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Genotypic differentiation of *Monilinia* spp. populations in Serbia using a high-resolution melting (HRM) analysis

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Abstract: *Monilinia laxa*, *Monilinia fructicola* and *Monilinia fructigena* are the three main causal agents of brown rot, which is one of the most important diseases of stone fruits in pre- and postharvest conditions. Nowadays, the need for the precise genotyping of these *Monilinia* species in terms of the genetic diversity of their populations or differences in their pathogenicity and host range is a prerequisite for any efficient disease management. In our study, the genetic structure of *Monilinia* populations in Serbia from three geographically distinct regions was investigated employing a high-resolution melting (HRM) analysis which is a sensitive and rapid molecular approach in fungal genotyping and diagnostics. Using species-specific primer pairs genotype-specific HRM melting curve profiles were generated allowing to efficiently decipher the genetic diversity of the *Monilinia* populations. The *Monilinia* genotypes could be easily distinguished according to their melting curves. The isolates from the northern region were assigned to distinct genotypes and grouped rather independently compared to the isolates of the other two regions among all three tested *Monilinia* spp. *M. fructicola* and *M. fructigena* showed a higher genetic diversity among their populations (44%) compared with the genetic diversity among the *M. laxa* populations (7%). In contrast, the genetic variance within the pathogen populations was higher in the case of *M. laxa* (93%). Our data revealed an absence of host specificity in the *Monilinia* spp. populations.

Keywords: brown rot; population genotyping

Brown rot caused by *Monilinia* spp. is one of the most destructive diseases affecting the stone-fruit production worldwide (Ortega et al. 2019). *Monilinia laxa* (Aderhold & Ruhland) Honey, *Monilinia fructicola* (G. Winter) Honey, and *Monilinia fructigena* Honey are the three most widespread agents associated with brown rot (Côté et al. 2004; Wang et al. 2018). These species are differentially distributed around the world and their prevalence depends on the geographic region and host preference (Berrie & Holb 2014). *M. laxa* and *M. fructicola* are the most prevalent species in European countries, whereas *M. laxa* is considered an endemic species (Villarino et al. 2013). *M. fructigena*, instead, is more prevalent in pome fruits across Europe and Asia (Martini & Mari 2014). The economic losses caused by *Monilinia* spp. in orchards and storage

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facilities across Europe range from 7 to 25% and from 0.6 to 8%, respectively (Berrie & Holb 2014).

In Serbia, stone fruits are thoroughly cultivated all over the country. An intensive survey, conducted from 2010 to 2012, revealed that *M. laxa* was rather endemic and, by far, the most frequently isolated species (96%) (Hrustić et al. 2015). In 2011, the presence of *M. fructicola* on stone fruit in Serbia was detected for the first time (Hrustić et al. 2013). After the first detection, more isolates of *M. fructicola* were derived from different locations and from different stone fruit hosts, indicating the spread of the pathogen. The current brown rot management measures are mainly based on the usage of synthetic fungicides that are applied up to five times per growing season, depending on the host species, cultivars, locations, and weather conditions. However, the observed changes in the *Monilinia* spp. prevalence are expected to change the efficiency of the fungicides and raise the cost of the currently applied control measures (Hrustić et al. 2015).

An intensive knowledge-based disease management system is a key factor in preventing blossom blight and to reduce the risk of fruit infections. Genotyping the genetic diversity and structure of fungal populations in agricultural environments is of high importance to improve control strategies. Among the main factors causing changes in the genetic structure of fungal populations is the rapid evolution of novel and hybrid races that might quickly adapt themselves to environmental changes, and thereby overcome the resistance responses of the hosts, as well as developing resistance to fungicides (Stukenbrock 2016).

Different molecular markers, focused on specific genetic regions, such as a random amplified polymorphic DNA (RAPD) and a sequence characterised amplified region (SCAR) primers, as well as single sequence repeats (SSRs), have been deployed to assess the genetic diversity among *Monilinia* isolates (Côté et al. 2004; Miessner & Stammler 2010; Hily et al. 2011; Hu et al. 2011; Fan et al. 2014). Furthermore, different polymerase chain reaction (PCR) approaches have been developed to differentiate the *Monilinia* species (Ioos & Frey 2000; Gell et al. 2007a; Guinet et al. 2016; Wang et al. 2018). However, all of these molecular genotyping approaches are quite time-consuming and they involve laborious post-PCR analyses (Zambounis et al. 2015a).

