Characterization of *M. laxa* and *M. fructigena* isolates from Hungary with MP-PCR

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Abstract

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Monilinia laxa (*Monilia laxa*), *Monilinia fructicola* (*Monilia fructicola*) and *Monilinia fructigena* (*Monilia fructigena*) are the causal agents of brown rot on pome and stone fruits in Hungary. Forty-five isolates collected from different hosts, different years in several orchards were used for characterization of the *M. laxa* and *M. fructigena* population in Hungary. The isolates were identified on species level based on morphological and molecular biological methods; out of these 24 were *M. laxa*, 20 were *M. fructigena* and 1 was *M. fructicola*. Populations of the three *Monilinia* species were studied with microsatellite primers and the degree of genetic diversity within the species was measured. The population structure analysis revealed that genetic diversity within *M. laxa* subpopulations was H_S = 0.1599, while within *M. fructigena* subpopulations was H_S = 0.2551. The total genetic diversity was H_T = 0.3846, while genetic diversity between *M. laxa* and *M. fructigena* subpopulations was D_{ST} = 0.1771. No clustering relationship was observed among isolates by the different years or hosts.

Keywords: brown rot; intraspecific diversity; microsatellite primers

Four species of *Monilinia*: *Monilinia laxa* (anamorph: *Monilia laxa*), *Monilinia fructigena* (anamorph: *Monilia fructigena*), *Monilinia fructicola* (anamorph: *Monilia fructicola*), and *Monilinia linhartiana* (anamorph: *Monilia linhartiana*) cause brown rot in *Prunus* spp. (stone fruits), *Malus* and *Pyrus* spp. (pome fruits) (Wormald 1954; Byrde, Willets 1977; Ogawa, English 1960), including cash crop cherry, apple, plum and several ornamental plants (van Leeuwen et al. 2002).

Monilia laxa and *Monilia fructigena* have long been known in Hungary, while *M. fructicola* was first isolated from imported fruits in 2005 (Petróczy, Palkovics 2006). The national survey conducted by the Central Agriculture Office Central Laboratory for Pest Diagnosis in 2006 found *M. fructicola* in the orchards (Kiss 2007).

M. fructicola is listed as a quarantine pathogen in the European Union (Council Directive 77/93/CEE, 1976; 2000/29/EC) and *M. fructigena* is listed as a quarantine pathogen in the USA (Code of Federal Regulations 1996).

M. laxa and *M. fructicola* can cause blossom blight, twig blight and fruit-rot (Rozsnyay, Vajna 2001; Holb 2003). *M. laxa* can only infect fruits with damaged cuticle while *M. fructicola* is capable of infecting fruits through the intact tissues. *M. fructigena* primarily causes fruit rot mainly in pome fruits (Ubrizsy 1965).

The precise distinction between *Monilinia* species based on the morphological characters requires great expertise, however, nowadays a plenty of molecular biological methods are available for the identification of the species. Fulton and

Brown (1997) located a group I intron in the ribosomal small subunit (SSU) gene of *M. fructicola*. Ioos and Frey (2000) developed a rapid identification method of the *Monilinia* species in a single PCR-run. In recent times, with the development of the multiplex PCR method, the identification of *Monilinia* species based on the size of the PCR products became easier (Cote et al. 2004).

Gell et al. (2007) were the first in Europe to study *M. laxa* populations from several orchards in Spain, using RAPD markers revealing the highest genetic diversity within subpopulations. Gril et al. (2008) analyzed the genetic diversity and relationships of *Monilinia laxa* isolates obtained from different host plants with amplified fragment length polymorphism (AFLP) marker system. Their results showed host restricted specialization of isolates, with *M. laxa* isolates from apple clearly distinguished.

In the present study, the genetic variation in the populations of three *Monilinia* species (*M. laxa, M. fructigena* and *M. fructicola*), present in Hungary on different host-plants and in different locations was investigated for the level of host-specialisation by applying microsatellite primers (MP-PCR).

MATERIAL AND METHODS

Collection and description of isolates. Isolates obtained from natural infections were collected from several orchards in various years and from different hosts in Hungary. Altogether 44 isolates of the 2 *Monilinia species* (*M. laxa* and *M. fructigena*) from 9 different hosts, collected between 2002 and 2007 were used in this study (Table 1). The only *M. fructicola* isolate was from isolates collected during the national survey in 2006 (Kiss 2007).

