Monilinia spp. Causing Brown Rot of Stone Fruit in Serbia

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Abstract

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Brown rot is one of the most important pre- and postharvest fungal diseases of stone fruit worldwide. In Serbia, where production of stone fruit is economically important, *Monilinia laxa* and *M. fructigena* are widely distributed. In surveys from 2011 to 2013, 288 isolates of *Monilinia* spp. were collected from 131 localities in 16 districts and from six hosts in Serbia. Using multiplex polymerase chain reaction, phylogenetic analysis, and morphological characterization, three species of *Monilinia* were identified as the causal agents of brown rot of stone fruit: *M. laxa* (89% of isolates), *M. fructigena* (3%), and *M. fructicola* (8%). In 2011, *M. fructicola* was reported for the first time on stone fruit in Serbia, with only one isolate detected. More isolates of *M. fructicola* were detected in 2012 (2 isolates) and 2013 (20 isolates). The presence of *M. fructicola*, as well as its increased frequency of detection during the survey, may indicate a change in the population structure of these pathogens, which could have an important impact on brown rot disease management in Serbia.

Brown rot is one of the most important pre- and postharvest fungal diseases of stone fruit (*Prunus* spp.), with a worldwide distribution in all major fruit-growing areas. Under favorable weather conditions, it can cause severe losses in fruit production (31). Three *Monilinia* spp. that cause brown rot are economically important on stone fruit: *Monilinia fructigena* Honey, *M. laxa* (Aderh. & Ruhland) Honey, and *M. fructicola* (G. Winter) Honey (4,31).

Occurrence and distribution of *Monilinia* spp. differs significantly worldwide. *M. laxa* and *M. fructigena* are the primary species causing brown rot of stone fruit in Europe (5), whereas *M. fructicola* is widespread in stone fruit grown in the Americas and some parts of Africa and Asia (4). In Europe, *M. fructigena* causes preharvest and storage fruit rot of pome fruit (1) whereas, in the United States and Australia, it is a quarantine pathogen (29,50). In contrast, *M. laxa* is more economically important on stone fruit and almond (*Prunus dulcis*), causing mainly blossom and twig blight (4), although it has also been detected on pome fruit (26). Finally, *M. fructicola* is the most destructive species causing blossom and twig blight, as well as fruit rot (11). It is classified as an A2 European and Mediterranean Plant Protection Organization quarantine organism (http://www.eppo.int/QUARANTINE/listA2.htm), and regulated in Annex IV, Part A, Section I of Council Directive 2000/29/EC4. However, over the last 10 years, this species has been detected in several European countries. For example, recent reports of this pathogen came from France (28), Hungary (34), the Czech Republic (8), Italy (33), Spain (7), Switzerland (3,15), Slovenia (25), Slovakia (32), Germany (30), Poland (37), and Serbia (17,46). Although several reports on the occurrence of *M. polystroma* have been published (35,37,45,51), its economic impact and significance is still to be established.

Monilinia spp. are reportedly able to infect fruit of their host plants only through wounds (16,41,44,49). There are a few reports of *M. fructicola* and *M. laxa* infecting intact fruit (38,41) but this important epidemiological issue still needs to be clarified.

Stone fruit species are by far the most important for total fruit production in Serbia in terms of number of fruit trees and quantity of produced fruit (27), with plum being the most important. Total annual plum production amounted to over 500,000 tons in the last five years, which places Serbia among the three top producers in the world and the first in Europe (9). However, plum and other stone fruit are severely affected by brown rot every year, resulting in significant losses (2). *M. laxa* and *M. fructigena* are widely distributed (2), with first reports of these fungi in Serbia and their significance originating from the middle of the 20th century (40). Both pathogens appear on stone fruit every year, causing high yield losses when rain coincides with bloom or fruit ripening. As a consequence of blossom blight and fruit rot, losses in stone fruit production are estimated to be up to 100% (2). In 2011, *M. fructicola,* a pest on the 1A part I list of quarantine pest organisms in Serbia, was detected on stored apple and nectarine fruit (17,46). The recent discovery of *M. fructicola* and its potential to develop fungicide resistance (23,24), suggests possible changes in population structure of brown rot fungi in Serbia that could affect stone fruit production. Therefore, the aims of this study were to (i) identify the *Monilinia* spp. occurring in six of the most commonly grown stone fruit species in Serbia using conventional morphological and molecular methods; (ii) determine the relative abundance of each *Monilinia* sp. for each stone fruit crop; and (iii) characterize selected isolates in terms of virulence, growth rate, sporulation ability, and other morphological and molecular features.

Materials and Methods

Surveys. In order to determine the presence and distribution of *Monilinia* spp. on stone fruit in Serbia, surveys were carried out from 2010 to 2013. Samples of mummified fruit, infected twigs, and rotted fruit of apricot (*P. armeniaca*), peach (*P. persica*), nectarine (*P. persica* var. *nectarina*), plum (*P. domestica*), sweet cherry (*P. avium*), and sour cherry (*P. cerasus*) were collected. Two to five samples were collected from each of 131 commercial orchards in 16 administrative districts of Serbia, depending on orchard size (0.2 to 2 ha), tree age (5 to 10 years), and presence and incidence of symptoms resembling those caused by *Monilinia* spp. In addition, some samples were collected from green markets and house yards.

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Fungal isolation. After collection, symptomatic plant samples were individually packed in paper bags, marked, and transported to the Laboratory for Applied Phytopathology at the Institute of Pesticides and Environmental Protection in Belgrade, where they were examined further. Plant samples were surface disinfected for 2 min with a commercial bleach solution (0.5% sodium hypochlorite), and small pieces (2 to 3 mm^2) from the border of healthy and diseased tissue from each sample were aseptically excised and placed onto the surface of potato dextrose agar (PDA; 200 g of potato, 20 g of dextrose, 17 g of agar, and 1 liter of H_2O). The plates were incubated at 24°C in

Table 1. Isolates of *Monilinia* spp. identified in this study and retrieved from the GenBank database, affected host plant, and GenBank accession numbers

