Intraspecific Diversity of *Monilinia fructicola* and *M. laxa* Populations from Blossoms and Fruit of Different Hosts in Greece

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Abstract

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The genetic variation among 145 isolates of *Monilinia fructicola* and 156 isolates of *M. laxa* collected from two distinct regions of Greece (Imathia and Larissa) was analyzed using intersimple-sequence repeat markers. The *Monilinia* spp. isolates had been collected from infected fruit or blossoms of peach, apricot, sweet cherry, and plum. Calculation of Nei's gene diversity and Shannon's diversity indices showed that *M. fructicola* populations had higher genetic diversity compared with *M. laxa* populations in both regions sampled. The levels of genetic diversity were similar between populations obtained from diseased blossoms and fruit for each species and the main variances were all from within rather than between

populations for the respective regions, hosts, and organ of origin. Genetic distance (Nei's analysis) was lower between peach and apricot populations than between cherry and plum populations of *M. fructicola*. *M. fructicola* isolates from peach and apricot and from sweet cherry and plum were clustered together, while *M. laxa* isolates clustering based on the host of origin was not possible. The analysis of index of association showed the absence of sexual recombination for both species. The derived data support the hypothesis of a long presence of *M. fructicola* in Greece, and provide evidence of specialization of *M. fructicola* populations based on their host of origin.

Greece is one of the most important countries in the world for stone-fruit production, ranking fifth worldwide for peach production. Stone fruit are cultivated in the regions of Thessaly (central Greece), Western Macedonia (northern Greece), and Central Macedonia (northern Greece), with the latter being the most important region. Brown rot is one of the most destructive diseases of stone fruit in temperate regions of the world, including Greece (Byrde and Willettes 1977; Papavasileiou et al. 2015). The disease initially appears on the blossoms of the trees, causing blossom blight. On blighted blossoms, the fungi sporulate and the inoculum spreads to leaves and shoots, causing twig blight, or to the newly developed fruit, causing fruit blight (Holb et al. 2013; Hong et al. 1997). However, the most severe damages occur due to fruit rots that become evident at ripening, either pre- or post-harvest (Byrde and Willettes 1977). Fruit rots are the outcome of infections that may be either latent or direct (Keske et al. 2011). Latent infections may occur during most stages of fruit development, while direct infections may occur on ripe fruit just before harvest or during storage (Luo and Michailides 2001; Luo et al. 2005).

Four different *Monilinia* spp. (*Monilinia laxa*, *M. fructicola*, *M. fructigena*, and *M. polystroma*) are associated with brown rot of stone fruit in European countries. *M. laxa* (Aderh. & Ruhland) Honey (anamorph *Monilia laxa* (Ehrenb.) Sacc. & Voglino) has a worldwide distribution and, until recently, was considered to be the main agent of brown rot in Southern and Central European countries. *M. fructicola* (Winter) G. Honey (anamorph *Monilia fructicola* L. R. Batra) is the main agent of the disease in North and South America, and has also been reported in Australia, China, and Japan. *M. fructicola* is a quarantine pathogen for European Union (EU) countries (EPPO 2013), although it has been reported recently in several European countries. *M. fructigena* has also a wide distribution in Europe and Asia but has not been reported yet, in North America and Australia

(Batra 1991; Byrde and Willettes 1977). *M. polystroma* was recently isolated from stone fruit and currently was detected in a limited number of European countries and in China (Poniatowska et al. 2013; Zhu and Guo 2010; Zhu et al. 2011).

A recent study showed that *M. fructicola* had been introduced into Greece, although it is still considered as a quarantine pathogen (Papavasileiou et al. 2015). *M. fructicola* was found to be prevalent on maturing peach and plum fruit and occurred at high frequencies in cherry and apricot orchards. In contrast, *M. laxa* was found to be predominant in blighted blossoms of peach, apricot, sweet cherry, and plum. *M. fructigena* was not isolated from stone fruit, although it has been reported in apple fruit showing postharvest rots (Konstantinou et al. 2011). *M. fructicola* is now known to exist in several other European countries, including Switzerland, Spain, Italy, and Poland (Bossard et al. 2006; De Cal et al. 2009; Pellegrino et al. 2009; Poniatowska et al. 2013). This species may replace *M. laxa* eventually, due to its higher reproduction capacity, higher dispersal ability, and higher risk for fungicide resistance development (Jänsch et al. 2012; Michailides et al. 1987; Papavasileiou et al. 2015; Villarino et al. 2013).

