cydonia oblonga*	Siófok, HU	09.05. 2013	LN908896	LN908895	LN908894
MLH2	*Monilinia linhartiana*	*Cydonia oblonga*	Harkány, HU	01.05. 2013	LN908897	LN908901	LN908905
MLH3	*Monilinia linhartiana*	*Cydonia oblonga*	Budapest, HU	02.05. 2014	LN908898	LN908902	LN908906
MLH4	*Monilinia linhartiana*	*Cydonia oblonga*	Kecskemét, HU	10.05. 2014	LN908899	LN908903	LN908907
MLH5	*Monilinia linhartiana*	*Cydonia oblonga*	Şirince, Turkey	24.04. 2015	LN908900	LN908904	–
2010/PS78	*Monilinia polystroma*	*Malus domestica*	Poland	2010	LT615168	LT615156	–
2014/PS36	*Monilinia polystroma*	*Prunus domestica*	Zsámbék, HU	21.08. 2014	LT615178	LT615164	–
2013/FG73	*Monilinia fructigena*	*Cydonia oblonga*	Pincehely, HU	07.11. 2013.	LT615170	LT615158	–
2014/FG42	*Monilinia fructigena*	*Pyrus communis*	Abasár, HU	15.08. 2014	LT615177	LT615163	–
2013/LX13	*Monilinia laxa*	*Prunus triloba* 'Multiplex'	Baracska, HU	10.05. 2013.	LT615173	LT615160	–
2013/LX5	*Monilinia laxa*	*Prunus armeniaca* 'Ceglédi bíbor'	Kápolnásnyék,HU	09.05. 2013.	LT615171	LT615159	–
2014/FC32	*Monilinia fructicola*	*Prunus persica* 'Cresthaven'	Érd, HU	29. 08. 2014.	LT615174	LT615149	–
2014/FC48	*Monilinia fructicola*	*Prunus persica* var. *nucipersica*	market, HU	30.08. 2014.	LT615175	LT615151	–

Table 2. PCR parameters.

Primer	Sequence	PCR cycle	Length	Reference
ITS5	5'-GGAAGTAAAAGTCGTAACAAGG-3'	94°C, 30 s 47°C, 30 s 72°C, 60 s	1115 bp	White *et al.*, 1990.
NL4	5'-GGTCCGTGTTTCAAGACGG-3'			Kurtzman, 1997.
BT-forw	5'-ATGCGTGAGATTGTACGTAT-3'	94°C, 30 s 47°C, 30 s 72°C, 90 s	1587 bp	present paper
BT-rev	5'-GTACCAATGCAAGAAAGCCT-3'			
UniMon-F	5'-ATCGGCTTGGGAGCGG-3'	94°C, 30 s 51°C, 30 s 72°C, 30 s	341 bp	Petróczy *et al.*, 2012.
UniMon-R	5'-GAGCAAGGTGTCAAAACTTCCAT-3'			

the 10 million generation of the MCMC (Markov chain Monte Carlo) each 10000th was stored. Three replicates of each analysis were run. Phylogenetic trees were composed with 25% burn-in and support was assessed based on posterior probabilities (70%), summarising the results of the three parallel run (Gelfand and Smith, 1990). Interspecific and intraspecific diversities were calculated by the CLUSTAL O (1.2.1) multiple sequence alignment program (Sievers *et al.*, 2011).

Statistical analyses. The statistical analyses were performed using the IBM SPSS Statistics 22.0 software. The size (length and width) of the conidia and the length of disjunctors were analysed by one-way ANOVA with factor "isolate" (MLH1, MLH2, MLH3, MLH4, MLH5). Normality of the conidial length was assumed based on the skewness and kurtosis values of unstandardized residuals (Tabachnick and Fidell, 2007). The data on the conidial width were transformed by the method of Box and Cox (1964) with a lambda value of −0.8. The assumption of homogeneity of variances was met (Tabachnick and Fidell, 2007), therefore a Tukey post hoc test was run. Normality of the disjunctors was confirmed by a Shapiro-Wilk test ($K(94) = 0.979$; $p = 0.125$), but the mean square values differed, therefore the Games-Howell post hoc test was run.