Species, isolate	Host	Location	GenBank accession number
Monilinia laxa			
BPČO	Peach	Serbia	KC515383[y]
BPZK	Peach	Serbia	KC544793[y]
KPGG	Apricot	Serbia	KC544794[y]
KPGO	Apricot	Serbia	KC544795[y]
NPSV	Nectarine	Serbia	KC544796[y]
NPVI	Nectarine	Serbia	KC544797[y]
ŠPIV	Plum	Serbia	KC544798[y]
TPLČ	Sweet cherry	Serbia	KC544800[y]
TPRŠ	Sweet cherry	Serbia	KC544801[y]
VPJR	Sour cherry	Serbia	KC544802[y]
VPSV	Sour cherry	Serbia	KC544803[y]
MDA12	Unknown	United States	HQ846948[z]
BUL1A1	*Prunus insitita*	France	AF150676[z]
Hirodai number 3272	*P. mume*	Japan	AB125612
Sh675	Plum	Chile	EU042149
ES14	Unknown	Spain	EF153016[z]
CBS298.31	Unknown	United States	HQ846949[z]
1067.k	*Malus domestica*	Norway	Z73784
Hirodai number 2646	*P. mume*	Japan	AB125618
M. fructigena			
BŠPBA	Plum	Serbia	KC544804[y]
ŠPBA	Plum	Serbia	KC544805[y]
ŠPPR	Plum	Serbia	KC544806[y]
TPGO	Sweet cherry	Serbia	KC544807[y]
GENA4	Unknown	United Kingdom	HQ846945[z]
3FG	*Cydonia oblonga*	Hungary	AM937109
UASWS0643	*M. domestica*	Switzerland	HQ166417
IHEM	Pear	Belgium	FJ515296
40FG	*C. oblonga*	Hungary	AM937113
WASWS0333	*M. domestica*	Switzerland	EU098121
W17	Unknown	Spain	EF207424[z]
COY2M	*P. institia*	France	AF150680[z]
1079.k	*P. domestica*	Norway	Z73781
M. fructicola			
NPUD1	Nectarine	Serbia	KC544808[y]
NPUD2	Nectarine	Serbia	KC544809[y]
NPGM	Nectarine	Serbia	JX127303[y]
99.2.G5.04	Unknown	United States	DQ314730
Ft	Unknown	France	HQ846967[z]
LH01	Red bayberry	China	AM887528
W1	Unknown	Spain	EF207420[z]
Hirodai number 2636	*P. avium*	Japan	AB125615
M1PL	Unknown	Poland	JX312665[z]
782.k	*P. persica*	Norway	Z73777
M10020029	Peach	Slovenia	GU967379[z]
P164	Unknown	Italy	FJ411110[z]
THF-1	Peach	China	FJ515894
BHY1	Cherry	China	HQ846927[z]
SLT2	Peach	China	HQ846939[z]
NE18	Unknown	New Zealand	HQ846919[z]
LVN8	Unknown	United States	HQ846966[z]
DAOM231119	Wine grape	Canada	AY289185
Unknown	*M. domestica*	Serbia	JN176564[z]
M. polystroma			
HML3	*P. aitianli*	China	GU067539[z]
2319	Unknown	Japan	HQ856916[z]
UFT	*M. domestica*	Hungary	AM937114
AP1	*M. domestica*	Poland	JF820317
MP13	*M. domestica*	Serbia	JX315717[z]

[y] Sequences generated in this study.
[z] Sequences from peer-reviewed sources.

the dark and inspected after 3 to 5 days for gray, creamy yellow, or hazel zonate colonies with lobed or even margins, which are characteristic for *Monilinia* spp. Single-spore cultures were derived from each isolate and identified on the basis of their morphological and molecular features. Isolates were stored at −80°C in 20% glycerol for long-term storage, and at 4°C on PDA slants for short-term storage.

Morphological identification and characterization of *Monilinia* spp. isolates. For morphological characterization, 3-mm-diameter plugs of actively growing mycelium were cut from the edge of a 4-day-old colony on PDA and subcultured on fresh PDA. After 10 days of incubation at 22°C, colony color, colony margin appearance, rosetting pattern, sporulation, presence of concentric rings of spores, presence of black arcs (lines on substrate side of colonies), and qualitative growth rate were determined (20).

After identifying to species (see below), three representative isolates of each species from different host plants and districts of Serbia were selected for detailed characterization based on growth rate on three media: PDA, V8 agar (200 ml of V8 juice, 20 g of agar, and l liter of H_2O), and MALT (500 ml of industrial malt, 17 g of agar, and 500 ml of H_2O). Growth rate was calculated after 7 days of incubation at 24°C in darkness and expressed as millimeters per day. The mean value of three replicates was used to represent each isolate. Additionally, sporulation was quantified on MALT medium after incubation at 24°C in darkness. Ten-day-old cultures were flooded with 10 ml of sterile distilled water and conidia were gently removed from the media with a glass rod. The concentration of conidia was estimated using a hemocytometer, and the mean number of conidia was calculated from three replicate cultures. Conidial size, germ tube length, and morphology were also determined for each of the nine selected isolates. The suspension of conidia was spread onto water agar (WA; 17 g of agar and 1 liter of H_2O) with sterile cotton swabs and incubated at 24°C in the dark for 18 h. Germ tube

Table 2. Occurrence of *Monilinia* spp. on stone fruit in Serbia during 2010 to 2013

Year, districts	Number of fields[z]	Number of isolates	*Monilinia laxa*	*M. fructigena*	*M. fructicola*
2010					
South Bačka	1	2	2	0	0
Central Banat	1	1	1	0	0
Srem	5	8	7	1	0
City of Belgrade	2	4	3	1	0
Podunavlje	1	2	2	0	0
Subtotal = 5	10	17	15	2	0
2011					
Šumadija	1	1	0	0	1
Moravica	1	1	0	1	0
Jablanica	3	13	13	0	0
Subtotal = 3	5	15	13	1	1
2012					
South Bačka	4	11	11	0	0
South Banat	1	3	3	0	0
Srem	13	22	22	0	0
City of Belgrade	29	65	65	0	0
Mačva	2	2	2	0	0
Podunavlje	10	24	21	1	2
Braničevo	14	23	20	3	0
Moravica	7	20	19	1	0
Šumadija	10	19	19	0	0
Zlatibor	9	23	23	0	0
Rasina	2	3	3	0	0
Zaječar	1	1	1	0	0
Nišava	1	1	1	0	0
Subtotal = 13	103	217	210	5	2
2013					
City of Belgrade	4	8	6	0	2
Kolubara	2	3	0	0	3
Podunavlje	6	27	12	0	15
Zlatibor	1	1	1	0	0
Subtotal = 4	13	39	19	0	20
Total = 16	131	288	257	8	23

[z] Two to five samples of mummified fruit, infected twigs, and rotted fruit of apricot, peach, nectarine, plum, sweet cherry, and sour cherry were collected from each of 131 commercial orchards in 16 administrative districts of Serbia, depending on orchard size, tree age, and presence and incidence of symptoms resembling those caused by *Monilinia* spp.