Information on the genetic structure of fungal populations is of importance for a better understanding of fungal evolution in agricultural environments and may lead to the design of more effective control strategies (McDonald 1997). Few studies have been conducted to investigate genetic diversity of either *M. laxa* (Gell et al. 2007; Gril et al. 2008) or *M. fructicola* (Fan et al. 2010; Jänsch et al. 2012; Villarino et al. 2012) populations. Most of these reports focused on isolates obtained from a single host such as peach or apricot. However, different stone fruit can commonly be found in the same geographic region. *Monilinia* spp. are polytroph pathogens, attacking different stone fruit although a certain degree of host specialization seems to exist (Gril et al. 2008). In addition to host variability, *Monilinia* spp. also infect variable organs, causing blossom blight, twig blight, and pre- or postharvest fruit rots (Byrde and Willettes 1977). Despite this host and affected plant tissue variability, information on genetic diversity of *Monilinia* spp. from different hosts or different infected tissues is scarce.

Information about the genetic diversity of fungal populations can be obtained through the use of several molecular markers. Early

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attempts to obtain information about the genetic diversity of *Monilinia* spp. were mainly based on sequencing data of the internal transcribed spacer region (Fulton et al. 1999; Snyder and Jones 1999). Advances in the development of molecular techniques allowed the development of more powerful tools, enabling the assessment of genetic diversity in *Monilinia* spp. populations. Random amplified polymorphic DNA (RAPD) markers were used to access genetic diversity of *M. laxa* in Spain (Gell et al. 2007), while amplified fragment length polymorphism was used to estimate genetic variability of *M. laxa* populations in Slovenia (Gril et al. 2008). In addition, two more recent reports provided information on the genetic variability of *M. fructicola* populations from peach and apricot based on the use of intersimple-sequence repeat (ISSR) markers or microsatellite (simple-sequence repeat [SSR]) markers, respectively (Fan et al. 2010; Jänsch et al. 2012; Villarino et al. 2012).

The current study was conducted to (i) investigate the genetic diversity of both *M. fructicola* and *M. laxa* populations in Greece that had been obtained from different stone-fruit hosts (apricot, peach, plum, and sweet cherry) and different infected tissues (diseased blossoms and fruit of each host), (ii) identify the sources of genetic variation, and (iii) investigate the probability for sexual reproduction in the two fungal populations.

Materials and Methods

Isolate collection. The isolates used in the study were collected during spring 2012 from stone-fruit orchards in the two main stone-fruit cultivating regions of Greece, Central Macedonia (Imathia) and Thessaly (Larissa) (Papavasileiou et al. 2015). The sampled hosts included apricot, sweet cherry, peach, and plum. The isolates were collected during two distinct infection stages from blighted blossoms and from symptomatic fruit on the trees just before or during harvest. The DNA extraction procedure and the isolate identification to the species level were conducted as described by Papavasileiou et al. (2015). In total, 145 *M. fructicola* and 156 *M. laxa* isolates were selected for this study. The selection of isolates was based on the geographic origin, the host of origin and the plant tissue from which the isolates had been obtained. Details on the number of isolates and their origin are provided in Table 1. The number of isolates obtained from each region was uneven due to low availability of orchards for sampling (i.e., in the region of Larissa, plum were not cultivated) or unfavorable conditions for disease development leading to low disease incidence (i.e., absence of infections on peach blossoms in Larissa region).