RESULTS AND DISCUSSION

Symptoms and isolation. In 2013-2015, leaf blotches and shoot blights were observed on quince as specific symptoms of *M. linhartiana*. The first symptoms appeared on young leaves below the flowers (Fig. 1A). Affected leaves showed a reddish maroon or brown discolouration along the primary and secondary veins. On the necrotised tissues, a greyish coating of conidial chains appeared (Fig. 1B). The green shoots were colonised by the pathogen. As a consequence of shoot blight, the older leaves on the shoots were also necrotized, but conidial chains did not develop on them.

Floral infection has not been observed during our investigations, similarly to the observations of Alexandrescu and Morea (1964), although the sample collection period overlapped with the period from full bloom to petal fall. Prillieux and Delacroix (1893) mention that the flowers became brown and soft, upon ascospore infection, which occurred when the pathogens migrated from the shoots into the flower. Batra (1991) and Moral *et al.* (2011) reported that flower necrosis can be caused by a secondary conidial infection.

Morphological characteristics. *M. linhartiana* produced chains of conidia on the surface of the primary infected

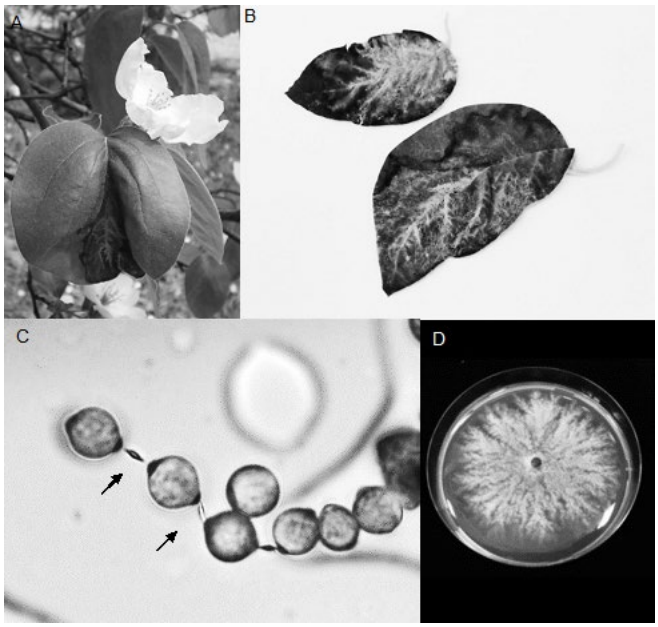

Fig. 1. Symptoms of monilinia disease of quince (A); conidia chains of *M. linhartiana* appear along the main veins of the leaves (B); conidia and disjunctors of the pathogen, 600× (C); cultural characteristics of the isolates (D).

leaves, while Moral *et al.* (2011) observed conidia chains also on the shoots. Conidial chains were extremely fragile due to the presence of some small modified conidia called disjunctors (Fig. 1C). Disjunctors connect two macroconidia and facilitate their dispersal. They are formed while the conidia are still enclosed by the host epidermis or cuticle (Batra, 1988). The structure of conidial chains suggests that the pathogen belongs to the section *Disjunctoriae* of the genus *Monilinia*.

The conidia were one-celled, hyaline, subglobose, less limoniform than those of *M. laxa* or *M. fructicola*. Significant differences in length and width were observed between isolates [length: $F(4,433)=34.4$; $p<0.001$; width: $F(4,431)=37.1$; $p<0.001$]. The average sizes of conidia are shown in Table 3. The size of 100 conidia matched the average value reported by other authors (Sumstine, 1913; Berkhout, 1923; Altinyay, 1972; Moral *et al.*, 2011). Disjunctors connected the tapered ends of the conidia; they were oblong, spindle-shaped. Significant difference was observed between the isolates ($F(4,89)=342.9$; $p<0,001$). The disjunctors of the Turkish isolate were the longest, 4.9 µm in length. *M. linhartiana* could be cultured on all culture media. PDA was the most suitable for colonization; the examined isolates had similar morphology (Fig. 1D).

