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SPECIFICITY OF SSR LOCI FOR *Melampsora* SPECIES ON POPLARS

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Two rust fungi, *Melampsora larici populina* and *Melampsora medusae* are common in all poplar growing regions worlwide. *M. larici populina* is native to Eurasia, while *M. medusae* is endemic to eastern Unated States on *Populus deltoides*. Poplar leaf rust (*Melampsora* sp.) is widely spread disease in our country, and can cause significant growing problems. Race that prevails in our growing region is *M. Larici populina*. This work initiated the research of population composition, i.e. qualitative participation of *Melampsora* races in popoluation of black poplars hybrid

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progenies using molecular methods. When five SSR markers were used the results showed that *M. larici populina* was found in three tested clones, while only *M. medusae* was determined in clone I-214.

Key words: genetic characterisation, *Melampsora* sp., microsatellites, poplar

INTRODUCTION

Melampsora species and their hybrids are obligate parasites of different poplar species worldwide. Two rust fungi, *Melampsora larici populina* and *Melampsora medusae* are common in all poplar growing regions. *M. larici populina* is native to Eurasia, but it also appears in Australia, New Zealand, western Unated States (NEWCOMBE *et al*., 2000), and in eastern Canada (INNES *et al.,* 2004). It belongs to dominant. European rust species on poplars which caused in the past decades severe economic losses in poplar growing regions (PINON and FREY, 2005). Although *M. medusae* is endemic to eastern Unated States on *Populus deltoides*, it was also introduced to Argentina, Australia, Africa, and western Unated States and according to OEPP/EPPO (1982) sources, this race was found also in Europe. Poplar leaf rust (*Melampsora* sp) is also widely spread in our country.

They occur each year in poplar nurseries and plantations, and they can cause significant growing problems depending on clone sensitivity and climatic conditions.

The consequences of pathogen attack are reduced assimilation surface, diminished photosynthesis, premature defoliation, and physiological weakness and reduced growth of cultivated plants.

Over the last century**,** during the 1960s VUJIĆ (1969) gave detailed description and studied developmental cycle of *Melampsora* species and its hybrids on black poplar leaves in his doctoral thesis. He determined that two rust fungi *Melampsora allii-populina* Kleb. and *Melampsora larici-populina* Kleb, the former being somewhat more widely spread, were present on black poplars in Danube basin, and probably in much wider poplar growing region.

In the years that followed, the sensitivity of greater number of black poplar genotypes to rust diseases under condition of spontaneous and artificial infection (AVRAMOVIĆ *et al*., 1992, 1995, 1998; PAP *et al*., 2006) was studied, and attention was paid to possibilities of controlling these fungi with pesticide application (AVRAMOVIĆ *et al*., 1997; KEČA, 2003; PAP *et al*., 2007). However, the population composition of rust diseases and their presence on black poplar genotypes in the poplar growing region in our country were not the subject of special investigation until now.

Numerous researches worldwide and in our country have determined that both studied races have shorten developmental cycle, and that fungi overwinter in bark of young sprouts and leaves (uredostadium), and multiply in spring by wind-borne uredospores (KLEBAHN, 1914; GÄUMANN, 1959; KIŠPATIĆ, 1959; CHIBA and ZINNO, 1960; SORAUER, 1962; VUJIĆ, 1969).

This explains the fact that rusts are present on black poplars each year, although in their immediate vicinity there are no transitional foster plants (*Allium* sp., *Arum* sp. and *Larix* sp.) for their development in haplophase, neither in nurseries nor in plantations. Both species show great similarity in dicariophase, and on the basis of their morphological-anatomical characteristics, and with no support of haplophase formed on transitional foster plants, determination of the species is not very reliable. In our previous studies (ORLOVIC *et al*., 2009, GALOVIC *et al*., 2008) it was proved that microsatellite DNA system is a reliable, informative and useful tool for wide range of genetic analysis. Due to difficulties relating to determination of this parasite, and often impossibility of their characterization, the molecular system of microsatellite, co-dominant in nature was chosen as the best choice for a detailed approach to this problem. Investigation of population composition, i.e. qualitative participation of *Melampsora* races in population of black poplar hybrid progenies was initiated using molecular methods with five microsatellite markers designed for both rusts (STEIMEL *et al.* 2005).

The aim of this study was to investigate if the markers applied could determine the specificity of *Melampsora* species on poplars in this growing region. These findings would be of importance to further detection and research of *Melampsora* species on poplars.

MATERIALS AND METHODS

Plant material, i.e. leaves with uredosoruses was collected at the end of September in 2009 in the genefund „Petrovaradinsko“ in experimental field of the Institute of lowland forestry and environment. For the purpose of investigation uredosoruses with spores originating from four clones of different taxonomic origin were isolated (Tab. 1).