Nowadays, a high-resolution melting (HRM) analysis is a molecular method that has been extensively used for DNA genotyping applications due to its high simplicity, sensitivity and specificity (Vossen et al. 2009). According to this technique, the double-stranded DNA fluorescence dissociation is measured using an intercalating dye. Consequently, the melting of the PCR products triggers specific changes in the fluorescence measurements and alters the shapes of the melting curve profiles. As a result, amplicon-specific melting curve profiles are generated representing the variation of the genotypes (Ganopoulos et al. 2012; Zambounis et al. 2016a). HRM has already been employed in the successful identification and differentiation of various pathogenic fungal species eliminating contamination risks during its deployment (Ganopoulos et al. 2012; Zambounis et al. 2015a, 2016a, b; Xanthopoulou et al. 2019). Additionally, this method has become increasingly applicable for the detection of sequence variations in plant fungal diagnostics (Zambounis et al. 2015b). In parallel, advances in molecular techniques have allowed the assessment of the genetic diversity and variation of *Monilinia* spp. and particularly of *M. laxa* and *M. fructicola* among their populations and hosts across European countries (Gell et al. 2007b; Gril et al. 2008; Fan et al. 2010; Jänsch et al. 2012; Villarino et al. 2012).

Since little is known about the genetic structure of the *Monilinia* spp. species in Serbia, the aim of this study was to genotype the populations of the *M. laxa*, *M. fructicola*, and *M. fructigena* isolates from various stone-fruit hosts across three geographic regions of the country using an HRM approach. This approach would also enable one to gain insights about the genetic diversity and the main sources of this variation for the three *Monilinia* spp. populations.

MATERIAL AND METHODS

Sample collection, fungal isolation and morphological identification of *Monilinia* spp. Samples of diseased fruits of apricot, peach, plum, sweet cherry, and sour cherry with distinct symptoms of brown rot, were collected from different commercial orchards located in the northern, central and southern parts of Serbia during the summer periods of 2015 up to 2019 (Table 1). The northern part is in the Vojvodina province, a flat area north of Sava and the Danube river, while the central and southern parts are hilly areas along the separated administrative regional provinces. The sampling locations across the three geographical regions of Serbia

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Table 1. *Monilinia* species, hosts of origin, and number of isolates per region used in the study

Monilinia spp.	Host	Regions (number of isolates)			Isolates abbreviations*
		southern	central	northern	
M. laxa	peach	2	1	1	PE
M. fructicola		ns	2	2	
M. fructigena		2	1	2	
M. laxa	plum	3	2	1	PL
M. fructicola		2	1	1	
M. fructigena		1	1	1	
M. laxa	apricot	1	ns	1	AP
M. fructicola		ns	1	2	
M. fructigena		ns	ns	ns	
M. laxa	sweet cherry	2	1	6	SwC
M. fructicola		ns	1	1	
M. fructigena		1	ns	ns	
M. laxa	sour cherry	2	1	2	SoC
M. fructicola		2	2	1	
M. fructigena		3	1	ns	

*Isolate abbreviations are according to their host origins: PE – peach; PL – plum; AP – apricot; SwC – sweet cherry; SoC – sour cherry ns – not sampled

are presented in a geographical map (supplementary Figure S1). The sampling fruits were individually packed in paper bags. Then, the fruits were surface sterilised for 2 min with a commercial bleach (0.5% sodium hypochlorite), and samples (2–3 mm^2) from the border of the healthy and diseased tissue were aseptically excised and placed onto the surface of a potato dextrose agar (PDA) medium. The plates were incubated at 24 °C in the dark and, after 3 to 5 days, the growth of grey, creamy yellow or hazel zonate colonies with lobed or even margins, was observed characteristic for *Monilinia* spp. A total of fifty-seven single spore isolates (26, 18 and 13 isolates for *M. laxa*, *M. fructicola*, and *M. fructigena,* respectively) were obtained (Table 1). The morphological characterisation was determined on the fresh PDA by adding plugs (3 mm diameter) from the edge of the colonies of the actively growing mycelia, 4 days after inoculation. After a 10-day-incubation at 22 °C, the colony colour, colony margin appearance, rosette pattern, sporulation, presence of concentric rings of spores, presence of black arcs (lines on the substrate side of the colonies), and qualitative growth rate were determined according to Lane (2002). Three previously identified isolates (*M. laxa*: KC544795, *M. fructigena*: KC544807, *M. fructicola*: JX127303) from the Collection of Phytopathogenic Fungi of the Institute of Pesticides and Environmental Protection (Belgrade, Serbia) were used as the reference strains for the morphological identification of the *Monilinia* species in this study. The pathogenicity tests of all the obtained isolates were performed on the wounded fruits of the originating hosts. Briefly, the inoculated fruits were incubated for three days in randomly arranged separate plastic containers at 24 °C and 97% relative humidity (RH) in darkness. The morphological features of the re-isolated fungi were matched with the original ones used for the inoculation. The experiment was repeated twice. All the isolates were stored at –80 °C in 20% glycerol for long-term storage.