The colonies grew radially. Young colonies were pale yellow in colour with entire or slightly undulate margins. Brown stroma-like formations appeared around the inoculation point in 10-12 day old cultures. The average growth rates are listed in Table 3. On LMA and MEA plates, the isolates showed atypical growth patterns; they grew slower, and developed white colonies and large amounts of aerial mycelium in both media. Colonial morphology coincided with the descriptions by Berkhout (1923) and Moral *et al.* (2011). However Moral *et al.* (2011) observed microconidia production on the surface of colonies on PDA.

Table 3. *In vitro* mycelia growth rate and size of conidia of *Monilinia linhartiana* isolates (Values followed by the same letter are not significantly different).

Isolate	Average size (µm)			Growth rate (mm/day)
	conidia length	conidia width	disjunctors	
MLH1	14.2 (d)	13.8 (b)	2.5 (a)	5.6
MLH2	10.4 (a)	10.5 (a)	2.3 (a)	5.0
MLH3	11.4 (ab)	10.1 (a)	3.5 (b)	3.2
MLH4	11.9 (bc)	10.3 (a)	3.7 (b)	2.9
MLH5	12.8 (c)	9.8 (a)	4.6 (c)	2.1

Pathogenicity tests. All isolates were pathogenic to quince leaves and young fruits. Inoculation of mature fruits was unsuccessful. Brown necrosis developed on the petioles and leaf blades spreading from the base to the apex in a v-shape. The mesocarp and seeds of the young fruits became black and rotten, while mature fruits remained similar to control specimens. The same symptoms on young fruits, i.e. necrosis under the peel and mycelial formations were observed by Morel *et al.* (2011). No changes were shown by control specimens until the day of evaluation. Koch's postulates were fulfilled by re-isolating the fungus from symptomatic tissues and re-identifying the pathogen at the species level.

Molecular identification and characterization: ITS region and 28S rRNA gene. The ITS5-NL4 primers amplified a 1115 base-pair long sequence of the ITS region and the adjacent genes: a partial sequence of the 18S ribosomal gene, the ITS1 region, the 5.8S ribosomal gene, the ITS2 region and a partial sequence of the 28S ribosomal gene.

Phylogenetic analyses of the ITS1-5.8S-ITS2 region classified the *M. linhartiana* isolates in the section *Disjunctoriae* (Fig. 2). These species are listed according to host specificity; the *M. linhartiana* isolates could be classified into the group of species attacking pome fruits of rosaceous hosts. Holst-Jensen *et al.* (1997) refer to the hosts as dry stone fruits. The isolates MLH3, MLH4 and MLH5 were grouped together with the *M. linhartiana* sequences (HM581953, HM581954) found in the NCBI database, whereas MLH1 and MLH2 were more similar to *M. aucupariae* (Z73771, Z7377).

Carbone and Kohn (1993) claimed that the ITS1 and ITS2 regions are more variable than the adjacent genes (18S rRNA, 28S rRNA). The ITS1 of *Monilinia* spp. is 142-152 bp long, its ITS2 is 141-145 bp long. The ITS1 of isolates MLH1, MLH2, MLH3 and MLH5 contained 147 base pairs, as the majority of Spanish *M. linhartiana* isolates (Moral *et al.*, 2011). The isolate MLH4 contained 148 base pairs. Thymine was at position 116, and this substitution was present in a Spanish isolate (HM581953)