Table 3. Comparison of morphological features of *Monilinia laxa*, *M. fructigena*, and *M. fructicola* isolates

Species	Number of isolates	Colony color	Colony shape[v]	Colony margin[w]	Sporulation[x]	Concentric ring of spores[y]	Black arcs[z]
M. laxa	257	Gray	−	L	−	−	+/−
M. fructigena	8	White/Yellow	R	E	+	+	+/−
M. fructicola	23	Gray	−	E	++	+	−

[v] Colony shape: R = rosette and − = not rosette.
[w] Colony margin: L = lobate and E = entire.
[x] Sporulation: + = abundant and − = sparse.
[y] Concentric ring of spores: + = present and − = absent.
[z] Black arcs: + = present and − = absent.

length was measured with a stage micrometer using a light microscope at ×100 magnification. At minimum, 50 conidia and germ tubes were measured for each isolate. All data were analyzed by analysis of variance (ANOVA) at the 5% probability level, with individual pairwise comparisons made using Tukey's test (39). Each experiment was performed two times, with three replicates for each isolate.

Molecular identification. Total genomic DNA was isolated from mycelia of 7-day-old cultures grown on PDA using a method described by Harrington and Wingfield (14).

Molecular identification of all 288 *Monilinia* spp. isolates was performed with multiplex polymerase chain reaction (PCR), using the common reverse primer MO368-5, which is specific for *Monilinia* spp., and three species-specific forward primers—MO368-8R for *M. fructigena* and *M. polystroma*, MO368-10R for *M. fructicola*, and Laxa-R2 for *M. laxa* (6)—in order to amplify a noncoding region of *Monilinia* spp. with unknown function.

The PCR mix contained 12.5 μl of 2× Master mix with 2 mM $MgCl_2$ (Fermentas Life Sciences GmbH, Lithuania), 1 μl of 0.2 μM each primer, 1 μl of template DNA, and molecular-grade water up to a final volume of 25 μl. PCRs were performed in an Eppendorf Master Cycler (Eppendorf, Germany) with the following reaction conditions: an initial denaturation at 95°C for 2 min; followed by 35 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 1 min; and a final extension at 72°C for 3 min. Negative controls were included by replacing template DNA with molecular-grade water. The PCR products were separated by electrophoresis in 2% agarose gels run in 1× Tris-borate EDTA buffer at 100 V constant voltage. The gels were stained with ethidium bromide and the products were visualized and photographed under UV light.

Sequencing of ribosomal DNA internal transcribed spacer region. The identity of a subset of 18 *Monilinia* spp. isolates was further confirmed by amplification and sequencing of the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) using primers ITS1 and ITS4 (48) and the same reaction mixture as described above. PCR amplifications were performed with an initial denaturation for 90 s at 94°C, followed by 29 cycles consisting of a denaturation step for 30 s at 94°C, primer annealing for 30 s at 55°C, and extension for 30 s at 72°C. The final extension step was performed at 72°C for 9 min 30 s (48).

Amplified products were purified using the mi-PCR Purification Kit (Metabion International, Germany), according to the manufacturer's instructions, and sequenced directly on automated equipment (Macrogen Inc., Korea) in both directions using the same primers as for amplification. For each isolate, the consensus sequence covering the partial rDNA-ITS region was reconstructed using the ClustalW program (43) and deposited in GenBank (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences of Serbian *Monilinia* spp. isolates were compared with the respective sequences available in the GenBank using the ClustalW program (43) and MEGA5 software (42).

Phylogenetic analysis. A phylogenetic tree was reconstructed based on 18 ITS sequences generated in this study and 38 sequences representing *M. fructicola*, *M. laxa*, *M. fructigena*, and *M. polystroma* isolates from different hosts and geographic origins retrieved from GenBank (Table 1) using the Maximum Parsimony algorithm implemented in MEGA5. The tree was evaluated with 1,000 bootstrap replications to test clade stability, and bootstrap values <50% were omitted. Sequences of both *Botryotinia fuckeliana* (GenBank accession number HQ846943) and *Sclerotinia sclerotiorum* (HQ846942) were used as outgroup reference species.

Pathogenicity test and reisolation. Pathogenicity of all isolates of *M. laxa* (n = 257), *M. fructigena* (n = 8), and *M. fructicola* (n = 23) was confirmed on wounded fruit of the originating host plant. The fruit were surface disinfected with 0.5% NaClO for 5 min, rinsed three times with sterile-distilled water, and air dried. Each fruit was wounded with a carpenter nail (4 mm in diameter and 3 mm in depth), and a mycelial plug (3 mm in diameter) from the margin of a 6-day-old colony grown on PDA was placed on each wound. Five fruit were used for each isolate. Fruit inoculated with sterile PDA plugs served as a negative control. Inoculated fruit were incubated for 3 days in randomly arranged separate plastic containers at 24°C and 97% relative humidity (RH) in darkness. Inoculated fruit were inspected daily for the occurrence of brown rot symptoms. After the appearance of disease symptoms in the inoculated fruit, the pathogen was reisolated on PDA. Then, the morphological features of the isolated fungi were compared and matched with the original ones used for inoculation. The experiment was repeated twice.

Pathogenicity on nonwounded fruit. Pathogenicity of three selected representative isolates (one *M. fructigena*, one *M. laxa*, and one *M. fructicola*) was tested on nonwounded mature fruit of six stone fruit species: nectarine, peach, apricot, sour cherry, sweet cherry, and plum. Mycelial plugs of all three isolates, prepared using the previously described procedure, were placed upside down on the intact cuticle of the surface-sterilized fruit. For each isolate and each fruit species, five fruit were used. Fruit inoculated with sterile PDA plugs served as a negative control. Occurrence of brown rot symptoms and sporulation was observed after incubation at 24°C and 97% RH in darkness for 7 days.

Virulence to different host plants. The virulence of selected representative isolates of *M. fructigena* (four isolates), *M. laxa* (four isolates), and *M. fructicola* (one isolate) originating from different fruit species was tested on mature wounded fruit of the originating host plant as well as other stone fruit, including nectarine, peach, apricot, and plum. Pathogenicity tests were conducted as described above. In all, 5 fruit in three replicates (total of 15 fruit) for each host plant and isolate were used. The lesion size was measured 3 days postinoculation, and the values [(width + length)/2] were calculated. The experiment was repeated twice and results were analyzed for effects of isolate in each fruit species separately by ANOVA at the 5% probability level, with individual pairwise comparisons made using Tukey's test (39).