DNA extraction. The total fungal genomic DNA was extracted using 20 mg of dry mycelia from the fresh subcultures of the 5–7 day-old cultures grown in a potato dextrose broth medium (PDB) using a DNeasy Plant Mini Kit (Qiagen, Germany) and following the manufacturer's instructions. The DNA quality and concentration were estimated on a NanoDrop spectrophotometer (Thermo Scientific, USA) and the final concentrations were adjusted to 20 ng/uL.

Genotyping with high-resolution melting analysis. The *Monilinia* spp. isolates were previously identified with species-specific primers as designed by Gell et al. (2007a). Thus, the primer pairs

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ILaxaS/ILaxaAS were selected to genotype the *M. laxa* isolates, the primer pairs IColaS/IColaAS were selected to genotype the *M. fructicola* isolates, and the primer pairs IGenaS/IGenaAS were selected to genotype the *M. fructigena* isolates. The primer sequences and amplicon lengths are shown in supplementary Table S1. The PCR reactions for the amplification of the genomic fragments of all *Monilinia* isolates with the species-specific primers were performed in 20 uL volumes containing 20 ng of the fungal genomic DNA, 2.5 mM of $MgCl_2$, 0.2 mM of each dNTP (deoxyribonucleotide triphosphate), 300 nM of each primer, 1.5 mM of the Syto®green fluorescent stain, and 1 U of the Kapa Taq DNA polymerase (Kapa Biosystems, Inc., South Africa). The PCR amplification, DNA melting and end point fluorescence measurements were performed on a Rotor-Gene 6000 real-time 5P HRM PCR Thermocycler (Corbett Research, Australia). A rapid PCR protocol was established for each species-specific primer pair and the PCR reactions were conducted at an initial denaturing step of 94 °C for 3 min, followed by 34 cycles of 94 °C for 30 s, annealing at 51 °C for 30 s and extension at 72 °C for 30 seconds.

The HRM analysis and the fluorescent data were acquired at the end of each extension step after the PCR amplification. The HRM analysis was performed at a temperature ramping from 72 to 99 °C, increasing by 0.1 °C increments every 2 seconds. The melting curves of all the samples, which represent the melting of the specific PCR amplicons, were normalised during the major fluorescence decrease. All the samples were plotted according to their normalised melting profiles (Hewson et al. 2009) and temperature shifted melting curves displayed as difference plots. The data for each PCR-HRM run were further interpreted by conventional genotype plots by assigning melting curves as reference genotypes. Particularly, the ML-1 reference genotype (for *M. laxa*) was assigned based on the melting curve profile of an isolate from the sweet cherry, while the MFC-1 (for *M. fructicola*) and the MFG-1 (for *M. fructigena*) reference genotypes based on the melting curve profiles of the respective isolates from the peach. All three isolates were sampled from the northern region of Serbia. The melting curve profiles of all the samples were assigned to a genotype and plotted within an average threshold of the genotype confidence percentage (GCP) value up to 80%. The reproducibility of the HRM method, the consistency of the melting profiles, as well the genotype-specific assignment of all the isolates among the *Monilinia* spp. runs were repeated three times for each species. The results were graphically displayed with the Rotor-Gene 6000 software (version 1.7), TeeChart Office. All the PCR products were visualised after electrophoresis in 1× Tris/Borate/EDTA (TBE) in 1.5% agarose gels to confirm their expected amplicon sizes. Finally, all the PCR amplicons were sequenced using the respective forward primers for each *Monilinia* spp.

In order to determine the genetic structure of each *Monilinia* spp. at the population hierarchical levels, an analysis of molecular variance (AMOVA) was performed along with the plotting of the isolate groups in the principal coordinates analysis (PCoA) using the GenAlEx software (version 6.502b) (Peakall & Smouse 2012). The standardised option for a binary data set which is based on the conversion of the distance matrix to a covariance matrix was followed by employing Nei's standard genetic distance between the populations.

RESULTS

In the present study, a total number of fifty-seven *Monilinia* spp. isolates was collected from all the studied host plants across northern, central and southern parts of Serbia (Table 1). After their morphological identification and pathogenicity confirmation, the PCR-HRM analysis allowed the accurate genotyping and differentiation of these isolates at a species level. All the HRM specific melting profiles consisted of one main defined peak. All the isolates were assigned to twenty distinct HRM genotypes using an average good clinical practice (GCP) threshold of 80% confidence to consider the melting profiles as identical genotyping matches. Thus, eight HRM genotypes were assigned for the *M. laxa* (ML-1 up to ML-8) isolates, six were assigned for the *M. fructicola* (MFC-1 up to MFC-6) isolates and six were assigned for the *M. fructigena* (MFG-1 up to MFG-6) isolates based on their HRM profiles after the implementation of the specific primer pairs. After normalisation and the temperature shift, a clear variation was evident among the HRM genotypes which were clearly discriminated each other sharing different melting curve peak values. This polymorphism was visualised using fluorescence difference plots, where the relative melting curves of the different genotypes were normalised upon their distinctive HRM profiles, facilitating the

