Preliminary Testing of SRAP Primers in Order to Establish Genetic Diversity of *Astragalus exscapus* L. subsp. *transsilvanicus* (Schur) Nyár.

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BulletinUASVM Horticulture 72(1) / 2015

Print ISSN 1843-5254, Electronic ISSN 1843-5394

DOI:10.15835/buasvmcn-hort:11070

ABSTRACT

The Carpathian List of Endangered Species contains eight *Astragalus* species mentioned to be vulnerable or endangered. One of them is the endemic *Astragalus exscapus* L. subsp. *transsilvanicus* (Schur) Nyár., a rare perennial plant with only 24 populations remaining in the Transylvanian basin. Analysis of genetic diversity and population structure of this threatened subspecies is an important prerequisite for its conservation. The present study investigates eight different populations of *A. exscapus* using sequence related amplified polymorphism (SRAP) molecular markers. This technique is a relatively new PCR-based system, more reliable than random amplified polymorphic DNA (RAPD) markers, and suitable for population structure analysis. This essay reports the screening of 64 SRAP primer combinations on 3 to 4 samples from every population with the aim of selecting the most reliable ones for subsequent analysis. PCR amplification products were assessed on 2% agarose gel and a total number of 1473 DNA fragments were visualized by EtBr staining. We selected fourteen primer combinations for further research based on clarity, reproducibility of amplified bands and high rates of polymorphism. The most efficient primer combination was Me8/Em3 which generated 42 visible DNA fragments.

Keywords: *Astragalus exscapus*, genetic diversity, primer testing, SRAP

INTRODUCTION

Astragalus exscapus L. is a rare melliferous perennial plant characterized by particularly narrow habitat requirements (Becker, 2010) and it is marked as vulnerable in the Carpathian List of Endangered Species (Witkowski, 2003). The subspecies *transsilvanicus* (Schur) Nyár. is localized in the Transylvanian basin (Podlech, 1988), with only 24 remaining populations. This local endemism, mapped by Bădărașu *et al.* (2000, 2001), is considered to be of national interest, requiring strict protection in accordance with annex 4B of the Government Emergency Ordinance 57/2007.

The main objective of our work is to assess the genetic diversity at molecular level and to find out

the range of polymorphism within and between populations of *A. exscapus* in order to establish some conservation strategies. Molecular markers are commonly used in genetic diversity analysis, thereby our study will implement Sequence-related amplified polymorphism (SRAP) markers. This technique is a relatively new PCR-based molecular marker system introduced by Li and Quiros (2001), presenting a higher reproducibility than RAPD and a simpler approach than AFLP. The preliminary testing of SRAP primers is a necessary step for selecting the most appropriate combinations in large-scale use of the specified technique, to characterize genetic diversity, which can help the establishment of more efficient conservation strategies.

MATERIALS AND METHODS

Plant material was collected from 126 individuals belonging to eight natural populations of *A. exscapus* subsp. *transsilvanicus* (Table 1) situated in the Transylvanian basin. The obtained leaves were stored at -20 °C until further processing. To find out the most suitable SRAP primer combinations we used four randomly selected samples (B13, G12, S11, V13).

Genomic DNA was isolated applying the CTAB method – as published by Pop *et al.* (2008). We optimized the protocol by using TissueLyser II – Qiagen for 30 seconds as an initial grinding stage with liquid nitrogen. The properties of the extracted DNA were determined using a NanoDrop1000 spectrophotometer.

The degree of polymorphism within and between populations is an important indicator in the survival potential of vulnerable species and it can be investigated by numerous techniques. SRAP molecular marker system (Li and Quiros, 2001) specifically amplifies polymorphic junction fragments between exons and flanking DNA. For PCR reaction a specific forward and a reverse

primer are employed, both consisting three elements: (1) an arbitrary filler sequence of 10 to 11 bases at the 5' end; (2) sequence motifs CCGG and AATT in the forward and reverse primer, respectively; (3) three selective bases at the 3' end. A special PCR running program was designed, that starts at 35 °C annealing temperature in the first five cycles, and then increases to 50 °C for the rest of the cycles. In order to select the most reliable primer combinations for further analysis of *A. exscapus* eight forward and eight 8 reverse primers were tested (Table 2).

Amplification for each of the 64 primer combinations was carried out on a Corbette Research PalmCycler. Every 15 µl PCR reaction mix contained 1 × Green Buffer, 1,5 mM MgCl₂, 200 µM of dNTPs, 0,3 µM of both forward and reverse primer, 1 U of Taq DNA polymerase (Promega) and approximately 50 ng of template DNA. After DNA amplification, the PCR products were electrophoresed on a 2% (w/v) agarose gel, in 1.0 × TAE buffer, at 115 V, for approximately 2 hours. The gel was stained in 1µg/mL EtBr for 20 to 30 minutes and photographed with Biospectrum AC.