Results

Presence and distribution of brown rot fungi in Serbia. During the 4-year survey, infected plant parts showing characteristic symptoms were collected from 131 fields (Table 2). In total, 288 isolates resembling *Monilinia* spp. were recovered: 232 isolates from fresh fruit, 45 from mummified fruit, and 11 from twigs. *M. laxa* was by far the most frequently isolated species from mummified fruit and

Table 4. Colony growth rate and sporulation of *Monilinia laxa, M. fructigena*, and *M. fructicola* isolates[y]

	Colony growth rate (mm/day) on[z]					
	PDA		V8 juice agar		MALT	
Species	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
M. laxa	8.7 ± 0.4b	8.0–9.1	6.9 ± 0.2a	6.6–7.1	12.0 ± 0.2a	11.9–12.3
M. fructigena	2.6 ± 0.4c	2.3–3.3	3.7 ± 0.2b	3.4–4.0	11.9 ± 0.2a	11.4–12.1
M. fructicola	11.4 ± 1.0a	10.1–12.4	2.9 ± 0.4c	2.6–3.6	8.8 ± 0.7b	8.1–10.3

(*continued on next page*)

[y] The same letter within the column indicates that the difference is not significant. Mean value ± standard deviation (SD).
[z] PDA = potato dextrose agar and MALT = malt extract agar.

twigs. *M. laxa* occurred in all but the Kolubara district, *M. fructigena* was found in 5 districts, and *M. fructicola* was detected in 4 of the 16 sampled districts. All isolates were identified using both morphological characteristics and molecular methods.

Morphological identification and characterization of *Monilinia* spp. isolates. On PDA, 257 isolates that were light to dark gray with lobed margins and no sporulation were identified as *M. laxa*. Eight isolates with cream to yellow colonies, rare sporulation, and entire margins were identified as *M. fructigena*. Twenty-three of 288 isolates that developed hazel-colored zonate colonies with more or less even margins and abundant sporulation, and concentric rings of spores on the surface were identified as *M. fructicola* (Table 3). Of 257 isolates of *M. laxa*, 44 originated from mummified fruit, 202 from fresh fruit, and 11 from twigs. A total of 6 isolates of *M. fructigena* and 18 isolates of *M. laxa* developed black arcs on the undersurface of the culture; none of the isolates of *M. fructicola* exhibited this feature. One of eight *M. fructigena* isolates was derived from mummified and seven from fresh fruit. All isolates of *M. fructicola* originated from fresh fruit.

In-depth study of the nine representative isolates of *Monilinia* spp. show that growth rates on PDA, MALT, and V8 juice medium differed significantly among isolates and species as well as isolates from the same species ($P < 0.05$; Table 4). On PDA, *M. fructicola* had the highest growth rate (10.1 to 12.4 mm/day) and *M. fructigena* had the lowest (2.3 to 3.3 mm/day). On MALT medium, *M. fructigena* (11.4 to 12.1 mm/day) and *M. laxa* (11.9 to 12.3 mm/day) grew significantly ($P < 0.05$) faster than *M. fructicola* (8.1 to 10.3 mm/day). In general, growth rates of *M. laxa* and *M. fructigena* isolates were faster on MALT medium compared with PDA and V8 juice, whereas growth rates of *M. fructicola* was greatest on PDA medium.

The conidia of *M. fructigena* were 22.0 μm long (20.0 to 25.0 μm) and 13.0 μm wide (10.0 to 15.0 μm), *M. laxa* were 11.2 by 8.2 μm (7.5 to 12.5 by 5.0 to 10.0), and *M. fructicola* were 16.0 by 10.7 μm (12.5 to 17.5 by 7.5 to 12.5). Conidia of *M. fructigena* germinated into multiple germ tubes, whereas *M. laxa* and *M. fructicola* consistently germinated into one germ tube. *M. laxa* was also distinguished from *M. fructigena* and *M. fructicola* by the characteristic short-distance germ tube elongation from the conidium to the first germ tube branch (less than 60 μm). Germ tube elongation for *M. fructicola* and *M. fructigena* was 365 and 197 μm, respectively. The most abundant sporulation was recorded for *M. fructicola* (3.0×10^4), followed by *M. fructigena* and *M. laxa* (1.5×10^4 and 0.3×10^4, respectively) (Table 4).

PCR identification. Further confirmation of *M. laxa*, *M. fructigena*, and *M. fructicola* on stone fruit in Serbia was done by using multiplex PCR. All primers were able to amplify one clear band of predicted size of 535 bp for *M. fructicola*, 402 bp for *M. fructigena*, and 352 bp for *M. laxa*, providing preliminary identification of 257 isolates as *M. laxa*, 8 isolates as *M. fructigena*, and 23 isolates as *M. fructicola*.

Sequence analysis and phylogeny of brown rot fungi. BLAST analysis showed that the ITS sequence of 11 Serbian *M. laxa* isolates had 100% nucleotide identity with GenBank *M. laxa* sequences, and sequences of 4 *M. fructigena* isolates had 100% nucleotide identity to *M. fructigena* from other parts of the world. Sequences of three Serbian *M. fructicola* isolates were identical to each other and to 17 isolates of *M. fructicola* from GenBank originating from different parts of the world, including 4 from Europe (FJ411109, FJ411110, GU967379, and JN176564). Comparison of ITS sequences revealed 100% nucleotide identity among isolates of the same species, except for the sequence of VPSV (*M. laxa*) isolate derived from sour cherry sampled in Slankamenački Vinogradi, which showed difference in one nucleotide (99.8% identity) from other *M. laxa* isolates obtained in this study.

A maximum parsimony tree, reconstructed using the ITS sequence of 54 *Monilinia* isolates, revealed the presence of two main clusters, each containing two well-supported disjunct groups (Fig. 1). In one clade, isolates of *M. laxa* originating from the Americas (United States and Chile), Asia (Japan), and Europe (France, Norway, and Spain) and 11 Serbian *M. laxa* isolates were present in one group, and 3 *M. fructicola* isolates from Serbia and 16 from Japan, New Zealand, the United States, Canada, China, Spain, Norway, Slovenia, Poland, France, Italy, and Serbia (from stored apple fruit JN176564) were present in the other group. In the second clade, *M. polystroma* isolates from Hungary, Poland, Serbia, and Asia occurred in one group, and four isolates of *M. fructigena* from Serbia obtained in this study (BŠPBA, ŠPBA, ŠPPR, and TPGO) together with nine isolates of *M. fructigena* from Europe (Switzerland, Norway, Belgium, the United Kingdom, Hungary, Spain, and France) were in the second group.

Species composition of brown rot fungi in Serbia. Based on morphological characteristics, molecular identification, and ITS sequence analysis, 89% of isolates obtained in this study were *M. laxa*, 3% were *M. fructigena*, and 8% were *M. fructicola. M. fructicola* was isolated in the second (1 isolate), third (2 isolates), and fourth (20 isolates) year of the survey. *M. laxa* was detected in all years, on all hosts, and in all surveyed districts of Serbia, and it was isolated from all plant parts studied. Seven of eight *M. fructigena* isolates were found on fresh fruit of plum and sour and sweet cherry from central parts of the country (Belgrade City, Podunavlje, Braničevo, Šumadija, and Moravica). In addition, one isolate of *M. fructigena* was derived from mummified plum fruit originating from the Moravica region in central Serbia. *M. fructicola*, a quarantine pathogen in Serbia, was detected on fresh fruit of nectarine, apricot, plum, and peach in four regions in central Serbia (Šumadija region, Podunavlje, Kolubara, and Belgrade City) (Table 2).