Primer selection and polymerase chain reaction amplification conditions. In total, 16 ISSR primers—(AAG)8, (AG)8C, (AG)8YC, (AC)8T, (AG)8YT, (AG)AYC, (AC)8G, (AC)8YC, (AAG)6, (AC)8YT, T3B, (GACA)4, VHV(GT)7, (GTC)6, (GTG)5, and M13—that have been used in previous studies for DNA fingerprinting of other fungi, including *M. fructicola* (Ma and Michailides 2005; Villarino et al. 2012), were initially screened in a pilot experiment for their ability to amplify reproducible and polymorphic DNA bands. The primers were tested on five randomly chosen *M. fructicola* and five *M. laxa* isolates that had been obtained

from different geographic regions and hosts. The primers were synthesized by Life Technologies Corporation, (Carlsbad, CA). Polymerase chain reaction (PCR) was performed using an Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany) in a 50- μ l volume containing 80 to 100 ng of template DNA, 2 μ M microsatellite primer, 0.2 mM each dNTP, 2.0 mM $MgCl_2$, 1 $\times$ *Taq* polymerase buffer (10 mM Tris-Cl [pH 9.0], 50 mM KCl, and 0.1% Triton X-100), and 1.5 U of *Taq* polymerase. PCR was performed using the following thermal parameters: an initial preheat for 3 min at 95°C; followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1.5 min; and terminated with a final extension at 72°C for 10 min. The reactions were conducted twice for each isolate. PCR products were separated by electrophoresis in 1.5% agarose gels in Tris-acetate buffer, stained with ethidium bromide (0.1 μ g/ml), and viewed under UV light.

Data analysis. For analysis of the ISSR data sets, the gels were examined visually. The presence or absence in the same position of each amplified band was scored as 1 or 0, respectively, and a binary matrix was constructed. Amplified fragments ranged in size from 0.2 to 1.5 kb and only intense and reproducible bands were recorded. For the genetic analysis of the population, isolates were classified within the two *Monilinia* spp. in three different ways, taking into account their geographic origin, their source host, and the host tissue (blossoms or fruit) of origin. Nei's gene diversity (Nei 1973) and Shannon's diversity index (Shannon and Weaver 1949) were used to estimate the genetic diversity of each population, using the GenAlEx6 software (Peakall and Smouse 2006). An analysis of molecular variance (AMOVA) within the different groups of *M. fructicola* and *M. laxa* isolates was conducted in order to determine the main sources of variation, using the same software. To determine the similarity of the two populations of *Monilinia* spp. populations among the sampled hosts, the Nei's genetic identity and distance were calculated using the GenAlEx6 software (Peakall and Smouse 2006). A dendrogram was constructed based on Nei's genetic distances between *Monilinia* populations from different hosts, using the unweighted pair-group method with arithmetic average for multiple populations by the program NEIGHBOR of PHYLIP, version 3.5c, by Joe Felsenstein, of the software package PopGene. In order to analyze the reproductive mode of the two sampled *Monilinia* populations, the index of association (I_A) tests were used (Ma and Michailides 2005). This test is used to measure the extent of linkage equilibrium within a population by detecting statistical associations between alleles at different loci (Agapow and Burt 2001).

The I_A is defined as follows: $I_A = V_O/V_E - 1$, where V_O is the observed variance of pairwise distances and V_E is variance expected in the absence of linkage disequilibrium (Agapow and Burt 2001). In the presence of linkage equilibrium because of frequent recombination events, the expected value of I_A is zero. Populations with a clonal mode of reproduction are identified by an I_A value that differs significantly from zero.

Results

Primer selection. Among the 16 primers tested, 8 of them—namely, (AAG)8, (GTG)5, M13, (AG)8C, (AC)8T, (AG)8YT, (AG)AYC, and (AC)8YC—consistently generated stable band profiles. These eight primers were used for DNA fingerprinting of the 145 *M. fructicola* and 156 *M. laxa* isolates. Each of these primers amplified 3 to 10 polymorphic bands per isolate. In total, 63 bands were amplified and 52 of them were polymorphic. The data revealed the presence of 140 and 136 distinct genotypes within the *M. fructicola* and *M. laxa* populations tested, respectively.

