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MOLECULAR CHARACTERIZATION OF *MONILINIA LAXA* AND *M. FRUCTIGENA* CAUSING BROWN ROT OF SWEET CHERRY IN ZEMGALE REGION OF LATVIA

Julija Volkova, Liga Vilka, Lasma Stanke, Regina Rancane, Anna Bazenova

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In 2012 samples of rotted sweet cherry fruits were collected in Dobele in sweet cherry plantations of Latvia State Institute of Fruit – Growing. Causal agents of fruit rots were detected and identified by sequencing of ribosomal RNA gene region containing two internal transcribed spacers and the 5.8S rRNA gene (ITS1-5.8S-ITS2). In majority of samples *Monilinia laxa* Aderh. & Ruhland Honey was identified as causal agent of fruit rot, although in some samples also *M. fructigena* Aderh. & Ruhland, Honey occured. Other causal agents of fruit rot – *Botrytis cinerea* Pers., *Colletotrichum acutatum* Simmonds, *Phomopsis* spp. were identified as well.

Fungal isolates belonging to genus *Monilinia* were analysed in comparison with isolates from other plantations nearby. The quarantine pathogenic fungus *M. fructicola* (G. Winter) Honey was not found in the samples from sweet cherry. The DNA sequences of ITS region from samples of *M. laxa* from Zemgale were highly similar with only a few polymorphisms and the sequences were also quite similar to isolates of *M. laxa* from stone fruits in other countries.

This study provides first description of *M. fructigena* detected in stone fruits in Latvia.

Key words: *Monilinia laxa*, *Monilinia fructigena*, sweet cherry.

Julija Volkova, Lasma Stanke. University of Latvia, Faculty of Biology, Kronvalda bv.4, Riga, LV-1010, Latvia: e-mail: julija.volkova@laapc.lv, lasma.stanke@gmail.com
Liga Vilka. Latvian University of Agriculture, Liela iela 2, Jelgava, LV-3001: e-mail: liga.vilka@laapc.lv
Julija Volkova, Regina Rancane, Anna Bazenova. Latvian Plant Protection Research centre, Struktoru str. 14a, Riga, LV-1039, Latvia, e-mail: regina.rancane@laapc.lv, anna.bazenova@laapc.lv

INTRODUCTION

Most important species from fungal genus *Monilinia* in Europe are M. *fructigena* Aderh. & Ruhland, *M. laxa* Aderh. & Ruhland Honey, *M. fructicola* G. Winter, Honey and also *M. polystroma* G. Van Leeuwen (EPPO 2009). Fungi from genus *Monilinia* cause brown rot of numerous fruit crops from plant genus Rosaceae, but the most economically important damages occur in pome fruits, as well as in stone fruits. Traditionally *M. fructigena* has been considered as causal agent for pome fruits, but in stone fruits most widespread species is *M. laxa*, as well as the highly destructive quarantine fungus *M. fructicola*. First described in Japan, but nowadays occurring also in Europe is *M. polystroma*, which is closely related to *M. fructigena*. The differences between these two species were identified, when isolates from Asian countries were compared to isolates from Europe (Van Leeuwen et al. 2002).

A number of studies suggest that fungi from genus *Monilinia* are not strictly specialized to pome fruits and stone fruits, and can cross-infect both groups of plants. *M. fructigena* has been reported as causal agent of brown rot of stone fruits in South Africa (Carstens et al. 2010), in Hungary (Petróczy et al. 2011), in China (Zhu, Chen 2011), in France and Spain (Ioos, Frey 2000), in Lithuania (Valiuškaite et al. 2005) and in Poland (Poniatowska et al. 2013).

Information about genetic diversity of *Monilinia* isolates is contradictory. Earlier research (Fulton et al. 1999) based on ITS region data indicated that there is no apparent distinction between isolates from pome fruits (*Malus* spp.) and isolates from stone fruits (*Prunus* spp.) and that isolates do not cluster according to geographical region of isolation. Later it was found that isolates of *M. fructigena* may differ depending on origin, e.g., Chinese and European isolates are genetically distinct (Zhu & Chen 2011). In contrast, isolates of *Monilina laxa* can be variable, but are randomly distributed worldwide (Fulton et al. 1999). Analysis of amplified fragment length polymorphism (AFLP) patterns in *M. laxa* found significant differences between isolates from apple trees and isolates from other host plants (Gril et al. 2008). No further grouping according to any other host plant was observed.

Numerous methods for studying genetic diversity have been used in genus *Monilinia*. Several species specific primers for molecular identification and differentiation of *M. laxa*, *M. fructigena, M. fructicola* and *M. polystroma* have been developed based on sequencing data of ITS region of rDNA (Ioos & Frey 2000, Côté et al. 2004, Gell et al. 2007), and the protocol developed by R. Ioos (2000) is suggested as reference in EPPO guideline Nr. PM 7/18 (2).

