

GENETIC VARIABILITY OF *Verbascum* POPULATIONS FROM METAL POLLUTED AND UNPOLLUTED SITES

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Danilović G., F. Morina, Z. Satovic, Lj. Prokić, and D. Panković (2015):
Genetic variability of Verbascum populations from metal polluted and unpolluted sites.- Genetika, Vol 47, No. 1, 245-251.

Mullein (*Verbascum*) plants have extensive distribution and can grow in variable environmental conditions. Seed was collected from mullein plants grown at 4 locations, two metals contaminated and two metal uncontaminated areas. Genetic variability of progeny was examined. Populations collected from unpolluted areas were genetically more similar than those collected from polluted areas as revealed by RAPD and SSR markers and UPGMA analysis. The results indicate that there is genetic differentiation between examined populations and therefore they represent suitable material for further investigation of plant adaptation mechanisms to increased metal content.

Key words: metal tolerance, mullein, RAPD, SSR, UPGMA

INTRODUCTION

Mullein plants (*Verbascum*), native to Europe and Asia, have extensive distribution and can grow in a range of diverse conditions often on disturbed sites, such as roadsides and industrial areas, as pioneer species (KUMSCHICK *et al.*, 2013). However, mullein was introduced and spread over North America, where it is one of model plant species for the examination of invading properties. Main factors that influence invading process of a plant species are rapid adaptation and/or physiological tolerance and plasticity (PARKER *et al.*, 2003). By investigating

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relevant morphological and physiological traits of different *V. thapsus* populations and their genetic relatedness, DLUGOSCH and PARKER (2007) showed that the invasion at high elevations was caused by phenotypic plasticity rather than rapid adaptation. Other studies indicated that local adaptations might contribute to widespread invasion of multiple habitats by *V. thapsus* (KUMSCHICK *et al.*, 2013). It has been shown that the ability of *V. thapsus* to colonize Zn contaminated industrial waste disposal and accumulate high concentrations of Zn was followed by increased antioxidative status of both leaves and roots (MORINA *et al.*, 2008, JOVANOVIĆ *et al.*, 2007; MORINA *et al.*, 2010). The ability of mullein to colonize metal contaminated areas might be the result of adaptation and/or phenotypic plasticity. In order to get first information on this mechanism we have examined genetic diversity between 4 mullein populations, two from metal contaminated and two from uncontaminated soils, by the use of RAPD and SSR markers.

MATERIALS AND METHODS

Seed collection

Seeds from 2 controls *V. thapsus* L. populations (NM1 and NM2) were randomly collected in metal-uncontaminated areas: NM1 in a ruderal meadow where the soil was degraded by erosion, on mountain Zlatibor and NM2 population that was found in an urban area, on a ruderal meadow close to a preserved park-forest Kosutnjak, near Belgrade, Serbia. Third population of *V. thapsus* L. (M1), was an early colonizer species on a jarosite smelter, which has been in use since 2000, coming from a zinc production process (M1) in the Zorka Sabac Industry. The fourth population (M2), *V. lychmitis* L., was among the few species present on open cast copper mine deposits since 1990 in Majdanpek. Metal contents in soils from locations in Sabac and Majdanpek were 20 to 200 folds higher than in unpolluted soil (JOVANOVIĆ *et al.*, 2007). Progeny of the collected populations was grown as in JOVANOVIĆ *et al.*, (2007) and used for genetic analysis.

Experimental conditions

DNA extraction and PCR amplifications

Leaf tissue, bulked from 8-10 plants, was frozen in liquid nitrogen, grinded with mortar and pestle and genomic DNA was extracted using a DNeasy mini plant kit (Quiagen, Germany).

Data on molecular markers and gene sequences in *Verbascum* are scarce, therefore we used RAPD and sunflower SSR markers to determine genetic distances between examined populations. RAPD-PCR amplifications were performed with 7 decamer primers (Eurofins MWG Operon, Germany) in a 20 μ l volume of reaction mixture containing: 30 ng μ l⁻¹ gDNA, 0.4 mM dNTP, 1U TaqDNA polimerase (Thermo Fisher Scientific- Fermentas), 1 \times Taq buffer, 0.2 μ M RAPD primer, 3 mM MgCl₂ and 2.5 μ g μ l⁻¹ BSA. The thermocycler (Eppendorf, Germany) was programmed as follows: 5 min at 95°C, 45 cycles consisting of 1 min at 95°C, 1 min 36°C, 2 min at 67°C, followed by the final extension at 67°C for 5 minutes.