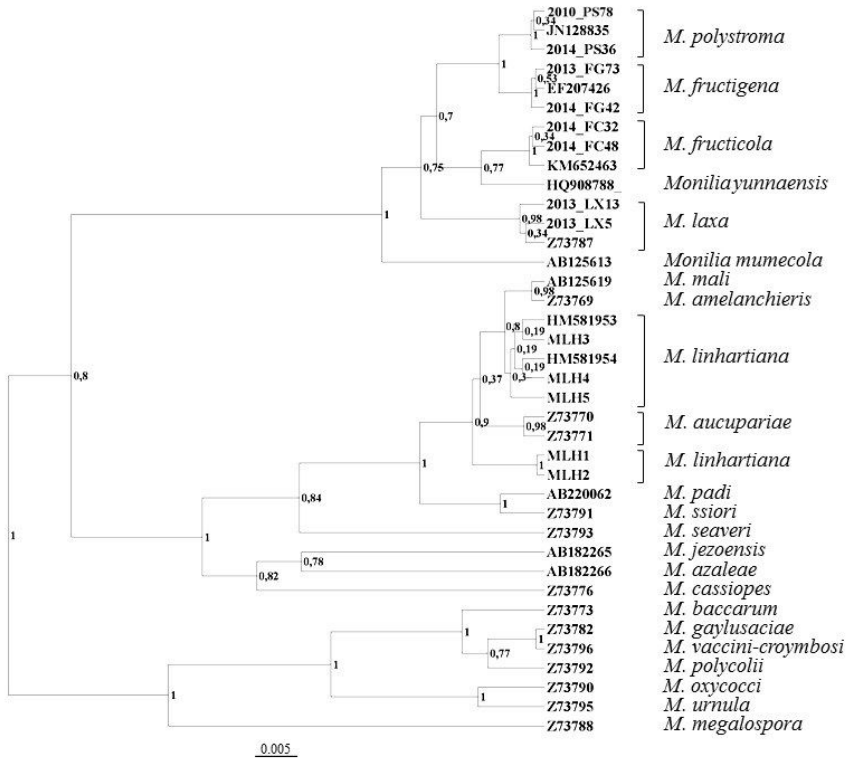

Fig. 2. Phylogenetic tree of *Monilinia* and *Monilia* species. 452 bp of the ITS1–5.8S rDNA–ITS2 region were analysed based on Bayesian inference using HKY85 substitution model. Numbers close to the branches show the parsimony value.

as well. The ITS2 was 143 bp long for all five isolates. The analysis of the two non-coding regions detected a higher variability in the ITS1 than in ITS2 region. ITS1 contained four variable sites whereas only two were found in ITS2, but this variance lags far behind the variance observed in *M. linhartiana* isolates by Moral *et al.* (2011). In this study, *M. linhartiana* isolates were found to have 42 variable sites in the ITS1, and 38 variable sites in the ITS2 region.

The ribosomal genes of *M. linhartiana* isolates were highly similar. The 31 base pairs of the 18S rRNA gene did not contain any variability; further analysis of this short region has not been carried out. The variable sites of the ITS region were even shorter, because no variation was shown in the 5.8S rDNA gene the same as reported by Gell *et al.* (2007a). *M. linhartiana* isolates contained 157 base pairs. Holst-Jensen *et al.* (1997) examined the 5.8S rDNA gene of 17 *Monilinia* species, and found low interspecific variation. The gene contained only two parsimony informative sites among the species of the genus. The partial sequence of the 28S ribosomal gene of *M. linhartiana* isolates was 637 bp long, and contained one base substitution. The 637 base pairs of the 28S rDNA gene contained cytosine at position 572 in the isolates MLH1 and MLH2, while in the case of MLH3, MLH4 and MLH5 adenine was present there. These are the first sequence data of the 28S gene of the species.

Partial sequence (319 bp) of the 28S rRNA of several *Monilinia* species have been published. The phylogenetic studies of this region could not separate *M. linhartiana* from other closely related species.

Analysis of the 28S rRNA gene separated *M. linhartiana* isolates from other members of the genus, supported by high bootstrap values (Fig. 3). The highest similarity (99%) was shown with isolates of *M. aucupariae* (Z73744) and *M. padi* (Z73755). The sequence of the 28S rRNA gene separated the species more accurately than the ITS region, but this feature could not separate all species. *M. aucupariae*, *M. padi*, *M. baccarum* and *M. polycodi* had the same sequence, and the species of the group *Junctoriae* could not be separated either. In the study of Holst-Jensen *et al.* (1997), 67 out of 318-319 base pairs of the 28S rDNA gene were variable, 32 of which were parsimony informative. We recommend the use of this gene for identification, for the examination of the complete gene may lead to a more valuable phylogenetic analysis of the section *Disjunctoriae*.