Small tissue pieces were dissected from the edges of single lesions of fruits or twig cancers. The infected tissue samples were incubated in Petri dishes on potato-dextrose agar (PDA) medium amended with 0.5 g/l of chloramphenicol at 25°C for 2 days. Sporulation was induced by growing mycelia at 5°C for 3 months on tomato agar (1% glucose, 2% agar, 14% tomato pulp with concentration of 2 %). Isolates were stored on PDA under mineral oil at 5°C. Species identification of the *Monilinia* isolates was based on morphological and conidia characteristics (Batra 1991; Lane 2002).

DNA extraction. All fungal isolates were grown on potato-agar plates covered with sterile fabric discs to facilitate the subsequent removal of the mycelium from the agar. DNA was extracted from 20 mg mycelium that was pulverised in a mortar in liquid N_2 using the DNA extraction KIT (Fermentas, St. Leon-Rot, Germany). The genomic DNA pellet was suspended in 20 µg/ml Rnase (Fermentas). The concentrations of the samples were quantified with spectrophotometry (SmartSpec Plus Spectrometer; Bio-Rad Laboratories, Inc., Budapest, Hungary) and the final concentration was set to 50 ng/µl. It was diluted 50 ng/µl for PCR used.

Species-specific PCR. Primer-pairs identified by Ioos and Frey (2000) were used for the determination of the species.

MP-PCR amplification reactions. The following seven microsatellite primers were used for MP-PCR: $(CAC)_4RC$, $(GTG)_5$, $(GATA)_4$, $(GAG)_4RC$, $(GTC)_5$, T_3B and M13 (Intelmann, Spring 2002).

Amplification reactions were carried out in the volume of 25 µl solution containing 50 ng of template DNA; 1.5mM $MgCl_2$; 1× reaction buffer; 1U Taq polymerase (Fermentas); 100µM dNTP; 1µM of each primers and sterile milliQ water. There was also a negative control created without using DNA. All the reactions were repeated with all the isolates three times with each primer.

The PCR amplification reactions were performed in PCR System 2700 (Applied Biosystem, Forest City, USA) according to the following steps: an initial denaturation (at 94°C for 3 min) followed by 38 cycles of denaturation (at 94°C for 30 s), annealing (at 55°C for 30 s), extension (at 72°C for 2 min), and a final extension (at 72°C for 7 min).

The PCR products were separated with electrophoresis in 1.5% agarose gel in 0.5× TBE buffer at 50 V for 50 min and the gels were stained with ethidium bromide and photographed under UV light (F1- F2 Fuses type T2A; Bio-Rad Laboratories, Inc., Milan, Italy).

Data analysis. Genetic variation among all pairs of isolates was calculated in a way that presence or absence of each amplified band was marked with 1 or 0, and all bands were weighed equally. The data were grouped into a matrix and – using the Treecon program pack (Van de Peer, De Wachter 1997) – a dendrogram was made with UPGMA method considering the simple matching coefficient.

Analysis of gene diversity and structure of population. Gene diversity in the total population (H_T) was divided into gene diversities within (H_S) and between (D_{ST}) subpopulations, $H_T = H_S + D_{ST}$ (Nei 1987; Takezaki, Nei 1996).

Genetic differentiation relative to the total population was calculated by the coefficient of gene