Out of several *Helianthus* SSR primers, previously used for the investigation of cultivated and wild sunflowers (SAFTIĆ-PANKOVIĆ *et al.*, 2005, PANKOVIĆ *et al.*, 2007), 6 primers (Thermo Fisher Scientific- Fermentas) were used for PCR amplifications in 20 μ l volume of

reaction mixture containing: 40 ng gDNA, 0.4 mM dNTP, 1U *Taq* polymerase (Thermo Fisher Scientific- Fermentas), 1 × *Taq* buffer, 0.6 μM SSR primers, 3 mM MgCl₂ and 2,5 μg μl⁻¹ BSA. The following PCR touch-down program was used: 3 min at 95°C, 7 cycles of 30 seconds at 94°C, 30 seconds at 64°C, with the a decrease of temperature by 1 °C in each cycle, 30 seconds at 67°C, 33 cycles of 30 seconds on 94°C, 30 seconds at 57°C, 30 seconds at 67°C, followed by the final extension at 67°C for 20 minutes (TANG *et al.*, 2002).The sequences of RAPD and SSR primers are given in Table 1.

Amplified PCR products were separated by agarose gel electrophoresis (1% and 2% agarose for RAPD and SSR primers, respectively) in 1 × TBE running buffer, at 120 V for 60 min. Bands were visualized by in-gel ethidium bromide (0.5 μl ml⁻¹) under UV light, by a Bio Print Analysis System (VILBER LOURMAT, France).

Molecular data analyses

Interpopulational relationships of the *Verbascum* species were analyzed by distance-based cluster analyses, similar to those in NEJSUM *et al.* (2005). Amplified fragments were scored for the presence (1) or absence (0) of homologous bands to create binary matrices. Pairwise genetic distances were calculated using Jaccard's coefficient (JACCARD, 1901). A hierarchical cluster procedure was performed using a UPGMA algorithm as implemented PAST ver. 2.01 (HAMMER *et al.*, 2001). The statistical support of the branches was tested with bootstrap analysis using 1,000 replicates (FELSENSTEIN, 1985). The characteristics of RAPD and SSR loci analyzed in *Verbascum* gDNA are shown in Table 1.

RESULTS

Molecular marker analysis

Polymorphism of genomic DNA was analyzed by RAPD and SSR markers. The number of amplified fragments by primers varied from 3 to 7. Size of RAPD markers varied from 500 to 3000 bp. SSR markers were smaller they ranged from 50 to 700 bp. Two of the highly polymorphic SSR markers, SSR 317 and SSR 595 (Table 1) were mapped in loci for simple inherited traits in sunflower; branching (i.e. multiple flowers) and chlorophyll deficiency, respectively (HAHN and WIECKHORST, 2010).

Twenty-six out of 47 polymorphic fragments were populations specific (unique fragments). While *V. thapsus* populations originating from uncontaminated areas (NM1 and NM2) had 5 unique fragments each, the populations from the metal-contaminated area (*V. thapsus*; M1 and *V. lychnitis*; M2) showed 8 unique fragments each. Pairwise genetic distances between populations varied from 0.58 (NM1 / NM2) to 0.72 (M2 / NM2), with an average of 0.67. As expected, the populations *V. thapsus* populations originating from uncontaminated areas (NM1 and NM2) clustered together in the UPGMA tree supported by a bootstrap value of 63% (Figure 1), while *V. lychnitis* population from the metal-contaminated area (NM2) was the most distant from the rest of the populations.

Table 1. Characteristics of RAPD and SSR loci analyzed in *Verbascum gDNA*

Primer name	Primer sequence (5'-3')	Number of amplified fragments	Fragment size range (bp)	Number of polymorphic fragments
RAPD OPA 14	TCTGTGCTGG	7	500-1850	6
RAPD OPA 16	AGCCAGCGAA	5	1200-1800	5
RAPD OPO 19	GGTGCACGTT	4	950-1950	4
RAPD OPC 19	GTTGCGAGCC	3	600-1700	1
RAPD OPO 02	ACGTAGCGTC	4	650-2500	4
RAPD OPO 07	CAGCACTGC	3	1500-3000	2
RAPD OPO 04	AAGTCCGCTC	6	500-2500	6
SSR 166	F CAGCCACATGCCCTCTGA R TGTTAAGAACCGCGACAACCTG	3	50-350	1
SSR 1021	F AACCCCTAATCCAACCAGATACG A R CAACGAAAAGACAGAATCGAA A	5	50-700	4
SSR 317	F TTTGGCAGTTTGGTGGCTTA R GGTCGTATGCTTAATTCTTTCTC	3	50-250	3
SSR 509	F CAACGAAAAGACAGAATCGAA A R CCGGGAATTTTACAAGGTGA	5	50-300	4
SSR 595	F TGATGGTAATGCATCGGGTA R CACACCATCCCTTGTAATAATCA	4	50-150	4
SSR 499	F CACGGTTGGTCAATCACATC R TTTAGGCTGCACATTATGGACT	3	50-240	3