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accurate identification of each genotype (supplementary Figure S2). Each of them was represented by a distinct melting curve profile, implying the existence of a high genetic diversity among the isolates. These results were emphasised by using the fluorescence difference plots, where the assigned genotypes were clustered around a baseline representing a reference genotype (supplementary Figure S3). The results were confirmed by sequencing all the PCR products, where different SNP variations were observed across the nucleotide alignments of the HRM genotypes for each *Monilinia* spp. (supplementary Figure S4).

Our results indicate that the HRM approach was capable of efficiently genotyping the three most economically important brown rot causal agents across the three geographic regions of Serbia. Particularly for *M. laxa*, all twenty-six isolates based on their HRM profiles were clustered in eight distinct ML genotypes, which were region-specific with each one containing isolates of the same region or being comprised of unique isolates (Figure 1A). The same clustering pattern of the isolates was not entirely maintained among the isolates of the other two *Monilinia* species. Thus, genotypes MFC-2 and MFG-2 mainly contained isolates from the southern region, but also contained isolates from the central region (Figures 1B and C).

According to the population distances matrices of the inter-population AMOVA analysis for each *Monilinia* species, there were significant ($P < 0.001$) genetic differences in the populations (supplementary Table S2). Particularly, a high percentage of variation due to the population subdivision (93%) was observed for *M. laxa*, while only 7% of the total genetic diversity was attributed to the among-population differentiation. In contrast, the AMOVA revealed a reduction in the percentage of the variation due to the population subdivision (56%), and the increase in the total diversity was attributed to the among-population differentiation (44%) for *M. fructicola* and *M. fructigena* compared to those of the *M. laxa* species (supplementary Table S2). The genetic relationships were further validated by the principal coordinate analysis (PCoA), in which, the individual distance matrices were used as an input for each *Monilinia* spp. (Figure 1). In all the cases among the three *Monilinia* species, the isolate populations from the northern region were rather more distant from the populations of both the southern and central regions.

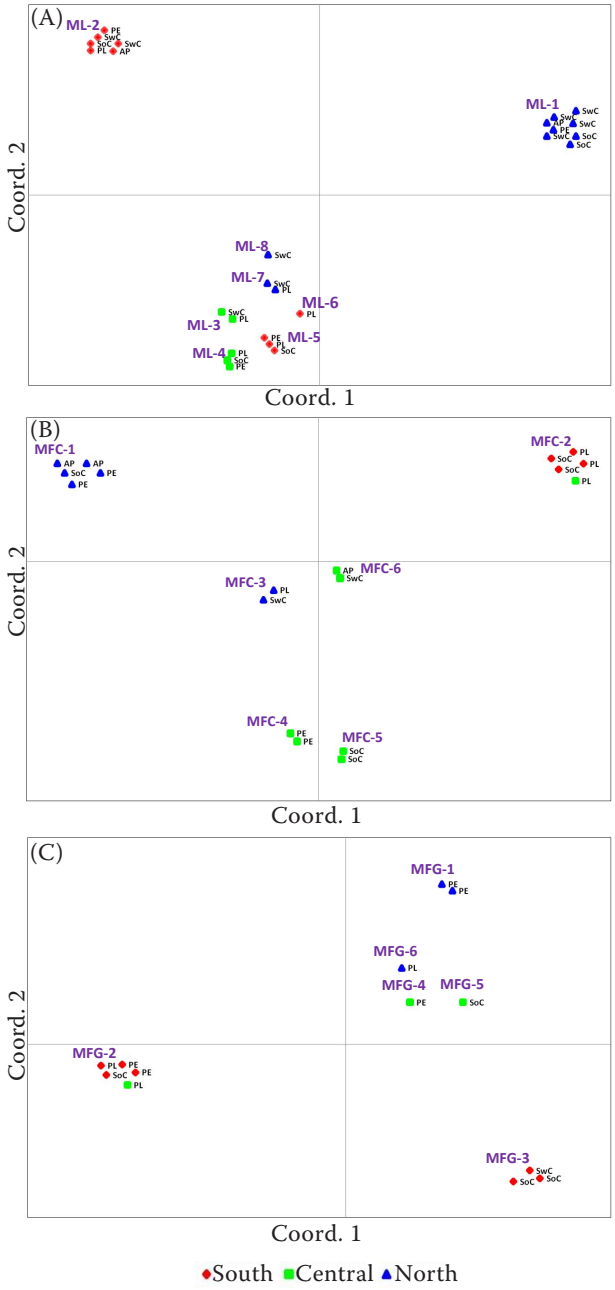

Figure 1. Principal coordinate analysis (PCoA) plots revealing the genetic relationships of the three populations (southern, central and northern Serbia) of *Monilinia* spp. based on the HRM genotyping analysis