Tab. 1. Geographical origins and sample size of eight natural populations of *Astragalus exscapus* L. subsp. *transsilvanicus* (Schur) Nyár.

Population code	Location	Sample size	Longitude E	Latitude N
M	Milășel	14	24° 26' 55.94"	46° 44' 52.03"
B	Beleni	15	23° 51' 52.38"	46° 45' 57.65"
G	Gădălin	19	23° 50' 50.40"	46° 51' 28.38"
SI	Silivașu de campie	15	24° 17' 4.08"	46° 46' 41.58"
S	Săbed	15	24° 26' 46"	46° 39' 52"
V	Viișoara	18	23° 54' 35"	46° 33' 28"
SG	Sărmășel Gară	15	24° 12' 45.04"	46° 47' 3.11"
VF	Valea Florilor	15	23° 47' 48.75"	46° 40' 25.19"

Tab. 2. SRAP primers used in the present study

No.	Forward primers		Reverse rpimers	
1	Me1	TGA GTC CAA ACC GGA TA	Em1	GAC TGC GTA CGA ATT AAT
2	Me2	TGA GTC CAA ACC GGA GC	Em2	GAC TGC GTA CGA ATT TGC
3	Me3	TGA GTC CAA ACC GGA AT	Em3	GAC TGC GTA CGA ATT GAC
4	Me4	TGA GTC CAA ACC GGA CC	Em4	GAC TGC GTA CGA ATT TGA
5	Me5	TGA GTC CAA ACC GGA AG	Em5	GAC TGC GTA CGA ATT AAC
6	Me6	TGA GTC CAA ACC GGA CA	Em6	GAC TGC GTA CGA ATT GCA
7	Me7	TGA GTC CAA ACC GGA CG	Em7	GAC TGC GTA CGA ATT CAA
8	Me8	TGA GTC CAA ACC GGA CT	Em8	GAC TGC GTA CGA ATT CAC

RESULTS AND DISCUSSION

The 64 combinations of the eight forward and eight reverse primers generated over 200 electrophoretic profiles. The images (Figure 1) were processed with Total Lab 120 software.

The main criteria by which the primer selection was made are: clarity, reproducibility of

amplified bands and high rates of polymorphism. For example, lane-group 33 and 34 are similar - if we consider the number of generated bands - but lane 34 is more polymorphic than lane 33. In this case, we decided to continue working with the primers that generated higher rates of

Electrophoretic profiles of 3 samples on 2% agarose gel; primer combinations 21-36

Electrophoretic profiles of 3 samples on 2% agarose gel; primer combinations 37-52

Fig. 1. Representative PCR amplification products with 32 different primer pair combinations.

Tab. 3. The 14 combinations selected

Primer combination	Number of amplified fragments			Total generated bands	Average
	B3	G12	S11		
Me1/Em6	9	12	9	30	10.00
Me2/Em1	10	10	11	31	10.33
Me2/Em6	12	8	9	29	9.67
Me3/Em3	7	10	10	27	9.00
Me4/Em2	11	10	9	30	10.00
Me4/Em4	8	11	1	3	10.00
Me4/Em5	9	11	11	31	10.33
Me5/Em2	9	10	10	29	9.67
Me5/Em6	10	10	9	29	9.67
Me6/Em1	11	9	12	32	10.67
Me6/Em8	6	11	11	28	9.33
Me8/Em2	10	13	13	36	12.00
Me8/Em3	13	15	14	42	14.00
Me8/Em6	16	15	11	42	14.00
Total	141	155	150	446	
Average	10.07	11.07	10.71	31.86	10.59

polymorphism even if the produced bands are the same numerically in both situations.

To assure reproducibility of amplified bands, the basic steps were performed successfully in two repetitive experiments. After screening the PCR amplification products (1473 in total) for reproducibility and polymorphism, 50 combinations were discarded out of the 64 tested. The remaining 14 combinations produced total 446 DNA fragments with an average of 10.59 clear bands per primer combination.

CONCLUSION

The selected SRAP marker combinations represent a powerful tool to analyze genetic diversity in *A. exscapus* subsp. *transsilvanicus*. We are going to perform large-scale PCR amplification with the 14 primer combinations (Table 3) of SRAP markers on numerous individuals of the studied subspecies, in order to analyze genetic diversity and population structure. We expect that the revealed molecular diversity will help to design future conservation strategies concerning this endangered species.

Aknowledgement. This paper was published under the frame of European Social Fund, Human Resources Development Operational Programme 2007-2013, project no. POSDRU/159/1.5/S/132765.

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