Population diversity. Calculation of the Nei's and Shannon's diversity indexes showed that, in total, genetic diversity in the *M. fructicola* population was higher than that in *M. laxa* population, with values of 0.24 and 0.17 for Shannon's information index and 0.16 and 0.11 for Nei's diversity index, respectively (Table 2). Similar were the values of the Nei's and Shannon's diversity indexes within the two *Monilinia* spp. populations, when the isolates were grouped according to their plant tissue of origin (Table 2). Higher genetic diversity was found in the region of Imathia within the *M. fructicola*

Table 1. Host, tissue of origin, and number of *Monilinia laxa* and *Monilinia fructicola* isolates used in the study^a

<i>Monilinia</i> spp.	Host	Imathia		Larissa	
		Blossom	Fruit	Blossom	Fruit
<i>M. laxa</i>	Peach	15	5	N.S.	0
<i>M. fructicola</i>	Peach	16	19	N.S.	16
<i>M. laxa</i>	Cherry	10	14	15	16
<i>M. fructicola</i>	Cherry	0	18	0	0
<i>M. laxa</i>	Apricot	17	18	14	15
<i>M. fructicola</i>	Apricot	0	33	8	0
<i>M. laxa</i>	Plum	8	9	N.S.	N.S.
<i>M. fructicola</i>	Plum	17	18	N.S.	N.S.

^a N.S. = not sampled

population with Nei's and Shannon's diversity index values of 0.16 and 0.24, respectively, than in the region of Larissa, where the respective values were 0.13 and 0.2 (Table 2). In contrast, within the *M. laxa* populations, no differences were observed among the two different geographic regions (Table 2). When the two *Monilinia* spp. populations were grouped according to their host of origin, higher levels of genetic diversity were found in the population originated from apricot for both *M. fructicola* and *M. laxa*, while the levels of genetic diversity in the populations that originated from peach, cherry, and apricot were similar (Table 2). Specifically, for the population of *M. laxa* collected from apricot, higher levels of genetic diversity were found in isolates from both infected blossoms and fruit whereas, in the *M. fructicola* population, higher levels were mainly found in the isolates that had been obtained from infected apricot fruit compared with that of isolates obtained from apricot blossoms (Table 2).

AMOVA. The AMOVA showed that, in the *M. fructicola* population, 76% of the genetic variance was found within the source hosts and 24% was due to separations based on the different hosts (Table 3). In contrast, in the *M. laxa* population, 95% of the total variation occurred within the populations obtained from the four different hosts and only 5% of the observed differentiation was due to host separation (Table 3). Factors such as the geographic origin and infected tissue contributed even less to the total variation of both *Monilinia* spp. (Table 3).

Calculation of Nei's genetic identity and distance between *M. fructicola* populations originating from the four different sampled hosts showed values of 0.9273 to 0.9791 and 0.0212 to 0.0755, respectively, indicating high genetic similarity among the population (Table 4). The highest genetic identity was found between populations originating from peach and apricot and from cherry and plum, with values of 0.9791 and 0.9759, respectively (Table 4). AMOVA showed that only 10% of the observed genetic variation was found among *M. fructicola* populations obtained from peach and apricot, while 28 and 33% of the observed variation was found among populations originating from peach and either sweet cherry or plum, respectively (Table 5). Similarly, AMOVA showed that only 14% of the observed variation was among the sweet cherry and plum

populations, while 24% of the observed variation was found among the populations from sweet cherry and apricot (Table 5). Regarding the population of *M. laxa* originating from the four different hosts, the values of Nei's genetic identity and distance varied from 0.9866 to 0.9977 and from 0.0134 to 0.0023, respectively, suggesting high genetic similarity among the populations (Table 4). The above results were consistent with the dendrogram based on Nei's genetic distances between the populations originating from the different hosts (Fig. 1). The dendrogram showed that, within *M. fructicola*, the population from peach and apricot and the populations from plum and sweet cherry were very similar to each other (Fig. 1). In the case of *M. laxa*, all populations were very similar and grouped together (Fig. 1).

I_A . The observed I_A of the 145 isolates of *M. fructicola* and 156 isolates of *M. laxa* was 0.294 and 0.279 ($P < 0.001$), respectively. I_A values were significantly different from zero, indicating a clonal mode of reproduction for both fungal populations tested.