Table 1. Origin of the *Monilia* isolates, the PCR reaction

Isolate name	Origin				Amplification with the primer pair specific for [(+) specific reaction; (–) no specific reaction]		
	host	part of the plant	year of isolation	source	*M. laxa*	*M. fructigena*	*M. fructicola*
Sz1, Sz6	*Armeniaca vulgaris*	fruit	2004	Kunmadaras	+	–	–
Sz2	*Cerasus vulgaris*	fruit	2004	Kunmadaras	+	–	–
Sz5	*Cerasus avium*	fruit	2004	Dunaföldvár	+	–	–
Sz12	*Prunus domestica*	fruit	2004	Kunmadaras	+	–	–
Sz14	*Cerasus vulgaris*	fruit	2004	Tiszabura	+	–	–
Sz19	*Cerasus avium*	fruit	2002	Vas county	+	–	–
Sz20	*Cerasus vulgaris*	fruit	2002	Vas county	+	–	–
Sz38, Sz40, Sz43, Sz44	*Amygdalus communis*	shoot	2004	Érd	+	–	–
Sz53	*Malus domestica*	fruit	2004	Gödöllő	+	–	–
Sz54	*Cerasus vulgaris*	fruit	2005	Budapest	+	–	–
Sz66	*Cerasus vulgaris*	fruit	2005	Érd	+	–	–
FC1	*Armeniaca vulgaris*	fruit	2006	Csongrád county	+	–	–
Sz78	*Armeniaca vulgaris*	fruit	2005	Kunmadaras	+	–	–
Sz128	*Cerasus vulgaris*	fruit	2007	Kunmadaras	+	–	–
B1, B6, B25	*Prunus domestica*	fruit	2004	Kisvárda	+	–	–
B28a	*Persica vulgaris*	fruit	2004	Kisvárda	+	–	–
Sz8	*Malus domestica*	fruit	2004	Kunmadaras	–	+	–
Sz35	*Malus domestica*	shoot	2004	Gödöllő	–	+	–
Sz37	*Cydonia oblonga*	fruit	2004	Szada	–	+	–
FC4	*Prunus domestica*	fruit	2006	Vas county	–	+	–
Sz70, Sz71, Sz72, Sz75	*Armeniaca vulgaris*	fruit	2005	Kunmadaras	–	+	–
Sz81, Sz83	*Pyrus communis*	fruit	2004	Kunmadaras	–	+	–
Sz86, Sz87, Sz89	*Prunus domestica*	fruit	2004	Kunmadaras	–	+	–
Sz90	*Armeniaca vulgaris*	fruit	2004	Zsámbok	–	+	–
Sz96	*Malus domestica*	fruit	2004	Zsámbok	–	+	–
B5, B12	*Prunus domestica*	fruit	2004	Kisvárda	–	+	–
B20	*Malus domestica*	fruit	2004	Kisvárda	–	+	–
FC2	*Persica vulgaris*	fruit	2006	Szolnok county	–	–	+

differentiation (Nei 1987; Takezaki, Nei 1996): $G_{ST} = D_{ST}/H_T \times G_{ST}$ can take values between 0.0 (no differentiation between subpopulations) and 1.0 (complete identity within each subpopulation and complete differentiation between subpopulations).

RESULTS AND DISCUSSION

Out of 45 isolates 24 were *M. laxa*, 20 were *M. fructigena* and 1 was *M. fructicola* (Table 1). Seven microsatellite primers were used to chart the 45 isolates. In the case of two primers [T_3B and $(GATA)_4$] out of the seven primers, reactions did not give amplification products. In the course of the testing of the 45 isolates altogether 52 bands were obtained in the case of five primers [$(GAG)_4RC$, $(CAC)_4RC$, $(GTG)_5$, M13 and $(GTC)_5$]. Thirteen bands were generated with GAG_4 primer. The *M. laxa* and *M. fructigena* isolates gave a well-distinguishable pattern with this primer. The bands marked with circles in Fig. 1 had the size

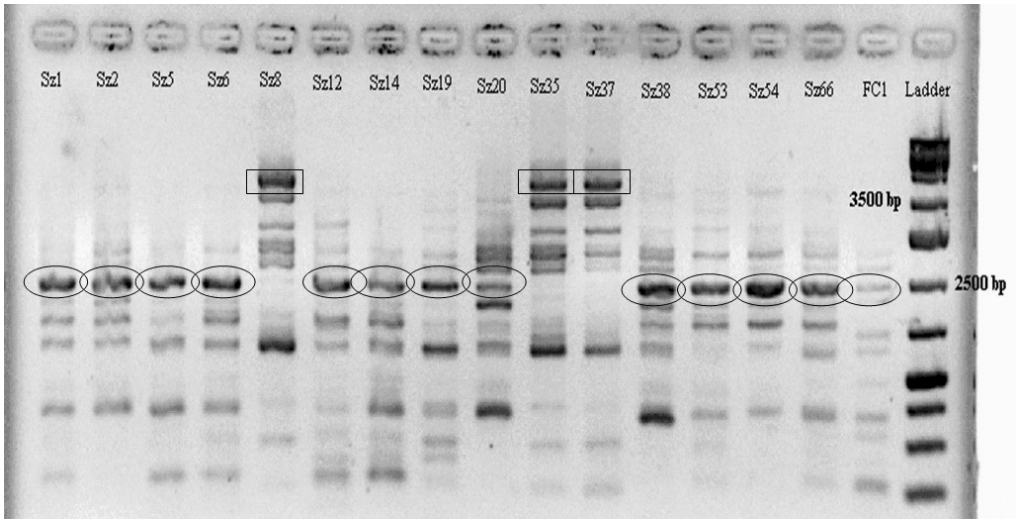

Fig. 1. PCR product from *Monilia* isolates generated by (GAG_4)RC
From left to right the gel contains the following isolates: Sz1, Sz2, Sz5, Sz6, Sz8, Sz12, Sz14, Sz19, Sz20, Sz35, Sz37, Sz38, Sz53, Sz54, Sz66, FC1. The bands marked with circles are characteristic of the *M. laxa* isolates and the bands marked with squares are characteristic of the *M. fructigena* isolates

of 2,500 bp and they are only characteristic of *M. laxa*, whereas the bands marked with squares have the size of 3,500 bp and they are only characteristic of *M. fructigena*.