Pathogenicity test and reisolation. After 3 days of incubation on inoculated fruit, all *Monilinia* spp. isolates caused brown rot symptoms on their respective host species. Koch's postulates were fulfilled by successful reisolation of the pathogen from all inoculated fruit. Colony morphology of the original and recovered isolates was identical.

Pathogenicity on nonwounded fruit. Artificial inoculation of nonwounded mature fruit of nectarine, peach, apricot, sour cherry, sweet cherry, and plum with selected isolates of all three *Monilinia* spp. resulted in the development of brown rot symptoms in all fruit–isolate combinations. However, sporulation on inoculated fruit was different among *Monilinia* spp.; *M. laxa* and *M. fructigena* isolates did not sporulate on any of the hosts, whereas *M. fructicola* produced intense sporulation on all inoculated fruit.

Virulence to different host plants. After 3 days of incubation, all inoculated wounded fruit of four stone fruit species tested showed typical brown rot symptoms, whereas none of the fruit inoculated with sterile agar plugs developed symptoms. Artificial inoculation

Table 4. (*continued from previous page*)

Conidial size (μm)		Germ tube length (μm)			
Mean	Range	Mean	Range	Number of conidia (cm^2)	Germination (%)
11.2 × 8.2	7.5–12.5 × 5–10	40	20–70	0.3×10^4c	88.0
22.0 × 13.0	20–25 × 10–15	197	70–800	1.5×10^4b	83.3
16.0 × 10.7	12.5–17.5 × 7.5–12.5	365	210–600	3.0×10^4a	88.7

of wounded stone fruit revealed significant differences in virulence among isolates of *Monilinia* spp., as well as among isolates of the same species ($P < 0.01$). *M. fructigena* was most destructive on peach and nectarine fruit, whereas two different *M. laxa* isolates (BMLE and VGRSE) were most virulent on plum and apricot, regardless the original host of the isolate that was used for inoculation. Thus, on nectarine and peach fruit, the highest virulence was recorded for the isolate of *M. fructigena* originated from plum (ŠPPR) whereas, on plum fruit, the largest necrotic zone was caused by the *M. laxa* isolate from peach (BMLE). On apricot fruit, isolates of *M. laxa* caused

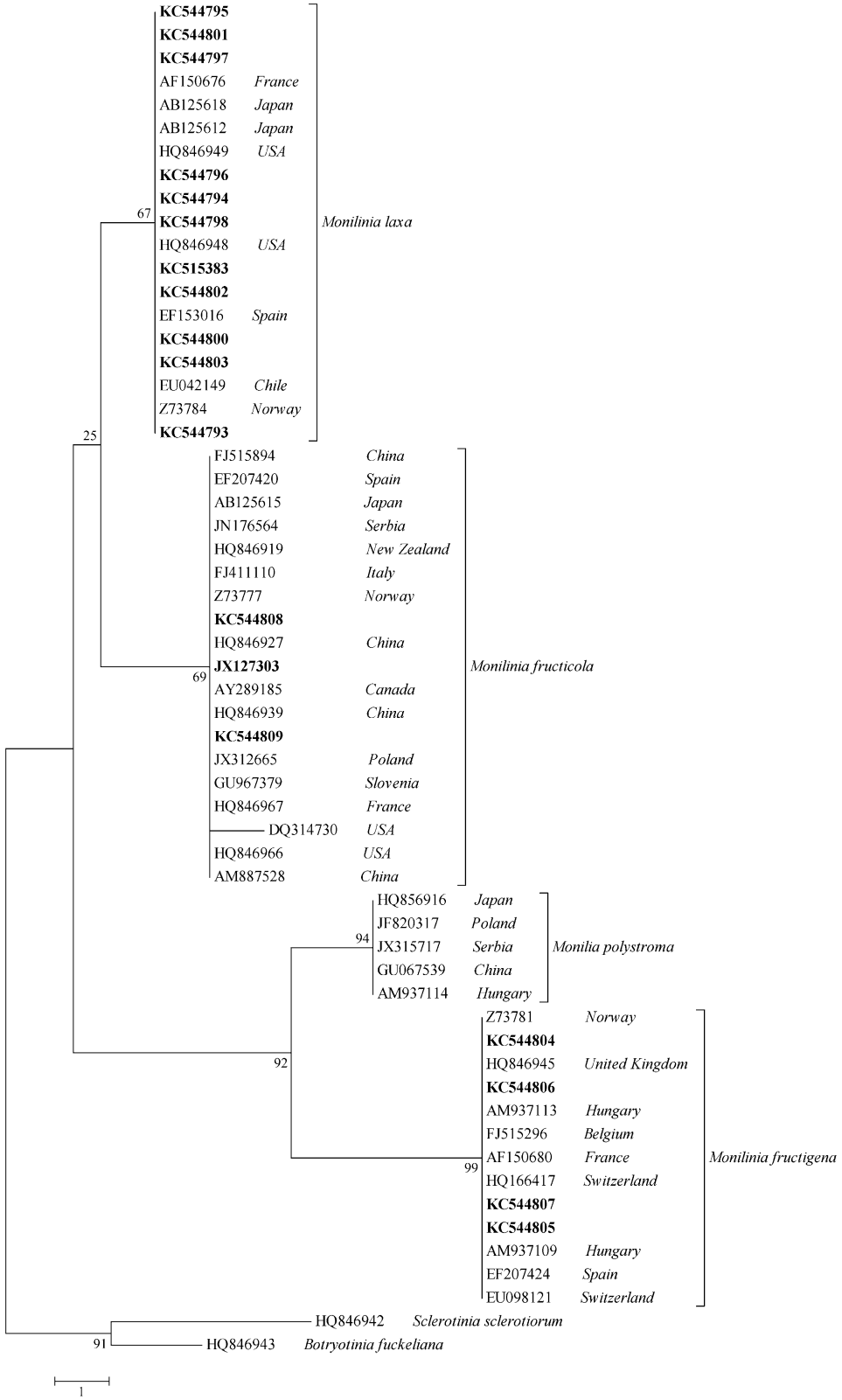

Fig. 1. Phylogenetic relationships among *Monilinia laxa*, *M. fructigena*, and *M. fructicola* isolates obtained from DNA isolated from stone fruit from Serbia, based on nuclear ribosomal DNA internal transcribed spacer (ITS) region. The tree was constructed using the maximum parsimony algorithm, with the *Botryotinia fuckeliana* and *Sclerotinia sclerotiorum* ITS sequences as outgroup. Bootstrap values are shown above or below branches. The numbers near each branch represent percentages out of 1,000 bootstrap replications. Bold accession number were sequenced in this study and others were retrieved from GenBank.

the largest (VGRSE) and smallest (ŠPSV) necrotic zones. None of the isolates, however, originated from apricot (Fig. 2).