The aim of the study was to identify general causal agents of brown rot in sweet cherry plantations under protective cover in Latvia.

MATERIAL AND METHODS

Sample collection and pathogen isolation

During survey samples of rotted sweet cherry fruits were collected in sweet cherry plantations under protective rain cover VOEN in Dobele, Latvia State Institute of Fruit – Growing (LSIFG) in 2012**.** Rotted fruits were collected randomly over plantation from cultivars 'Iputj', 'Krupnoplodnaja', 'Karmela','Tiki', 'Meelika','Radice' and 'Kadrin'**.** 56 samples were collected in total. Typical sporodochia of brown rot from infected fruits were aseptically transferred to Petri dishes with the potato dextrose agar (PDA) and grown for 5-7 days at 22°C in the dark. After 7 days cultures were examined for morphological characteristics, and the pure cultures without infection of secondary fungi or bacteria were transferred to 50 ml tubes with 25 ml liquid malt extract broth (MEB) and grown until appropriate amount of mycelium was obtained, typically for 7-10 days at 22°C in the dark. Afterwards mycelium was collected, grinded in mortar, placed in a 2 ml Eppendorf tube, covered with 96% ethanol and stored at +5°C until further processing. DNA was extracted using DNeasy Plant Mini kit (Qiagen, Hilden, Germany) protocol and stored at -20°C until

Table 1. Species-specific primers used for PCR amplification

Primer name	Sequence 5'-3'	Specific to species	Author, year
ITS1Mlx (forw)	TATGCTCGCCAGAGAATAATC	*M. laxa*	Ioos, Frey 2000
ITS4Mlx (rev)	TGGGTTTTGGCAGAAGCACACC		
ITS1Mfgn (forw)	CACGCTCGCCAGAGAATAACC	*M. fructigena*	
ITS4Mfgn (rev)	GGTGTTTTGCCAGAAGCACACT		
ITS1MFcl (forw)	TATGCTCGCCAGAGGATAATT	*M. fructicola*	
ITS4Mfcl (rev)	TGGGTTTTGGCAGAAGCACACT		

Table 2. Sequences of Monilinia from NCBI GeneBank used for construction of phylogenetic tree

Plant species	**GenBank accesion**	**Origin**	**Author, year**	**Fungal species**
Prunus insititia	AF150673	France	Ioos, 1999	*M. laxa*
Stone fruit	EF153015	Spain	Gell, 2007	*M. laxa*
Malus domestica	Z73784	Norway	Holst, 1996	*M. laxa*
Prunus insititia	AF150680	France	Ioos, 2000	*M. fructigena*
Prunus domesticus	Z73781	Norway	Holst, 1996	*M. fructigena*
Pyrus sp.	FJ515296	Belgium	Fauche,2008	*M. fructigena*
Prunus persica	JN001480	Chile	Valencia, 2011	*M. fructicola*
Malus domestica	JX315717	Serbia	Vasic, 2012	*M. polystroma*

needed. Isolates used in this study have been put for long-term storage in freezer at -85ºC, covered with 15% glycerol, in culture collection of Latvian Plant Protection Research centre.

Identification with species-specific primers

As a first step, DNA from fungal isolates were analyzed with the primer set for conventional PCR specific for *M. laxa*, *M. fructigena*, *M. fructicola* developed by Ioos and Frey (2000) and established as standard EPPO method to distinguish these three species (Table 1.). Amplifications were performed in a 25 µl reaction mixture containing 12.5 µl Thermo Scientific DreamTaq PCR Master Mix, 0.5 µl 10 µM of each forward and reverse primers (IDTDNA, Germany), 0.375 µl of 20 mg ml^{-1} Bovine Serum Albumin (Thermo Scientific, Lafayette, USA), 10.125 µl molecular biology grade nuclease free water (Thermo Scientific) and 1 µl template DNA. PCR reactions were carried out in Eppendorf Personal Mastercycler at following conditions: initial denaturation – 5 min 94°C, followed by 30 cycles – 1.5 min 94°C, 2min 62°C, 3 min 72°C and final waiting 4°C. PCR products were separated by electrophoresis on 1% agarose gel in 0.5×TAE buffer. Gels were stained with Nancy 520 (Sigma – Aldrich) and photographed under UV. In total three PCR reactions for each sample were done.