Twenty species-specific HRM genotypes are depicted (ML-1 up to ML-8, MFC-1 up to MFC-6, and MFG-1 up to MFG-6) in the colour purple; the isolates are represented according to their host origins; (A) *M. laxa*, 54.69% of the total variance accumulated on the first two components (axis 1 = 33.42%, axis 2 = 21.27%); (B) *M. fructicola*, 66.48% of the total variance accumulated on the first two components (axis 1 = 35.71%, axis 2 = 30.77%); (C) *M. fructigena*, 65.1% of the total variance accumulated on the first two components (axis 1 = 40.63%, axis 2 = 24.47%); PE – peach; PL – plum; AP – apricot; SwC – sweet cherry; SoC – sour cherry

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DISCUSSION

Brown rot is one of the most destructive diseases of stone fruits in Serbia (Vasić et al. 2012; Hrustić et al. 2013). Therefore, understanding the patterns of the genetic diversity and population structure of *Monilinia* spp. and how they could be influenced by the host preference is crucial for the success of any disease management strategy. Among the other genotyping approaches, HRM-based techniques are rapid and are also low-cost in their deployment (Zambounis et al. 2016a; Sillo et al. 2017). The HRM approach has extensively been used in fungal species identification, genotyping and molecular diagnostics (Ganopoulos et al. 2012; Zambounis et al.2015a, b, 2016b; Papavasileiou et al. 2016; Garganese et al. 2018; Xanthopoulou et al. 2019). In the present study, in order to accurately genotype the populations of *M. laxa*, *M. fructicola* and *M. fructigena* across the different regions of Serbia, an HRM genotyping approach was employed using three species-specific primers for *Monilinia* spp. These primers were previously shown to be effective in distinguishing *Monilinia* spp. in stone fruit trees (Gell et al. 2007a), amplifying the well-separated and short amplicon sizes that are preferable for an HRM analysis.

The results of the present study confirmed that the HRM assay was sensitive and allowed the precise assignment of the *Monilinia* isolates of the main brown rot causing agents within the specific genotypes in each HRM run. Thus, all fifty-seven isolates across the three *Monilinia* species were clustered in 20 genotypes according to their distinct HRM profiles upon the shape fluctuations of the HRM melting curves. The GCP values were employed for assigning the isolates within the informative HRM genotypes ensuring reliable interpretations of the melting curve profiles (Zambounis et al. 2016a, b). For each *Monilinia* species and among the different HRM runs, the melting curves were genotype-specific and repeatable maintaining the same melting peaks, consistent with the haploid nature of the *Monilinia* species (van Brouwershaven et al. 2010). The sequencing of the *Monilinia* spp. PCR amplicons also confirmed the existence of different single-nucleotide polymorphism (SNP) variations across the *Monilinia* spp. HRM genotypes. The potential resolving impact of the HRM genotyping approach is much higher than a conventional gel or melting curve analyses, as different amplicons can be differentiated even by the fluctuations of the melting curve shapes, enabling an efficient standardisation that is crucial for a routine analysis (Ganopoulos et al. 2012). Previously, various molecular markers were employed to identify and genotype *Monilinia* spp. such as those spanning the region within the *cytb* gene intron, as well as inter-simple sequence repeat (ISSR) markers (Miessner & Stammler 2010; van Brouwershaven et al. 2010; Hily et al. 2011; Papavasileiou et al. 2016; Tran et al. 2019).

The availability of genomic sequences across the *Monilinia* species would provide novel opportunities through genotyping-by-sequencing (GBS) approaches to identify and evaluate potential markers for population genetic studies towards an efficient genotyping of the genetic variation in *Monilinia* populations (Lu et al. 2013). Furthermore, HRM-based genotyping techniques are promising approaches in order to decipher the genetic diversity among fungi species and populations. A deeper understanding of the population dynamics and diversities will contribute to more durable disease management strategies against *Monilinia* spp. Towards this prospective, the choice of proper markers with sufficient resolution is crucial for discriminating the multilocus genotypes and identify the sub-clonal variations in *Monilinia* spp. populations.

In our study, the genetic analysis of the *M. laxa* populations originated from the different regions in Serbia revealed a high genetic variation within the populations (93%), while only 7% of the total diversity was attributed to the among-population differentiation. These results are in accordance with previous studies in *M. laxa* populations conducted in Europe and Asia (Gell et al. 2007b; Zhu et al. 2010; Fazekas et al. 2014). The relative low diversity (7%) among the three *M. laxa* populations might be explained by the fact that this species has been a rather endemic species for a long period in Serbia, and thus a high rate of gene flow may homogenise its populations. In parallel, the PCoA grouped the isolates of *M. laxa* into three sub-populations groups, whereas the isolates from the northern and southern regions were mainly clustered in two groups according to the ML-1 and ML-2 genotypes, respectively, and independently from their plant host preferences. A third sub-population group consisting from the isolates of all three regions, which were clustered in the other six ML genotypes, was evident and it might contribute to the absence of differences among the three populations.