Discussion

Until 2011, the general belief was that *M. laxa* and *M. fructigena* were widely distributed on stone fruit in Serbia and the only fungi causing brown rot. In 2011, *M. fructicola* was detected for the first time in Serbia, first on apple (46) and later in the same year on nectarine (18). Significant changes in population structure of *Monilinia* spp. were recorded in California (23), and in some European Union countries after the introduction of *M. fructicola* (19,36,37). Studies on the diversity of *Monilinia* spp. on stone fruit in Serbia were undertaken because changes in species prevalence and distribution are expected to influence disease epidemiology, cause greater yield losses, change efficacy of fungicides, and raise the cost of control measures.

In most European countries, *M. laxa* and *M. fructigena* are the predominant species (26,36,37). Poniatowska et al. (37) reported that *M. fructigena* was the most frequently isolated species in Poland (60%), followed by *M. laxa* (26%) and *M. fructicola* and *M. polystroma* (4%). Recently, *M. fructicola* was detected in Hungary on peach fruit imported from Italy and Spain (34,36). In the 1950s, *M. laxa* was the most common brown rot species in California. However, according to Michailides et al. (23), when benomyl resistance in *M. fructicola* increased, the later species became the most prevalent (22). *M. fructicola* is also the most prevalent brown rot fungus on stone fruit in China (93%), followed by *M. fructigena* (5%), *M. laxa* (2%), and *M. polystroma* (<1%) (50). Our research suggests that a similar situation occurs with *Monilinia* spp. in Serbia. Among 288 isolates from 131 different locations and six hosts, three species of *Monilinia* were found, indicating a more complex species composition than was previously recognized. Among the *Monilinia* isolates recovered from necrotic twigs, rotted, and mummified fruit collected in stone fruit orchards in Serbia from 2010 to 2012, *M. laxa* was by far the most frequently isolated species (96%) compared with *M. fructigena* (3%), although previous data indicated its prevalence on stone fruit during the 1950s (40). Stojanović and Kostić (40) detected only *M. fructigena* and *M. laxa* at 59 and 41% incidence, respectively. Our data showed that, during a 50-year period, the population structure of *Monilinia* has changed significantly, with *M. laxa* becoming the most prevalent species in Serbia. However, in 2011, the presence of *M. fructicola* on stone fruit in Serbia was reported for the first time, with only one isolate detected (17). In further investigations, more isolates of *M. fructicola* were detected (2 in 2012 and 20 in 2013). The presence of *M. fructicola*, as well as its apparent increased detection during the survey, indicates that a change in the population structure of these pathogens may be occurring. This change could have an important impact on disease management, making it more difficult.

Accurate and timely identification and early detection is the cornerstone of successful disease management. Identification of *Monilinia* spp. used to be based on the host plant, symptoms, morphology, and cultural features (4). All three *Monilinia* spp. cause similar brown rot symptoms on their hosts and cannot be reliably distinguished based on symptoms alone. Therefore, precise identification to the species level is possible by combining different identification methods. Among recommended methods, morphological identification based on colony characters on PDA according to the key described by Lane (20) is the simplest and the most convenient way, which was confirmed by these studies. However, morphological characterization is time consuming, affected by environmental conditions, and not always reliable for accurate species discrimination (21,36). For these reasons, molecular methods have been developed to accurately identify *Monilinia* spp. (6,10,12,18,44); thus, primers and protocols described by Côté et al. (6) were tested in this research. The methods proved to be precise, fast, reliable, and easy to follow in a single-tube reaction. Using this protocol, the morphological identification of all 288 isolates of three *Monilinia* spp. was successfully confirmed.

For *Monilinia* spp., the ITS region is highly conserved and there are only a few nucleotide differences among these species (21). Phylogenetic analysis using this region of 18 isolates confirmed the close relationship between *M. fructicola* and *M. laxa*, and between *M. fructigena* and *M. polystroma*, as reported by Poniatowska et al. (37). As expected, all *M. laxa* and *M. fructicola* isolates clustered into main group I, whereas *M. polystroma* and *M. fructigena* isolates were clustered into main group II. The results also showed that the isolates of *M. laxa, M. fructigena*, and *M. fructicola* from Serbia did not differ from respective isolates originating worldwide.

There are conflicting opinions in the literature regarding modes of fruit penetration and infection establishment of *Monilinia* spp. Gibert et al. (13) and Sződi et al. (41) stated that *M. laxa* can infect fruit through wounds only. The results obtained by Sződi et al. (41) show that only *M. fructicola* is capable of infecting nonwounded fruit, whereas Holb (16) and Xu and Robinson (49) reported that *M. fructicola* can penetrate the fruit only through wounds. In contrast, Rungjindamai et al. (38) established that *M. fructicola* and *M. laxa* can infect both wounded and nonwounded fruit, whereas *M. fructigena* can infect

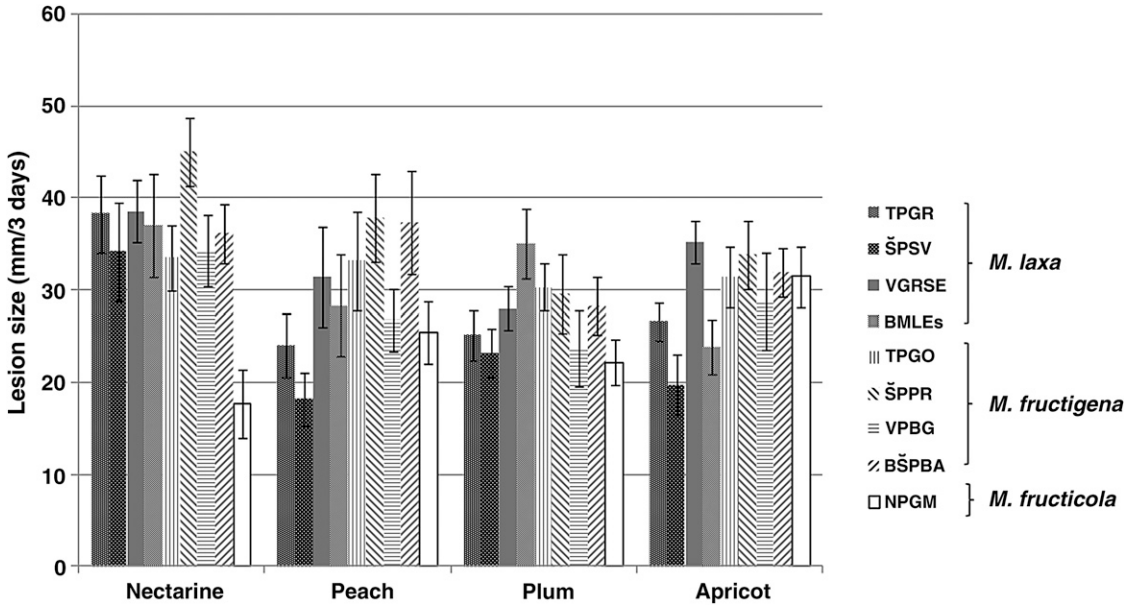

Fig. 2. Virulence of *Monilinia laxa*, *M. fructigena*, and *M. fructicola* on fruit of different stone fruit species.