PCR reaction with universal primers and sample sequencing

PCR amplification was performed in a 25 µl reaction mixture containing of 1 µl target DNA, *Taq* DNA polymerase 5 u/µl – 0.125 µl (Thermo Scientific), 2.5 µl of 25 mM $MgCl_2$, mixture of 2 mM dNTP - 2.5 µl in total, 10x *Taq* buffer with KCl – 2.5 µl, nuclease free water (Thermo Scientific) – 15.875 µl, 0.5 µl of each ITSF1 (Gardes, Bruns 1993) and ITS4 (White, Bruns

Table 3. Origin of samples used in this study, results of identification with species specific primers and according to sequencing result search in BLASTn

Origin	Cultivar	Legend	Fungal species according to BLASTn	PCR
	‘Kadrin’	DKa_I	*M. laxa*	*M. laxa*
		DKa_II	*M. laxa*	*M. laxa*
	‘Krupnoplodnaja’	DK_I	*M. laxa*	*M. laxa*
		DK_II	-	*M. fructigena*
		DK_III	-	-
		DK_IV	*M. laxa*	*M. laxa*
	‘Lapins’	DL_I	*M. laxa*	*M. laxa*
	‘Tiki’	DT_I	*M. laxa*	*M. laxa*
Dobele		DT_II	-	*M. laxa*
		DT_III	-	*M.laxa/M.fructigena*
		DT_IV	*M. fructigena*	*M. fructigena*
	‘Iputj’	DI_I	-	*M. laxa*
		DI_II	-	*M.laxa/M.fructigena*
		DI_III	-	-
		DI_IV	-	*M. laxa*
	‘Radice’	DR_I	*M. fructigena*	*M.laxa/M.fructigena*
	‘Tiki’	DT_IV	*M. fructigena*	*M.laxa/M.fructigena*
	‘Karmel’	DKm_I	-	*M. fructigena*
	‘Meelika’	LM_I	*M. laxa*	*M. laxa*
Kuldīga	‘Tjučevka’	LT_I	*M. laxa*	*M. laxa*
	Unknown	LN_I	*M. laxa*	*M. laxa*
Sesava	‘Krupnoplodnaja’	OK_I	*M. fructigena*	-
Pūre	‘Dragona dzeltenais’	PuD_I	*M. laxa*	-

1990) (IDTDNA, Germany) primer. The fungal ribosomal RNA gene region containing two internal transcribed spacers and the 5.8S rRNA gene (ITS1-5.8S-ITS2) was amplified. Before sequencing PCR products were treated with the Exo I/FastAP (Thermo Scientific) according to manufacturer guidelines. Sequencing was done using the same primers as for PCR at Macrogen Europe (Netherlands).

ITS data and phylogenetic analysis

DNA sequences were assembled using Staden Package GAP4 software (Staden et al. 1996) and aligned using multiple sequence alignment tool Clustal X2 (Larkin et al. 2007). Phylogenetic analysis of the sequence data was done with the MEGA 5.1. (Tamura et al. 2011) using Maximal Likelihood Method, and the quality of dendrograms was assessed by 100 bootstrap replicates.

Publicly available sequences of *Monilinia* ITS region were downloaded from the NCBI GeneBank (Table 2).

RESULTS

In total 18 isolates from LSIFG were analyzed and 5 isolates from other locations were analyzed and used for comparison (Table 2), all isolates were pure cultures. PCR analyses with species-specific primers were not always successful for accurate identification and differentiation between *M. laxa*, *M. fructigena* and *M. fructicola*, since double positive results were often observed with the primers used in this study. Increasing of the annealing temperature from +58ºC to +62ºC improved specificity, but still did not allow unambiguous species identification. Only one isolate was clearly identified as *M. fructigena* (DT_IV) with the species-specific primers, while most of the isolates were identified as *M. laxa*. Samples that yielded a clear single band in PCR with the species-specific primers were used for sequencing (Table 2). Isolate DKm_I were positive with the species specific primer set for *M. fuctigena*, but bands were not clear, and PCR product from this sample were not used for sequencing.

In total, ITS region was sequenced from 14 isolates. BLASTn homology search against NCBI Genbank database and phylogenetic analysis provided similar results as the PCR analysis with species-specific primers. Of all sequenced isolates of *Monilinia* three clustered together with reference isolates of *M. fructigena*, while