Discussion

The results of this study showed that the populations of *M. fructicola* obtained from stone-fruit orchards located in two geographically distinct regions of Greece had a higher genetic diversity compared with *M. laxa* populations originating from the same hosts and regions. *M. fructicola* is a quarantine pathogen that was detected in Greece for the first time in 2012 (Papavasileiou et al. 2015). However, interestingly, it was found in relatively high frequencies and, in the region of Imathia, it was the predominant *Monilinia* sp. on infected peach, plum, and apricot fruit (Papavasileiou et al. 2015). *M. laxa* is considered to be an indigenous fungal species in Europe and, thus, we would expect higher genetic diversity in its populations compared with populations of a newly introduced species (McDonald and Linde 2002). The high genetic diversity of the Greek *M. fructicola* population is comparable with that observed in countries with indigenous *M. fructicola* populations in which the pathogen exists for a long period of time and reproduces sexually (Fan et al. 2010; Fulton et al. 1999; Ma et al. 2003). Based on the high genetic diversity in *M. fructicola* populations, we hypothesize that this pathogen has been in Greece for a long time and remained unnoticed due to

Table 2. Genetic diversity within populations of *Monilinia fructicola* and *M. laxa* originating from different geographic regions, different hosts, and different infected plant tissue

Origin of isolates	<i>Monilinia</i> spp. ^a									
	<i>M. laxa</i>					<i>M. fructicola</i>				
	N	Na	Ne	I	h	N	Na	Ne	I	h
Total	158	1.24	1.17	0.17	0.11	143	1.2	1.28	0.24	0.16
Tissue										
Blossoms	82	1.16	1.17	0.16	0.1	38	1.08	1.26	0.23	0.15
Fruit	76	1.02	1.18	0.17	0.11	105	1.2	1.29	0.24	0.16
Geographic regions										
Larissa	46	1.04	1.18	0.16	0.11	23	1.04	1.24	0.2	0.13
Imathia	97	1.1	1.17	0.17	0.1	120	1.2	1.28	0.24	0.16
Host										
Peach	20	0.86	1.17	0.15	0.1	51	1.08	1.23	0.2	0.13
Apricot	63	1.16	1.18	0.18	0.11	42	1.02	1.27	0.22	0.15
Cherry	56	1	1.16	0.15	0.1	17	0.98	1.17	0.16	0.1
Plum	19	0.9	1.14	0.14	0.09	33	0.98	1.18	0.17	0.11
Blossom										
Peach	15	0.78	1.16	0.14	0.09	16	0.96	1.18	0.16	0.11
Apricot	32	1.04	1.15	0.16	0.1	7	0.84	1.19	0.16	0.11
Cherry	25	0.96	1.13	0.14	0.08	N.S.	N.S.	N.S.	N.S.	N.S.
Plum	10	0.82	1.13	0.13	0.08	15	0.94	1.16	0.17	0.11
Fruit										
Peach	5	0.74	1.14	0.12	0.08	35	1.06	1.24	0.2	0.13
Apricot	31	0.98	1.19	0.17	0.11	35	1.02	1.27	0.22	0.15
Cherry	31	0.96	1.15	0.14	0.09	17	0.98	1.17	0.16	0.1
Plum	9	0.74	1.13	0.11	0.07	18	0.88	1.16	0.15	0.1

^a N = number of isolates; Na = number of different alleles; Ne = number of effective alleles = $1/(p^2 + q^2)$; I = Shannon's Information Index = $-1 \times [p \times \ln(p) + q \times \ln(q)]$; h = diversity = $1 - (p^2 + q^2)$ where, for haploid binary data, p = band frequency and q = 1 - p; and N.S. = samples were not available.

either the absence of detailed studies or misidentifications. Similarly, Jänsch et al. (2012) observed a relatively high genetic diversity in *M. fructicola* isolates from apricot in Switzerland that had been collected during 2008, just a few years after the first report of the pathogen in Europe. They explained their findings by suggesting an earlier introduction of the pathogen in Europe that gave it enough time to diversify, possibly through sexual or parasexual recombination, accumulation of random mutations, or gene flow (Jänsch et al. 2012). In contrast, an early assessment of genetic variability in *M. fructicola* populations conducted just after the first report of the pathogen in Spain showed a low level of variability within the tested populations (Villarino et al. 2012). However, a later study conducted using *M. fructicola* isolates that had been collected several years after the first report of the pathogen in Spain showed an increase in the genetic variability of the population (De Cal et al. 2014).