only wounded fruit. Our study showed that all isolates of all three *Monilinia* spp. from Serbia were capable of infecting both wounded and nonwounded fruit. In addition, virulence of *Monilinia* spp. isolates on wounded fruit from different hosts showed that the origin of the isolate regarding host did not influence the level of isolate's virulence. Therefore, it can be expected that all investigated *Monilinia* spp. can spread from one host to the other easily, which should be taken into consideration during orchard establishment.

Using the multiplex PCR technique, phylogenetic analysis, and morphological characterization, three species (*M. laxa*, *M. fructigena*, and *M. fructicola*) were identified as the causal agents of brown rot of stone fruit in Serbia. This research revealed that *M. laxa* is the prevalent species (89%) and that it has almost completely displaced *M. fructigena* (3%). However, after the introduction and establishment of *M. fructicola*, this situation may change. The potential for *M. fructicola* to become the prevalent brown rot pathogen has been proven in California, where it partly displaced *M. laxa* in prune- and apricot-growing areas during the last several decades (22,23). Based on the limited number of *M. fructicola* isolates obtained in this study, it is difficult to estimate a possible impact of this new species on fruit production in Serbia. The risk of its spreading may be high because it is associated mainly with its adaptive capacity, widespread presence of susceptible host plants, and human activity. *M. fructicola* also exhibits higher growth and sporulation capability as well as a higher level of virulence in comparison with *M. laxa* and *M. fructigena* (47), and may be able to displace them in the orchards. Additionally, international trade can be responsible for spreading *M. fructicola* (19), raising the level of concern. Stone fruit imported into Switzerland from the United States and France tested positive for *M. fructicola* (3), and peach fruit imported into Hungary from Spain and Italy were positive for *M. fructicola* (34). Therefore, future population structure changes, especially the spread of *M. fructicola*, should be managed with special attention and in accordance with all available quarantine and control measures.