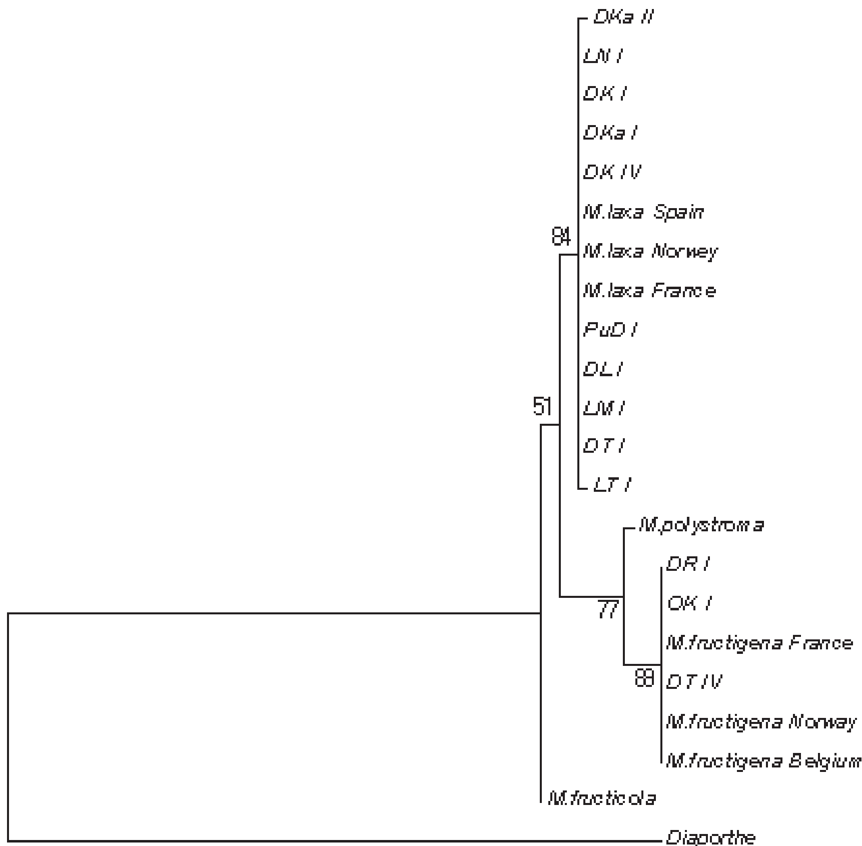

Fig.1. Phylogenetic analysis of *Monilinia* spp. isolated from sweet cherry and comparison with the sequences of identified isolates from other countries based on rRNA ITS 1 and ITS2 region.

the rest with *M. laxa* reference isolates. Multiple sequence alignment showed that sequences of *M. laxa* were mostly identical not just within one location – Dobele, but also if compared with the sequences from other locations – Sesava, Kuldīga and Pūre. A single nucleotide exchange (C/A) was observed at position 160 bp in the sequence of LT_I isolate (Kuldīga), and a C to T change was observed at position 90 bp in the DKa_II isolate sequence (Dobele).

Other species from genus *Monilinia*, such as *M. fructicola* or *M. polystroma* were not identified in this study. Reference sequences of *M. fructicola* and *M.polystroma* clustered separately with high bootstrap values in phylogenetic analyses.

Dendrogram was constructed with MEGA 5.1. Beta 3 software, Maximum Likelikehood Method. Bootstrap values are displayed at the branches. Sample codes are provided in Table 2. Phylogenetic tree is rooted by corresponding sequence from distantly related species *Diaporthe alni*.

DISCUSSION

Influenced by plant trade and also supported by climatic changes some fungi are increasingly distributed outside their natural biotopes and can be found well adapted worldwide. *M. fructicola* is one of the most typical examples. Originally it was found in the USA and until 1928 it was frequently reported as *M. fructigena* (Batra 1991). Later it was found that *M. fructicola* is more pathogenic and faster growing especially in areas with mild climate and it has been reported also in France since 2001 (EPPO 2009) and later has been found not just in Mediterranean region (Pellegrino et al. 2009) and Balkans, but also in Eastern Europe (Poniatowska et al. 2013).

M. fructicola has not been reported in Baltic countries. The importance of *M. fructigena* in stone fruit orchards is unknown. *M. fructigena* has been reported as causal agent of stone fruit brown rot (Ioos & Frey 2000, Valiuškaite et al. 2005, Poniatowska et al. 2013, Zhu & Chen 2011). In this study *M. fructigena* were isolated from rotted sweet cherry fruits in Latvia. Pathogenicity and difference between stone fruits and pome fruits is not known and genetic variation between isolates from both groups of plants is low. Sequences of rRNA ITS 1 and ITS2 region *of M. fructigena* sequenced in this study were identical to sequences downloaded from NCBI GeneBank, even though all three sequences were from different locations in Latvia.

Local observations of correct causal agents of brown rot are necessary not just in sweet cherry plantations but also in plum plantations and pome fruit orchards. Range of fungicides that could be used for chemical control of *Monilinia* fungi is limited in Latvia (LR Augu Aizsardzības līdzekļu saraksts, 2013), and fungicides with the same mode of action often are used for many years in one plantation, which may lead to developing and spread of *Monilinia* strains that are resistant to fungicides. Observations of genetic diversity in local fungal isolates and changes of diversity over time in combination with records of characteristic isolates could provide useful information about fungicide resistance genes and development of resistance in the future (Cox et al. 2009).

CONCLUSIONS

M. laxa and M. *fructigena* species were found in sweet cherry plantations in Latvia requiring further investigations to observe diversity and relative frequency of occurrance of each of the species.

Isolates of *M. laxa* were highly similar within ITS region, with just a few base pair changes observed, while isolates of *M. fructigena* were identical to reference sequences.

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