Taking into account sexual recombination as a source of variation, the presence of the teleomorphic stage in *M. fructicola* could explain the higher levels of genetic diversity, in contrast to *M. laxa*, for which the occurrence of sexual stage is rare (Byrde and Willetts 1977; Holtz et al. 1998). However, in the present study, the I_A analysis suggests that both *M. fructicola* and *M. laxa* populations have a clonal mode of reproduction in Greek orchards. In contrast to our findings, Jänsch et al. (2012) provided some evidence for sexual

recombination in *M. fructicola* isolates of European origin by analyzing SSR data; while, very recently, De Cal et al. (2014) induced apothecia production under laboratory conditions using *M. fructicola* isolates from Spain. However, currently no sexual reproduction of the pathogen in nature has been reported in any European country (De Cal et al. 2014; Jänsch et al. 2012; Villarino et al. 2012). Sexual reproduction in *M. fructicola* is a complex process, starting with the formation of stomatal tissue on mummified fruit and terminating with the dissemination of ascospores. These processes are strongly affected by several environmental factors such as temperature, relative humidity, soil humidity, and light (De Cal et al. 2014; Willetts and Harada 1984). Thus, the absence of sexual reproduction in the populations tested could be attributed to either the absence of conducive environmental conditions for apothecia development or cultural practices implemented by the growers to combat the disease such as removing the mummified fruit from the trees, controlling weeds in tree rows, or disrupting the orchard row middle by rototilling (De Cal et al. 2014).

Interestingly, differences were observed in diversity of *M. fructicola* populations originating from the two different geographic regions. The *M. fructicola* population originating from Imathia showed higher diversity compared with the respective population originating from Larissa. In contrast, no differences were observed

Table 3. Analysis of molecular variance among *Monilinia fructicola* and *Monilinia laxa* populations obtained from different hosts, geographic regions, and different infected plant tissue, using the intersimple-sequence repeat data set

Source of variation	<i>Monilinia</i> spp.									
	<i>M. fructicola</i>					<i>M. laxa</i>				
	Degree of freedom	Sum of squares	Variance component	Variation (%)	P^a	Degree of freedom	Sum of squares	Variance component	Variation (%)	P
Hosts ^b										
Among populations	3	114.85	1.02	24	0.001	3	21.688	0.128	5	0.001
Within populations	139	460.13	3.31	76	0.001	154	401.52	2.607	95	0.001
Total	142	574.98	4.331	100	...	157	423.2	2.735	100	...
Geographic regions ^c										
Among populations	1	15.038	0.287	7	0.001	1	3.131	0.008	0	0.001
Within populations	141	559.94	3.971	93	0.001	141	373.37	2.648	100	0.001
Total	142	574.98	4.258	100	...	142	376.5	2.656	100	...
Tissue of isolation ^d										
Among populations	1	8.375	0.078	2	0.001	1	5.591	0.037	1	0.001
Within populations	141	566.6	4.018	98	0.001	156	417.61	2.677	99	0.001
Total	142	574.98	4.097	100	...	157	423.2	2.714	100	...

^a P values are based on 1,000 permutations.

^b Sampled hosts included apricot, peach, plum, and sweet cherry.

^c Isolates originated from the regions of Imathia (central Macedonia) and Larissa (Thessaly).

^d Isolates originated from infected blossoms and fruit of each host.

Table 4. Nei's genetic identity (upper row for each host) and genetic distance (lower row) between populations of *Monilinia fructicola* and *M. laxa* from different hosts

Origin of population	Pathogen					
	<i>M. fructicola</i>			<i>M. laxa</i>		
	Apricot	Cherry	Plum	Apricot	Cherry	Plum
Peach	0.9791	0.9415	0.9273	0.9943	0.9977	0.9941
	0.0212	0.0603	0.0755	0.0058	0.0023	0.0059
Apricot	...	0.9461	0.9348	...	0.9918	0.9866
	...	0.0554	0.0674	...	0.0083	0.0134
Cherry	...	...	0.9759	...	...	0.9945
	...	...	0.0244	...	...	0.0056