Table 1. Poplar species included in investigation

Type of poplar clone:	Section
1.I-214 (*P. x euramericana*)	*Aigeiros* Duby
2.S11-8 (*Populus deltoides*)	*Aigeiros* Duby
3.1007 (*P. trichocarpa*)	*Tacamahaca* Spach
4. 9111/93 (*P. nigraxP. maximowicziix)xP.nigra var. Italica*	-

DNA isolation

Fresh innoculated plant material with uredospores was used for DNA extraction. Infected plant tissue, 100 mg of fresh mass, previously treated with liquid nitrogen was ground in a homogenzation apparatus (Retsch MM400, www.Retsch.com), 1min at 30 Hz. DNA extraction was acomplished according to instruction for Qiagen products users DNeasy Plant Mini Kit system (www.qiagen.com). DNA concentration was determined using spectrophotometric analysis, and quality by using electrohoretic system on 0.8% agarose gel. DNA was quantified to 20ng/ µl.

SSR markers

For microsatellite analysis five primer pairs were used for the following loci: *Mm*CAG-11, *Mm*CAT-30, *Mm*CAA-57, *Ml*CAG-30 and *Ml*CAG-101. Three microsatellite libraries enriched with CAT, CAG and CAA repetitive sequences were constructed for both rust fungi. Primers were designed from side regions of repetitive sequence and tested with DNA extracted from individual uredospores (*M. medusae*), or from several uredospores on individual poplar leaves (*M. larici populina*), Table 2.

Table 2. Loci, primers and accession number of microsatellite markers in gene bank for M. larici populina and M. medusa

Locus	Isolate origin	Primer sequence	Accession number to gene bank
*Mm*CAG-11	*M. medusae*	5'CCTTCATACACTTGCGAACTC3' 5'GGCCAGCATGTAATTGTTG3'	AY787478
*Mm*CAT-30	*M. medusae*	5'AAAGAAGTTCAAATGCCTTAC3' 5'GAAACGAGCTCATCTGTTC3'	AY787479
*Mm*CAA-57	*M. medusae*	5'GCTTACAAGTGAAAATTTG3' 5'TTAATGCAGATTGTAAATTAG3'	AY787480
*Ml*CAG-30	*M. larici populina*	5'ACCATATCCTGCCAGTCTTCTC3' 5'CGTCAGTGAGGGCGTAATG3'	AY787481
MlCAG-101	*M. larici populina*	5'TCCTTCTGGCTCCGCTGT3' 5'TATCTGTGGTTGCGAGTATTGG3'	AY787482

DNA amplification

In total volume of 25µl PCR the reaction mixture consisted of the following components: 1µl isolated gDNA, 0,2 µM each primer, 4mM $MgCl_2$, 0,2mM dNTPs, 0,3U of Taq DNA polimerase (Fermentas) and 1xTaq buffer as their final concentration. Fragments were amplified in Thermal cycler (Eppendorf) at initial denaturation temperature of 94^0C 1min and 25sec, 13 denaturation cycles at 95^0C 35sec, annealing temperature according to temperature of primer sequence melting point, 55 sec and elongation step at 72^0C 45sec. Then 13 denaturation cycles at 95^0C 35sec followed, annealing temperature according to the temperature of primer sequence melting point 55sec and elongation step at 72^0C 2min.

Then 9 denaturation cycles at 95^0C 35sec, annealing temperature according to temperature of primer sequence melting point 55sec and elongation step at 72^0C 3min. Followed by final elongation of 72^0C 1min.

PCR products were analyzed by electrophoresis with 2% agarose gel dyed EtBr submerged into 0,5xTBE buffer. Electrophoresis conditions for the separation were: half an hour at a constant voltage 80V. Visualization was done using DIAS system (SERVA Electrophoresis, GmbH, www.serva.de). PCR products that showed quality results were analyzed on Bioanalyzer2100 (Agilent Technologies, www.agilent.com)

using software for high DNA fragments separation with the aim to determine size of DNA and DNA fragments quantification (Tab.3).

RESULTS AND DISCUSSION

All loci except *Mm*CAA-57 were successfully amplified and showed polymorphism. Individuals in which amplification was omitted were marked as null alleles (Tab. 3). Twelve null alleles were observed, and they were not taken into consideration in statistical analysis. When statistically processed 5 SSR markers and 4 clones were used in this paper and total of 11 allels or 2,2 alleles per locus were detected (Tab. 4). Locus *Mm*CAG-11 amplified one allele of 214bp in length, *Mm*CAT-30 2 alleles 249bp and 364bp in length, while in locus *Mm*CAA-57 no amplification was occurred.