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In the cases of *M. fructicola* and *M. fructigena*, the genetic variation within the populations were, in both cases, less than that observed in *M. laxa*, whilst 44% of the total variation was attributed to the among-population differentiation. Particularly for the widespread pathogen *M. fructicola*, the increase in the among-population differentiation might be attributed to its higher reproduction capacity, possibly through sexual recombination, its higher sporulation and dispersal ability, as well as its higher risk for fungicide resistance which are all crucial sources of genetic diversity in *M. fructicola* (Villarino et al. 2013). Furthermore, the relatively high genetic diversity among the Serbian *M. fructicola* populations is in accordance with the diversity observed in countries where the pathogen co-exists for a long period of time and intensively reproduces sexually. Indeed, *M. fructicola* has been introduced in many countries in Europe long time ago (EFSA 2011), including Serbia (Vasić et al. 2012; Hrustić et al. 2013), where its population was rather diversified and possibly accumulated random mutations (Jänsch et al. 2012; De Cal et al. 2014). Toward this end, our HRM analysis was a rapid and inexpensive method capable of efficiently revealing the genotyping alterations in *M. fructicola* populations of Serbia. However, in order to more deeply uncover the genetic structure of such populations in broader geographical regions, a higher number of pathogen isolates would need to be tested by this approach in the future. Besides, taking into account that fungicide applications may alter the genetic structure of a fungal population, as well as that the *M. fructicola* populations are more prone to fungicide resistance (Villarino et al. 2013), we hypothesise that resistant sub-populations of this pathogen might be favoured and, thus, explaining the relatively high genetic diversity among its populations in the three different regions. Notably, isolates from the northern region, among all three tested *Monilinia* spp., were assigned to distinct genotypes compared to the isolates of the other two regions, and also grouped rather independently in the PCoA analyses from the other isolates. The northern region of Serbia is completely separated by physical barriers, such as mountains and rivers, and we hypothesise that the northern populations of all three *Monilinia* spp. were more distant from the other populations as the gene flow through conidia dispersal is quite obscure.

The host preference is also considered an evolutionary aspect that may direct the genetic structure of a fungal population. Even though *Monilinia* spp. are considered polytrophic, there are reports highlighting a certain degree of host specialisation particularly in *M. fructigena* (Gril et al. 2008). In contrast, other reports among *M. laxa* isolates from various hosts have not revealed any genetic differences (Gell et al. 2007b; Gril et al. 2008). In our study, there was not any evidence of host specificity in *Monilinia* spp. populations, perhaps because the *Monilinia* isolates across all three regions were collected from different stone-fruit hosts that were cultivated in adjacent orchards. Thus, we further hypothesise that a common adaptation to local environmental conditions was favoured in some degree for all the isolates of a particular region, putatively through the relative high gene flow among the different hosts.

Continuous monitoring is required to detect any possible changes in the population and the genetic structure of brown rot causal agents. Our results allow one to gain insights into the genotypic diversity of the *Monilinia* spp. populations obtained from different stone-fruit hosts across three regions in Serbia by employment of a fast and cost-effective HRM-based approach. The genotype-specific melting curve profiles were adequately assigned to all the isolates under investigation. The present study is the first attempt to discriminate *Monilinia* spp. populations in Serbia. In the long term, this information can be of high importance towards deciphering the population structures of *Monilinia* spp. in stone-fruit orchards and the design of more effective brown rot control strategies.

REFERENCES

Berrie A.M., Holb I. (2014): Brown rot diseases. In: Sutton T.B., Aldwinckle H.S., Angelo A.M., Walgenbach J.F. (eds): Compendium of Apple and Pear Diseases and Pests. St. Paul, American Phytopathological Society: 43–45.

Côté M.J., Tardif M.C., 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 Disease, 88: 1219–1225.

De Cal A., Eguen B., Melgarejo P. (2014): Vegetative compatibility groups and sexual reproduction among Spanish *Monilinia fructicola* isolates obtained from peach and nectarine orchards, but not *Monilinia laxa*. Fungal Biology, 118: 484–494.