among the two *M. laxa* populations originating from the two sampled regions. The absence of diversity among the two *M. laxa* populations can be explained by the fact that *M. laxa* is a native fungal species that exists for a long period of time in both regions. The two regions are not far away from each other but they are geographically completely separated by mountains and, thus, gene flow achieved through conidia dispersal via air, rain, or insects could be excluded. However, a low but constant gene flow can be achieved through planting infected material originating from Imathia, which is the center of stone-fruit production in Greece. Such constant gene flow between the two regions may have homogenized the fungal population, leading to absence of differences in the two populations. The higher diversity observed in *M. fructicola* populations originating from Imathia suggests that the pathogen has existed in the region for a significantly longer time than in the region of Larissa and, probably, the first invasion event of the pathogen in Greece may have taken place in the region of Imathia. Furthermore, because Imathia is the center of stone-fruit production in Greece, its numerous plant material production facilities have imported planting material for decades from other European countries for establishment of new orchards, and such imports might have contributed to pathogen introduction and increased genetic diversity in the country.

Host selection is considered to be one of the evolutionary forces that may shape the genetic structure of a fungal population (Leung et al. 1993). However, studies comparing the genetic diversity of either *M. fructicola* or *M. laxa* populations obtained from different hosts are either absent (in the case of *M. fructicola*) or scarce (in the case of *M. laxa*) (Förster and Adaskaveg 2000; Gell et al. 2007; Gril et al. 2008). The AMOVA of our data showed that, in the *M. fructicola* population, 24% of the total variation was due to separations based on the different hosts whereas, in *M. laxa*, it was only 5%. In addition, the values of the Nei's genetic identity for the *M. fructicola* population suggested that isolates from peach and apricot were strongly related and, similarly, isolates from cherry and plum were strongly related. Furthermore, isolates of *M. fructicola* from peach and apricot and from cherry and plum were grouped in separate clades in the constructed dendrogram. In contrast, isolates of *M. laxa* obtained from the four different sampled hosts were grouped together. Findings about the absence of genetic diversity among *M. laxa* isolates from the four different sampled hosts are consistent with an analysis based on RAPD and the presence or absence of the ribosomal DNA intron among isolates of *M. laxa* from a wide range of hosts in California that included apricot, almond, sweet cherry, peach, nectarine, plum, and prune (Förster and Adaskaveg 2000). In addition, in more recent studies, Gell et al. (2007) did not observe genetic diversity among *M. laxa* isolates originated from peach, apricot, and almond orchards in Spain, while Gril et al. (2008) found that *M. laxa* isolates obtained from apple fruit were genetically distinct from isolates obtained from apricot and sweet cherry but did

not observe any difference among the isolates originating from the two stone-fruit hosts. In Greece, all four stone-fruit species tested are cultivated in the same regions and very often in adjacent fields. Furthermore, *M. laxa* is considered to be an endemic species and, thus, the pathogen coexists with its hosts for a very long period of time. This ensures a high rate of gene flow among populations from different hosts that may contribute to a homogenization of the *M. laxa* populations (Delmotte et al. 1999).

The findings of genetic differentiation among *M. fructicola* isolates from peach and apricot or sweet cherry and plum could be related to several factors such as an adaptation to local environment, limited gene flow among different hosts, different origin, or different introduction time of the *M. fructicola* strains infecting the tested hosts. However, as explained earlier for *M. laxa*, the probability for an adaptation to the local environment or a limited gene flow among different stone-fruit hosts is restricted because these hosts are cultivated in the same region and in neighboring orchards and, thus, migration of strains can be easily achieved through natural means. Based on these assumptions, we can hypothesize that our data provide some indirect evidence of host specificity in *M. fructicola* populations. Species within the genus *Monilinia* are grouped into two distinct sections. *Juncetorae* species such as *M. fructicola*, *M. laxa*, and *M. fructigena* are considered polytrophic on cultivated or wild Rosaceae plants; however, there are some reports of a certain level of host preference in *M. fructigena* or *M. laxa* (Batra 1991). Interestingly, the clustering of the *M. fructicola* isolates based on their host of origin consisted of two distinct clades, one with isolates originating from fruit with smooth epidermis (plum and sweet cherry) and one with isolates originating from fruit with indumentum on the fruit surface (peach and apricot) (Fernández et al. 2011). It is well

Fig. 1. Dendrogram showing Nei's genetic distances in *Monilinia fructicola* and *M. laxa* populations originating from four different stone-fruit hosts. The dendrogram was constructed using intersimple sequence repeat data that clustered using the unweighted pair-group method with arithmetic average for multiple populations.