Table 3. Length of detected alleles (base pairs) in observed clones

Melampsora sp.	Genotypes			
Loci	I-214	S11-8	1007	9111/93
MmCAG-11	0	214	0	0
MmCAT-30	249, 364	0	0	0
MmCAA-57	0	0	0	0
MlCAG-30	297	298	0	299
MlCAG-101	287, 294	286, 427	0	288

According to literature data (STEIMEL *et al.*, 2005) SSR markers *Mm*CAG-11, *Ml*CAG-30 and *Ml*CAG-101 showed that it was possibile to differentiate between both rust fungi, while markers *Mm*CAT-30 and *Mm*CAA-57 were specific only for *M. medusae*.

Namely, loci *Mm*CAT-30 and *Mm*CAA-57 were detected only in *M. medusae*, while in *M. larici populina* there was no amplification, which could point out to non existence of these two loci in individuals infected with *M. larici populina.*

Table 4. Microsatellites (SSRs), range of fragment sizes and number of detected allels per locus

Locus	Size of fragments (base pairs)	Number of detected allele per locus
*Mm*CAG-11	214	1
*Mm*CAT-30	249-364	2
*Mm*CAA-57	-	-
*Ml*CAG-30	297-299	3
*Ml*CAG-101	286-427	5
Total alleles/locus	2,2	

Since amplification was determined with all three markers *Mm*CAG-11, *Ml*CAG-30 and *Ml*CAG-101 which were able to confirm hibridization between two species, it could be assumed that both rust races were present in clone S11-8. Locus MICAG-30 was polymorphic and was detected in three clones (I-214 S11-89111/93), while it was not detected in clone 1007. These results could reveal that race *M. larici populina* was detected in these clones, while this was not the case in clone 1007.

Fragment of 287 length in base pairs in clone I-214 was amplified using *Ml*CAG-101 marker which was in accordance with the results of STEIMEL *et al.*, 2005 who detected this allele in both rust races. In this paper this allele was present only in clone I-214. This revealed that there was possibility of presence of *M. medusae* in this clone, while in other clones detected fragments were of very similar length, and could reveal the presence of *M. larici populina.*

An interesting example was amplification of locus *Mm*CAT-30 only in clone I-214. This data could also point out the presence of *M. medusae* in clone I-214, while in other clones where amplification for this locus was omitted the presence of *M.larici populina* was suspected because this SSR marker was designed specifically for *M. medusae*. These assumptions could be corroborate with recent findings of GALOVIC *et al*., 2010 where authors according to NCBI blast and align sequences of *ITS1* and *ITS2* region indicated the existence of two *Melampsora* species.The first is the widespread *M. larici-populina,* while the other according to our preliminary results could be *M. medusae.*

M. medusae was detected in France in sporadic intervals in past 30 years, but according to PINON (1986) this species haven't spreaded significantly due to unfavourable environment. It is considered that forms present in Europe are not agressive and have no spreading tendencies and as that they are not threaten to other European countries. However, there is a risk of intrusion in EPPO region of one of well nown agressive species, that could cause huge losses especially in the regions with mild climate and no alternative host.

Concidering these findings it can be concluded that, locus *MmCAT-30* could be a specific marker for determination of *Melampsora* species in poplars. According to the preliminary results, for the first time in our plant material in clone I-214, *M. medusae* was determined. As this research work was recently initiated in the field of phytopathology it is necessary to be continued in order of additional characterization of this type of disease in the domestic material.

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SPECIFIČNOST SSR LOKUSA ZA *Melampsora* VRSTE KOD TOPOLA

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I z v o d

Dve vrste rđe, *Melampsora larici populina* i *Melampsora medusae* se sreću u svim regionima gajenja topola u svetu. *M. larici populina* je nativna vrsta za Evroaziju dok je *M. medusae* endemska vrsta istočnih Sjedinjenih država na *Populus deltoides* vrstama. Rđe na lišću topola (*Melampsora* spp.) su široko rasprostranjena oboljenja i u našoj zemlji i mogu da prouzrokuju značajne probleme u njihovom gajenju. Prevalentna vrsta u našem regionu gajenja je *M. Larici populina*. Ovim radom su započeta istraživanja populacionog sastava, odnosno kvalitativnog učešća *Melampsora* vrsta u populacijama hibridnih potomstava crnih topola korišćenjem molekularnih metoda. Korišćenjem 5 SSR markera rezultati su pokazali da je *M. larici populina* prisutna kod tri ispitivana klona dok je kod klona I-214 zabeleženo prisustvo druge vrste prouzrokovača rđe, neuobičajeno za naše gajeno područje, *M. medusae*.

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