Twelve bands were distinguished with the $(CAC)_4$RC primer. The pattern of *M. fructicola* was different from the patterns of both *M. fructigena* and *M. laxa*. The 2,000 and the 2,500 bp-long bands were only characteristic of *M. laxa*. Thirteen bands were obtained with $(GTG)_5$ primer. The double stripes of the 2,000 bp-long bands, characteristic of *M. laxa*, are well-distinguishable. Seven products were distinguished with each of the primers GTC and M13.

The three species could be distinguished from one another by analysing MP-PCR data. The dendrogram was obtained from the 52 amplicons (Fig. 2). *M. fructicola* formed a different branch in the genetic tree whereas *M. laxa* and *M. fructigena* proceeded from a common branch. In addition, both *M. laxa* and *M. fructigena* can be distinguished well on cluster level.

The studied year- and host- specialisations could not be distinguished on the genome level. Analysis of genetic structure applying the Nei's gene diversity (Nei 1987) based on 47 ISSR loci, the population structure analysis revealed that genetic diversity within *M. laxa* subpopulations was H_S = 0.1599, while within *M. fructigena* subpopulations were H_S = 0.2551. Diversity within *M. fructigena* was greater than in *M. laxa* populations. The total genetic diversity was H_T = 0.3846, while genetic diversity between *M. laxa* and *M. fructigena* subpopulations was D_{ST} = 0.1771. Förster and Adaskaveg (2000) reported low degree of genetic diversity within *M. laxa* populations in Californian orchards. Gell et al. (2007) conducted first European study to determine degree of genetic diversity in *M. laxa* within and among the orchards in Spain. It was concluded that the genetic diversity within subpopulations (orchards) was H_S = 0.567, while genetic diversity between subpopulations was D_{ST} = 0.018.

The relative magnitude of gene differentiation coefficient for *M. laxa* and *M. fructigena* subpopulations were G_{ST} = 0.4604. While Nei's unbiased genetic distances between *M. laxa* and *M. fructigena* were 0.5839 (Nei 1987).

Advances in molecular biology, particularly the PCR technique, provided new opportunities for the rapid detection of isolates of plant pathogens and the changes in their populations. Pathogens in different geographic regions usually differ in allele or genotype frequencies, and plant pathologists usually treat collections of isolates from different locations as separate populations. However, a high level of gene flow between two geographically distant populations may unite them into homogeneous genetic groups that are evolving together (Boeger et al. 1993).

In the course of the mapping of the genome, the similarities and the differences among the species can be revealed, which can also account for differences in the pathogenicity (Ogawa, English 1960).