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Literature Cited

1. Anonymous. 2004. *Monilinia fructigena* (brown rot). In: Crop Protection Compendium. CAB International, Wallingford, UK.
2. Balaž, J. 2000. *Monilia* spp. kao parazit voćaka. Biljni lekar. 2-3:155-162. (In Serbian)
3. Bosshard, E., Hilber-Bodmer, M., Scharer, H.-J., Bunter, M., and Duffy, B. 2006. First report of the quarantine brown rot pathogen *Monilinia fructicola* on imported stone fruits in Switzerland. Plant Dis. 90:1554.
4. Byrde, R. J. W., and Willettes, H. J. 1977. The Brown Rot Fungi of Fruit: Their Biology and Control. Pergamon Press, Oxford.
5. CABI. 2010. Crop Protection Compendium. Commonwealth Agricultural Bureau International (CABI), Wallingford, UK.
6. Côté, M. J., Tardif, M. C., and Meldrum, A. J. 2004. Identification of *Monilinia fructigena, M. fructicola, M. laxa*, and *Monilia polystroma* on inoculated and naturally infected fruit using multiplex PCR. Plant Dis. 88:1219-1225.
7. De Cal, A., Gell, I., Usall, J., Vinas, I., and Melgarejo, P. 2009. First report of brown rot caused by *Monilinia fructicola* in peach orchards in Ebro Valley, Spain. Plant Dis. 93:763.
8. Duchoslavová, J., Širucková, I., Zapletalová, E., Navrátil, M., and Šafárová, D. 2007. First report of brown rot caused by *Monilinia fructicola* on various stone and pome fruits in the Czech Republic. Plant Dis. 91:907.
9. FAOSTAT.2011. Food and Agriculture Organisation of the United Nations. Online publication. http://faostat3.fao.org/home/index.html
10. Förster, H., and Adaskaveg, J. E. 2000. Early brown rot infections in sweet cherry fruit are detected by *Monilinia*-specific DNA primers. Phytopathology 90:171-178.
11. Fulton, C. E., van Leeuwen, G. C. M., and Brown, A. E. 1999. Genetic variation among and within *Monilinia* species causing brown rot of stone and pome fruits. Eur. J. Plant Pathol. 105:495-500.
12. Gell, I., Cubero, J., and Melgarejo, P. 2007. Two different PCR approaches for universal diagnosis of brown rot and identification of *Monilinia* spp. in stone fruit trees. J. Appl. Microbiol. 103:2629-2637.
13. Gibert, C., Chadoeuf, J., Nicot, P., Vercambre, G., Genard, M., and Lescourret, F. 2009. Modelling the effect of cuticular crack surface area and inoculum density on the probability of nectarine fruit infection by *Monilinia laxa*. Plant Pathol. 58:1021-1031.
14. Harrington, T. C., and Wingfield, B. D. 1995. A PCR-based identification method for species of *Armillaria*. Mycologia 87:280-288.
15. Hilber-Bodmer, M., Bünter, M., and Patocchi, A. 2010. First report of brown rot caused by *Monilinia fructicola* on apricot in a Swiss orchard. Plant Dis. 94: 643.
16. Holb, I. J. 2006. Possibilities of brown rot management in organic stone fruit production in Hungary. Int. J. Hortic. Sci. 12:87-91.
17. Hrustić, J., Tanović, B., Mihajlović, M., Delibašić, G., Stanković, I., Krstić, B., and Bulajić, A. 2013. First report of brown rot caused by *Monilinia fructicola* on nectarine in Serbia. Plant Dis. 97:147.
18. Ioos, R., and Frey, P. 2000. Genomic Variation within *Monilinia laxa, M. fructigena*, and *M. fructicola*, and application to species identification by PCR. Eur. J. Plant Pathol. 106:373-378.
19. Jansch, M., Frey, J. E., Hilber-Bodmer, M., Broggini, G. A. L., Weger, J., Schnabel, G., and Patocchi, A. 2012. SSR marker analysis of *Monilinia fructicola* from Swiss apricots suggests introduction of the pathogen from neighbouring countries and the United States. Plant Pathol. 61:247-254.
20. Lane, C. R. 2002. A synoptic key for differentiation of *Monilinia fructicola, M. fructigena* and M. laxa, based on examination of cultural characters. Bull. OEPP/EPPO Bull. 32:489-493.
21. Ma, Z., and Michailides, T. J. 2007. Approaches for eliminating PCR inhibitors and designing PCR primers for the detection of phytopathogenic fungi. Crop Prot. 26:145-161.
22. Michailides, T. J., Luo, Y., Ma, Z., and Morgan, D. P.2007. Brown rot of dried plum in California: new insight on an old disease. APSnet Feature Story. Online publication. http://www.apsnet.org/publications/apsnetfeatures/Pages/BrownRot.aspx
23. Michailides, T. J., Ogawa, J. M., and Opgenorth, D. C. 1987. Shift of *Monilinia* spp. and distribution of isolates sensitive and resistant to benomyl in California prune and apricot orchards. Plant Dis. 71:893-896.
24. Miessner, S., and Stammler, G. 2010. *Monilinia laxa, M. fructigena* and *M. fructicola*: Risk estimation of resistance to QoI fungicides and identification of species with cytochrome b gene sequences. J. Plant Dis. Prot. 117: 162-167.
25. Munda, A., and Viršček Marn, M. 2010. First report of brown rot caused by *Monilinia fructicola* affecting peach orchards in Slovenia. Plant Dis. 94:1166.
26. Muñoz, Z., Moret, A., and Bech, J. 2008. Morphological and molecular characterization of *Monilinia* sp. isolates and pathogenicity on apple. Agrociencia 42:119-128.
27. Nikolić, D., Keserović, Z., Magazin, N., Paunović, S., Miletić, R., Nikolić, M., and Milivojević, J. 2012. Pages 3-22 in: Stanje i perspektive razvoja voćarstva u Srbiji. Zbornik radova i apstrakata 14. kongresa voćara i vinogradara Srbije sa međunarodnim učešćem. Vrnjačka Banja (In Serbian)
28. OEPP/EPPO. 2002. First report of *Monilinia fructicola* in France. EPPO Reporting Service 2002/003. Online publication. http://archives.eppo.int/EPPOReporting/2002/Rse-0201.pdf
29. OEPP/EPPO. 2009. PQR database. European and Mediterranean Plant Protection Organization, Paris. Online publication. https://www.eppo.int/DATABASES/pqr/pqr.htm
30. OEPP/EPPO. 2010. First report of *Monilinia fructicola* in Germany. EPPO Reporting Service 2010/016. Online publication. http://archives.eppo.int/EPPOReporting/2010/Rse-1001.pdf
31. Ogawa, J. M., Zehr, E. I., and Bird, W. 1995. Brown rot. Pages 7-10 in: Compendium of Stone Fruit Diseases. J. M. Ogawa, E. I. Zehr, G. W. Bird, D. F. Ritchie, K. Uriu, and J. K. Uyemoto, eds. American Phytopathological Society, St. Paul, MN.
32. Ondejkova, N., Hudecova, M., and Bacigalova, K. 2010. First report on *Monilinia fructicola* in the Slovak Republic. Plant Prot. Sci. 46:181-184.
33. Pellegrino, C., Gullino, M. L., Garibaldi, A., and Spadaro, D. 2009. First report of brown rot of stone fruit caused by *Monilinia fructicola* in Italy. Plant Dis. 93:668.
34. Petroczy, M., and Palkovics, L. 2006. First report of brown rot caused by *Monilinia fructicola* on imported peach in Hungary. Plant Dis. 90:375.
35. Petróczy, M., and Palkovics, L. 2009. First report of *Monilia polystroma* on apple in Hungary. Eur. J. Plant Pathol. 125:343-347.
36. Petróczy, M., Szigethy, A., and Palkovics, L. 2012. *Monilinia* species in Hungary: morphology, culture characteristics, and molecular analysis. Trees 26:153-164.
37. Poniatowska, A., Michalecka, M., and Bielenin, A. 2013. Characteristic of *Monilinia* spp. fungi causing brown rot of pome and stone fruits in Poland. Eur. J. Plant Pathol. 135:855-865.
38. Rungjindamai, N., Jeffries, P., and Xu, X. M. 2014. Epidemiology and management of brown rot on stone fruit caused by *Monilinia laxa*. Eur. J. Plant Pathol. 140:1-17.
39. Sokal, R. R., and Rohlf, F. J. 1995. Biometry: The Principles and Practice of Statistics in Biological Research, 3rd ed. W. H. Freeman and Company, New York.
40. Stojanović, D., and Kostić, B. 1957. Prilog proučavanju vrsta *Monilia* na jabučastom i koštičavom voću. Zaštita bilja 44:69-72. (In Serbian)
41. Sződi, S. Z., Komjáti, H., and Turóczi, G. 2012. Characterization of *M. laxa* and *M. fructigena* isolates from Hungary with MP-PCR. Hortic. Sci. 39: 116-122.
42. Tamura, K., Peterson, D., Peterson, N., Steker, G., Nei, M., and Kumar, S. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum

likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28:2731-2739.
43. Thompson, J. D., Higgins, D. G., and Gibson, T. J. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22:4673-4680.
44. van Brouwershaven, I. R., Bruil, M. L., van Leeuwen, G. C. M., and Kox, L. F. F. 2010. A real-time (TaqMan) PCR assay to differentiate *Monilinia fructicola* from other brown rot fungi of fruit crops. Plant Pathol. 59:548-555.
45. Vasić, M., Duduk, N., and Ivanović, M. S. 2013. First report of brown rot caused by *Monilia polystroma* on apple in Serbia. Plant Dis. 97:145.
46. Vasić, M., Duduk, N., Ivanović, M. M., Obradović, A., and Ivanović, M. S. 2012. First report of brown rot caused by *Monilia fructicola* on stored apple in Serbia. Plant Dis. 96:456.
47. Villarino, M., Melgarejo, P., Usall, J., Segarra, J., and De Cal, A. 2010. Primary inoculum sources of *Monilinia* spp. in Spanish peach orchards and their relative importance in brown rot. Plant Dis. 94:1048-1054.
48. White, T. J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: PCR Protocols. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds. Academic Press, San Diego, CA.
49. Xu, X. M., and Robinson, J. D. 2000. Epidemiology of brown rot (*Monilinia fructigena*) on apple: infection of fruits by conidia. Plant Pathol. 49:201-206.
50. Zhu, X. Q., Chen, X. Y., and Guo, L. Y. 2011. Population structure of brown rot fungi on stone fruits in China. Plant Dis. 95:1284-1291.
51. Zhu, X. Q., and Guo, L. Y. 2010. First report of brown rot on plum caused by *Monilia polystroma* in China. Plant Dis. 94:478.