Table 5. Analysis of molecular variance among *Monilinia fructicola* populations from different hosts independently

Hosts, source of variation ^a	Population					
	Apricot		Cherry		Plum	
	Variance component	Variation (%)	Variance component	Variation (%)	Variance component	Variation (%)
Peach						
Among populations	0.412	10	1.22	28	1.554	33
Within populations	3.565	90	3.197	72	3.161	67
Total	3.977	100	4.417	100	4.715	100
Apricot						
Among populations	...	...	1.096	24	1.371	29
Within populations	...	...	3.525	76	3.412	71
Total	...	...	4.621	100	4.783	100
Cherry						
Among populations	...	...	...	...	0.471	14
Within populations	...	...	...	...	2.827	86
Total	...	...	...	...	3.298	100

^a Isolates were from the regions of both central Macedonia and Thessaly and from both infected blossoms and fruit.

established that fruit epidermis and its composition plays a crucial role in *M. fructicola* interactions with its hosts (Bostock et al. 1999; Lee and Bostock 2007). Cross-inoculation studies conducted previously on blossoms and fruit of the four tested hosts using *M. fructicola* isolates that had been collected from the same four hosts in Greece showed that all the tested isolates were pathogenic on all four hosts independently of the host of origin (Papavasileiou et al. 2015). However, further research is required using a larger number of isolates to investigate whether there is a putative better adaptation of *M. fructicola* strains on fruit with a smooth epidermis or with indumentum on the fruit surface.

In a previous monitoring study of our group, it was found that, in regions where *M. laxa* and *M. fructicola* coexisted, *M. laxa* was the predominant agent of blossom blight while *M. fructicola* was predominant on fruit infections (Papavasileiou et al. 2015). These differences were not the outcome of differential aggressiveness of *M. laxa* and *M. fructicola* on blossoms and fruit of the stone fruit tested but of a better adaptation of *M. laxa* to lower temperatures prevailing during the bloom period (Papavasileiou et al. 2015, Tamm et al. 1995). In the current study, for both fungal species tested, it was shown that the tissue of origin did not contribute to the observed genetic diversity. Isolates of both *M. fructicola* and *M. laxa* originating from infected blossoms were genetically similar to those obtained from infected fruit. Such a finding suggests that there is no kind of specificity in *M. laxa* and *M. fructicola* isolates in regards to the plant tissue that they infect. Our results are in agreement with previously established hypotheses on the epidemiology of brown rot. According to these hypotheses, primary infections that occur on blossoms during early spring provide the secondary inoculum for infections causing fruit blight at the first stages of fruit growth or fruit rot that occurs on mature fruit at either pre- or postharvest stages (Holb et al. 2013; Hong et al. 1997).

The above-mentioned results represent the first detailed report on the genotypic diversity of *M. laxa* and *M. fructicola* populations obtained from different stone-fruit hosts in Greece. This information can be of high importance for the understanding of fungal evolution in stone-fruit orchards and the design of more effective brown rot control strategies. Fungicide applications may also be a selection factor shaping the genetic structure of a fungal population. Recent studies aiming to investigate the fungicide resistance profile of the *M. fructicola* and *M. laxa* populations have showed that the entire *M. fructicola* population is resistant to the benzimidazole fungicides (G. Karaoglanidis, unpublished data) whereas, in *M. laxa*, frequency of resistance to benzimidazoles was found to be either low or moderate (G. Karaoglanidis, unpublished data) (Malandrakis et al. 2012). Taking into account that *M. fructicola* grows faster, sporulates more abundantly, shows better dispersal ability compared with *M. laxa*, and is more prone to fungicide resistance development (Villarino et al. 2013), and that it is still a quarantine pathogen for EU countries, continuous monitoring is required to detect possible changes in the population and the genetic structure of brown rot causal agents.

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