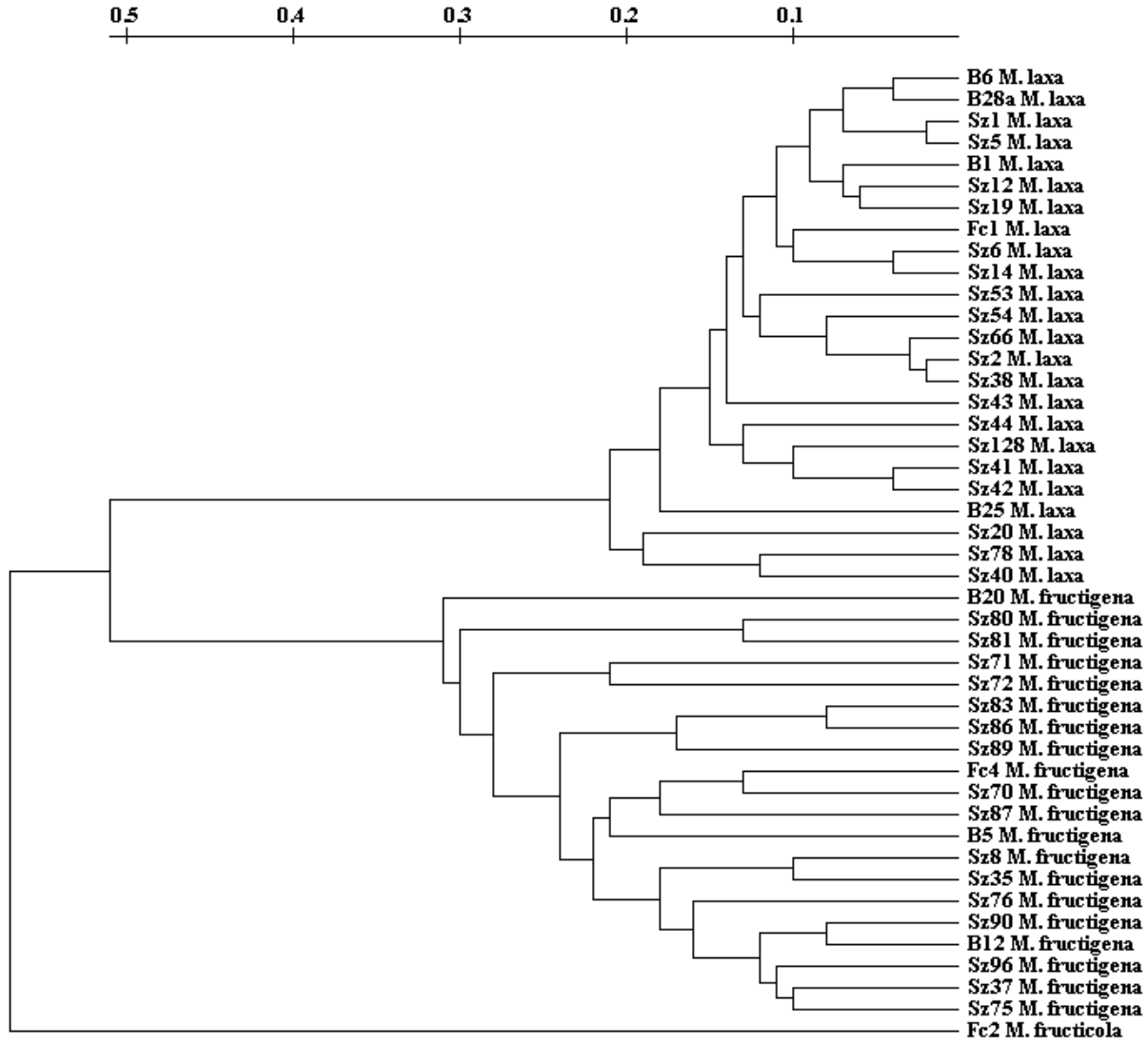

Fig. 2. Phenogram generated with the unweighted pair-group method with average cluster analysis of microsatellite-primer PCR data set from isolates of *M. laxa*, *M. fructigena* and *M. fructicola* from different host plants in Hungary

Snyder and Jones (1999) also assessed a microsatellite-primer PCR assay with primers $(GACA)_4$ and $(GTG)_5$ in order to differentiate *M. laxa* from *M. fructicola*. The obtained DNA fingerprints showed that there was a little intraspecific variation with both primers, but the banding patterns clearly distinguished *M. laxa* from *M. fructicola*. Förster and Adaskaveg (2000) found minor genetic variability among the Californian isolates of *M. laxa* with the analysis of the MP-PCR data set.

In another experiment the microsatellite primer M13 amplified a 735-bp DNA fragment from *M. fructicola* and a 732-bp fragment from *M. laxa* that were not differentiable in a 1.5% agarose gel, but the nucleotide sequences of these two fragments showed significant differences with an overall 58% dissimilarity (Ma et al. 2003). They found microsatellite primers that also generated unique bands for species other than *M. fructicola* (Weising et al. 1995; Ma et al. 2001).

In addition to the degree of the developing variability it is also important to deal with host specialization. It is worth observing the possible host specialisation during the genetic mapping and it is also worth studying of the genetic evolution of the populations. In the study, similarly to the one conducted by Gell et al. (2007), no relationship was found in clustering among isolates according to the year of isolation or original host plant. According to this fact and the considerable variability within the populations revealed with MP-PCR, it might be concluded that there was no host specialisation in

the investigated *Monilinia* population in Hungary. Gril et al. (2008) carried out an analysis with AFLP marker system which shows that *Monilinia* isolates were not grouped according to geographic origin.

The geographic isolation disappeared with globalisation presenting plant pathogens, their biotypes and physiological races within a species, with unlimited possibilities to spread among the continents and the countries (Vajna 2007). The conidia can easily be conveyed from one place to the other with cargoes or through the air. Considering the great variability within *Monilinia* isolates it is imperative to closely monitor the population of *Monilinia* isolates in order to act promptly once more pathogenic, aggressive, host-specific and perhaps fungicide-resistant genotype appears.

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