Beta-tubulin gene. The PCR reaction amplified a partial sequence of the β-tubulin gene. To our knowledge, this is the first sequence data about the β-tubulin gene of *M. linhartiana*. The forward primer starts from the first methionine-encoding triplet. The region contains 6 introns and 7 exons, accounting for 1587 base pairs between the primers. The sequences of the coding regions were identical, only the first intron differed by two base substitutions. The partial β-tubulin gene of *M. linhartiana* encoded 398 amino acids and was highly conserved. Three conserved domains were detected: a hypothetical domain

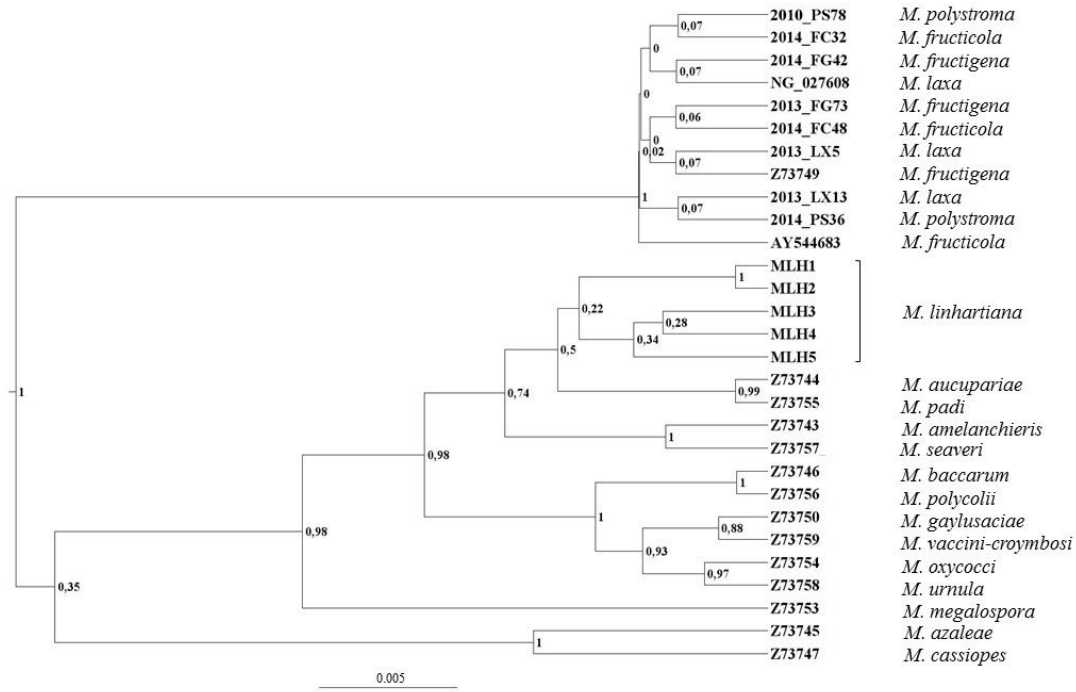

Fig. 3. Phylogenetic tree of *Monilinia* species. 285 bp of the 28S rDNA gene were analysed based on Bayesian inference using HKY85 substitution model. Numbers close to the branches show the parsimony value.