EFSA (2011): Pest risk assessment of *Monilinia fructicola* for the EU territory and identification and evaluation of risk management options. EFSA Journal, 9: 155. doi:10.2903/j.efsa.2011.2119

https://doi.org/10.17221/35/2020-PPS

Fan J.Y., Luo Y., Michailides TJ., Guo L.Y. (2014): Simultaneous quantification of alleles E198A and H6Y in the b-tubulin gene conferring benzimidazole resistance in *Monilinia fructicola* using a duplex real-time (TaqMan) PCR. Pest Management Science, 70: 245–251.

Fan J.Y., Guo L.Y., Xu J.P., Luo Y., Michailides T.J. (2010): Genetic diversity of populations of *Monilinia fructicola* (Fungi, Ascomycota, Helotiales) from China. Journal of Eukaryotic Microbiology, 57: 206–212.

Fazekas M., Madar A., Sipiczki M., Miklos I., Imre H. (2014): Genetic diversity in *Monilinia laxa* populations in stone fruit species in Hungary. World Journal of Microbiology and Biotechnology, 30: 1879–1892.

Ganopoulos I., Madesis P., Zambounis A., Tsaftaris A. (2012): High resolution melting (HRM) analysis allowed fast and accurate closed-tube genotyping of *Fusarium oxysporum* formae speciales complex. FEMS Microbiology Letters, 334: 16–21.

Garganese F., Ippolito A., di Rienzo V., Lotti C., Montemurro C., Sanzani S.M. (2018): A new high-resolution melting assay for genotyping *Alternaria* species causing citrus brown spot. Journal of the Science of Food and Agriculture, 98: 4578–4583.

Gell I., Cubero J., Melgarejo P. (2007a): Two different PCR approaches for universal diagnosis of brown rot and identification of *Monilinia* spp. in stone fruit trees. Journal of Applied Microbiology, 103: 2629–2637.

Gell I., Larena I., Melgarejo P. (2007b): Genetic diversity of *Monilinia laxa* populations in peach orchards in Spain. Journal of Phytopathology, 155: 549–556.

Gril T., Celar F., Munda A., Javornik B., Jakse J. (2008): AFLP analysis of interspecific variation between *Monilinia laxa* isolates from different hosts. Plant Disease, 92: 1616–1624.

Guinet C., Fourrier-Jeandel C., Cerf-Wendling I., Ioos R. (2016): One-step detection of *Monilinia fructicola*, *M. fructigena*, and *M. laxa* on *Prunus* and *Malus* by a multiplex real-time PCR assay. Plant Disease, 100: 2465–2474.

Hewson K., Noormohammadi A.H., Devlin J.M., Mardani K., Ignjatovic J. (2009): Rapid detection and non-subjective characterization of infectious bronchitis virus isolates using high-resolution melt curve analysis and a mathematical model. Archives of Virology, 154: 649–660.

Hily J.M., Singer S.D., Villani S.M., Cox K.D. (2011): Characterization of the cytochrome b (cytb) gene from *Monilinia* species causing brown rot of stone and pome fruit and its significance in the development of QoI resistance. Pest Management Science, 67: 385–396.

Hrustić J., Delibašić G., Stanković I., Grahovac M., Krstić B., Bulajić A., Tanović B. (2015): *Monilinia* species causing brown rot of stone fruit in Serbia. Plant Disease, 99: 709–717.

Hrustić J., Mihajlović M., Tanović B., Delibašić G., Stanković I., Krstić B., Bulajić A. (2013): First report of brown rot caused by *Monilinia fructicola* on nectarine in Serbia. Plant Disease, 97: 147. doi: 10.1094/PDIS-08-12-0718-PDN

Hu M.J., Cox K.D., Schnabel G., Luo C.X. (2011): *Monilinia* species causing brown rot of peach in China. PLoS ONE, 6:e24990. doi: 10.1371/journal.pone.0024990

Ioos R., Frey P. (2000): Genomic variation within *Monilinia laxa*, *M. fructigena* and *M. fructicola*, and application to species identification by PCR. European Journal of Plant Pathology, 106: 373–378.

Jänsch M., Frey J.E., Hilber-Bodmer M., Broggini G.A.L., Weger J., Schnabel G., Patocchi A. (2012): SSR marker analysis of *Monilinia fructicola* from Swiss apricots suggests introduction of the pathogen from neighboring countries and the United States. Plant Pathology, 61: 247–254.

Lane C.R. (2002): A synoptic key for differentiation of *Monilinia fructicola*, *M. fructigena* and *M. laxa*, based on examination of cultural characters.Bulletin OEPP/EPPO Bulletin, 32: 489–493.

Lu F., Lipka A.E., Glaubitz J., Elshire R., Cherney J.H., Casler M.D., Buckler E.S., Costich D.E. (2013): Switchgrass genomic diversity, ploidy, and evolution: novel insights from a network-based SNP discovery protocol. PLoS Genetics, 9: e1003215. doi: 10.1371/journal.pgen.1003215

Martini C., Mari M. (2014): *Monilinia fructicola*, *Monilinia laxa* (Monilinia rot, Brown rot). In: Bautista-Baños S. (ed.): Postharvest Decay-control Strategies. Cambridge, Academic Press.