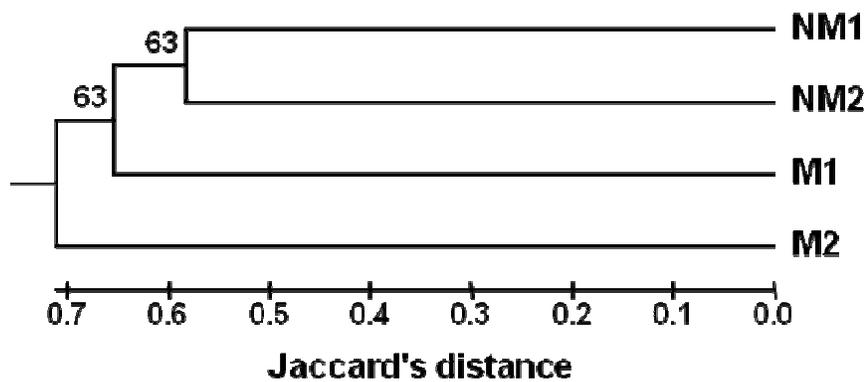


Figure 1. Distance-based cluster analyses between four mullein populations, from uncontaminated soils (NM1 and NM2) and from Cu and Zn contaminated soils (M1 and M2).

DISCUSSION

Unstructured, disturbed soils resulting from industrial and mining activities present an open space which can be colonized by pioneer species (LEHMANN and REBELE, 2004). Such soils are contaminated with metals, have poor nutrient availability and disturbed microflora (KE *et al.*, 2007). Under these unfavourable conditions, selective pressure can lead to favouring metal tolerant genotypes (LIU *et al.*, 2004). Here we investigated genetic variability of 4 mullein populations, 2 from metal uncontaminated and 2 from metal contaminated sites. Genetic distances based on the coverage of 55 loci, varied in the range of 0.14 distance units. Similarly, DLUGOSCH and PARKER (2007) have determined genetic relatedness among *V. thapsus* populations by AFLP markers and presented the variation of genetic distances between populations mostly below 0.1 distance units. In our work, cluster analysis clearly distinguished four populations, revealing higher genomic similarity between populations from metal uncontaminated, in comparison to populations from metal contaminated areas (Figure 1). DENG *et al.* (2007) have investigated the effect of heavy metal pollution on genetic diversity of *Sedum alfredii* populations by RAPDs and found that populations clustered according to their metal accumulation and tolerance capabilities. Our data are in favour of the effect of metal contamination as a strong selective pressure favouring tolerant genotypes and leading to differentiation between populations. Therefore these populations are promising model system for further investigations of plant adaptive mechanisms to metal tolerance.

ACKNOWLEDGEMENT

This research was supported by III43010 project funded by the Ministry of Education and Science, Republic of Serbia.

Received December 09th, 2014

Accepted February 25th, 2015

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**GENETIČKA VARIJABILNOST POPULACIJA IZ RODA *Verbascum*
SA LOKALITETA ZAGAĐENIH I NEZAGAĐENIH METALIMA**

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Izvod

Divizma (*Verbascum*) je široko rasprostranjena i može da raste u različitim uslovima životne sredine. U ovom radu je ispitana genetička varijabilnost na potomstvu populacija biljaka divizme koje su sakupljene sa četiri lokaliteta, dva zagađena metalima i dva nezagađena. Rezultati dobijeni sa RAPD i SSR markerima, kao i UPGMA analizom ukazuju na veću genetičku sličnost između populacija sa nezagađenih, u odnosu na populacije sa zagađenih lokaliteta. Genetička diferencijacija između ispitivanih populacija ukazuje da ove populacije predstavljaju pogodan model sistem za dalja ispitivanja mehanizama adaptacije biljaka na povećane sadržaje metala.

Primljeno 09. XII. 2014.

Odobreno 25 II. 2015.