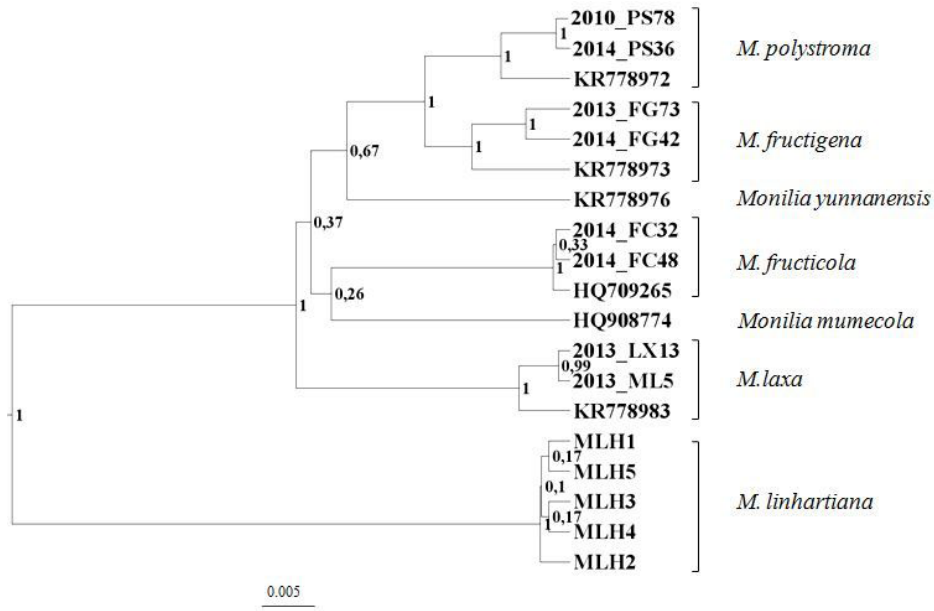

Fig. 4. Phylogenetic tree of *Monilinia* and *Monilia* species. 402 bp of the beta-tubulin gene were analysed based on Bayesian inference using HKY85 substitution model. Numbers close to the branches show the parsimony value.

(PLN00220), a cytoskeleton domain (COG5023) and another hypothetical domain (PTZ00010) (Delves *et al.*, 1989; Marchler-Bauer *et al.*, 2015). The β-tubulin protein chain shows homology with the proteins of closely related species such as *Botrytis cinerea* (P53373), *Monilinia fructicola* (AAP40256), *Sclerotinia homoeocarpa* (AHH01120) and *Sclerotinia borealis* (ESZ98925), and 98-99% sequence similarity with several other fungal pathogens.

The multiple sequence alignment of the β-tubulin gene of *M. linhartiana* isolates showed 99.9-100% identity. These sequences were allocated on a separate branch of the phylogenetic tree (Fig. 4). *M. linhartiana* could be separated clearly from the species of the section *Junctoriae* and, when compared with other *Monilinia* species, it showed the highest similarity with *M. mumecola* (91.6%), and the lowest similarity with *M. fructicola* (90.5%). This difference was found in the intron regions. Unfortunately, other species of the section *Disjunctoriae* were not available, thus the alignment could not be established.

The β-tubulin gene is a frequently studied genomic region of *Monilinia* spp. and is connected to fungicide resistance. Mutations of the gene are responsible for benzimidazole resistance. *M. fructicola* and *M. laxa* populations exhibit different levels of resistance depending on the site of the point mutation. Mutation of the E198A gene provides high resistance; this was found in *M. fructicola* and *M. laxa* populations (Ma *et al*., 2003a, 2003b). Mutations of the genes H6Y in *M. fructicola* and L240F in *M. laxa* populations account for low resistance (Ma *et al*., 2003a, 2003b). Our *M. linhartiana* isolates carried the sensitive triplets in all three positions, thus Hungarian isolates are sensitive to benzimidazoles.

Genomic region with unknown function. The primers amplifying this polymorphic region were designed for the easy recognition of the species *M. laxa*, *M. fructigena, M. polystroma* and *M. fructicola* (Petróczy *et al*., 2012). Identification of the species is possible based on the length of the target sequences. The DNA fragment lengths and sequences of *M. linhartiana* isolates were compared with those of other *Monilinia* species pathogenic to quince*: M. laxa* (FM994902), *M. fructicola* (FM994904), *M. fructigena* (AM937119) and *M. polystroma* (AM937120).

The original protocol of Petróczy *et al.* (2012) indicated 55°C as annealing temperature. No PCR product was produced with this parameter, but at a reduced temperature of 51°C the amplification was successful. Sequencing of the isolates MLH1-MLH4 showed that the polymorphic region contains 341 base pairs between the primers. To our knowledge, this is the first sequence data about this region of *M. linhartiana*.