Miessner S., 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. Journal of Plant Diseases and Protection, 117: 162–167.

Ortega SF., López M.P.B., Nari L., Boonham N., Gullino M.L, Spadaro D. (2019): Rapid detection of *Monilinia fructicola* and *Monilinia laxa* on peaches and nectarines using loop-mediated isothermal amplification. Plant Disease, 103: 2305–2314.

Papavasileiou A., Madesis P., Karaoglanidis G. (2016): Identification and differentiation of *Monilinia* species causing brown rot of pome and stone fruit using high resolution melting (HRM) analysis. Phytopathology, 106: 1055–1064.

Peakall R., Smouse P.E. (2012): GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. Bioinformatics, 28: 2537–2539.

Sillo F., Giordano L., Zampieri E., Lione G., De Cesare S. Gonthier P. (2017): HRM analysis provides insights on the reproduction mode and the population structure of *Gnomoniopsis castaneae* in Europe. Plant Pathology, 66: 293–303.

https://doi.org/10.17221/35/2020-PPS

Stukenbrock E.H. (2016): The role of hybridization in the evolution and emergence of new fungal plant pathogens. Phytopathology, 106: 104–112.

Tran T.T., Li H., Nguyen D.Q., Sivasithamparam K., Jones M.G.K., Wylie S.J. (2019): Genotypic structure of *Monilinia* populations in Western Australia two decades after incursion. Australasian Plant Pathology, 48: 167–178.

van Brouwershaven I.R., Bruil M.L., van Leeuwen G.C.M., Kox L.F.F. (2010): A real-time (TaqMan) PCR assay to differentiate *Monilinia fructicola* from other brown rot fungi of fruit crops. Plant Pathology, 59: 548–555.

Vasić M., Duduk N., Ivanović M.M., Obradović A., Ivanović M.S. (2012): First report of brown rot caused by *Monilinia fructicola* on stored apple in Serbia. Plant Disease, 96: 456.

Villarino M., Egüen B., Lamarca N., Segarra J., Usall J., Melgarejo P., De Cal A. (2013): Occurrence of *Monilinia laxa* and *M. fructigena* after introduction of *M. fructicola* in peach orchards in Spain. European Journal of Plant Pathology, 137: 835–845.

Villarino M., Larena I., Martinez F., Melgarejo P., De Cal A. (2012): Analysis of genetic diversity in *Monilinia fructicola* from Ebro valley in Spain using ISSR and RAPD markers. European Journal of Plant Pathology, 132: 511–524.

Vossen R.H., Aten E., Roos A., den Dunnen J.T. (2009): High-resolution melting analysis (HRMA) – more than just sequence variant screening. Human Mutation: 30, 860–866.

Wang J.R., Guo L.Y., Xiao C.L., Zhi X. (2018): Detection and identification of six *Monilinia* spp. causing brown rot using TaqMan real-time PCR from pure cultures and infected apple fruit. Plant Disease, 102: 1527–1533.

Xanthopoulou A., Ganopoulos I., Tryfinopoulou P., Panagou E.Z., Osanthanunkul M., Madesis P., Kizis D. (2019): Rapid and accurate identification of black aspergilli from grapes using high-resolution melting (HRM) analysis. Journal of the Science of Food and Agriculture, 99: 309–314.

Zambounis A., Samaras A., Xanthopoulou A., Osathanunkul M., Schena L., Tsaftaris A., Madesis P. (2016a): Identification of *Phytophthora* species by a high resolution melting analysis: an innovative tool for rapid differentiation. Plant Protection Science, 52: 176–181.

Zambounis A., Xanthopoulou A., Madesis P., Tsaftaris A., Vannini A., Bruni N., Tomassini A., Chilosi G., Vettraino A.M. (2016b): HRM: a tool to assess genetic diversity of *Phytophthora cambivora* isolates. Journal of Plant Pathology, 98: 611–616.

Zambounis A., Xanthopoulou A., Karaoglanidis G., Tsaftaris A., Madesis P. (2015a): A new accurate genotyping HRM method for *Alternaria* species related to fruit rot diseases of apple and pomegranate. International Journal of Phytopathology, 4: 159–165.

Zambounis A., Ganopoulos I., Chatzidimopoulos M., Tsaftaris A., Madesis P. (2015b): High-resolution melting approaches towards plant fungal molecular diagnostics. Phytoparasitica, 43: 265–272.

Zhu X.Q., Chen X.Y., Guo L.Y. (2011): Population structure of brown rot fungi on stone fruits in China. Plant Disease, 95: 1284–1291.

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