Electrophoresis reliably separated *M. linhartiana* from the species *M. fructicola*, *M. polystroma*, *M. fructigena* but not from *M. laxa*. The DNA fragment length differences between *M. linhartiana* and *M. laxa* were not distinguishable on agarose gel, but the species could be clearly separated based on the sequences. *M. laxa* contains one more base pair than *M. linhartiana* isolates, 342 base pairs in total.

Difficulties in the identification of the species of the section *Disjunctoriae*. *Monilinia* species are closely related, therefore they cannot be identified based on morphological and cultural characteristics alone, especially in the case of the rarely studied species of the section *Disjunctoriae*. Our data match to other authors' morphological and cultural observations of *M. linhartiana*, but these methods cannot identify the isolates in a trustworthy manner.

Host specification can also be used to identify the isolates. The members of the section *Disjunctoriae* infect only a single host (Schellenberg, 1923). *M. linhartiana* infection was only recorded on *Cydonia oblonga* (Batra, 1991; Farr and Rosmann, 2015), while the closely related *M. aucupariae* infects *Sorbus aucuparia* and *Sorbus commixta*, according to Farr and Rosmann (2015). The species cannot be separated reliably based on the size of the conidia either. Our data fit the size interval of *M. linhartiana*, but the conidial size distribution of *M. aucupariae* (9-12.5 × 7-10 µm) overlaps this range (Batra, 1991). A reliable classification is possible by molecular methods only (De Cal and Melgarejo, 1999).

Phylogenetic analysis of the ITS region confirmed that the isolates belong to the section *Disjunctoriae* infecting pome fruit hosts. In our study, three isolates (MLH3, MLH4, MLH5) were similar to the Spanish *M. linhartiana* isolates, showing 100% identity. Two isolates (MLH1, MLH2), showed the highest similarity (99.1%) with *M. aucupariae* isolates. *M. aucupariae* (Z73771 and Z7377) differed from MLH3 and MLH5 only in 3 and 4 base pairs on this fragment, while *M. mali* (AB125619) differed in 6-7 base pairs. The low variability of this region causes difficulties in the identification of other *Monilinia* species. *M. gaylussaciae* and *M. vaccinii-corymbosi* have identical sequences in the ITS region (Holst-Jensen *et al*., 1997). The recently described new species *M. polystroma* and *M. yunnanensis* differ from *M. fructigena* only in five (van Leeuwen *et al*., 2002) and eleven base pairs (Hu *et al*., 2011), respectively.

The ITS sequence of *Monilinia* species is highly conserved; intraspecific variation does not reach 2% (Ioos and Frey, 2000; Hu *et al*., 2011; Volkova *et al*., 2013), while intraspecific variation among other fungal species can reach 5% (Baura *et al*., 1992; Zambino and Szabo, 1993). The genera of the family *Sclerotiniaceae* show high similarity levels as well (Holst-Jensen *et al*., 1997). *M. fructicola* and *M. fructigena* show 96.6% sequence similarity with *Botrytis cinerea* in the ITS1 region, while *M. megalospora* and *M. oxycocci* are placed more closely to *Ciborinia erythonii* than to other *Monilinia* ssp. (Carbone and Kohn, 1993) on the phylogenetic tree.

Other sequences of *M. linhartiana* were not available for identification in the NCBI database. Not all isolates could be classified as *M. linhartiana* based on the ITS region, but all of them proved to be members of the section *Disjunctoriae* infecting rosaceous hosts that bear pome fruits. We believe that host specification and molecular evidence provide us with reliable species identification methods.

M. linhartiana shows high similarity with closely related species such as *M. aucupariae* and *M. padi*. The low rate of interspecific and intraspecific differences between *Monilinia* spp. (our data; Ioos and Frey, 2000, Hu *et al*., 2011; Volkova *et al*., 2013) suggests that the examination of the ITS region is not suitable for phylogenetic analysis. In our study, the analysis of the 28S rRNA gene clustered the species according to their hosts, which is the basis of classification in the section *Disjunctoriae*. We agree with the concept of Seifert *et al.* (1995), stating that for identification and phylogenetic study purposes, DNA analysis has to be combined with morphological studies as well. The analysis of the β-tubulin gene demonstrated that the gene sequences may be suitable for identification and phylogenetic purposes.

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