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PRELIMINARY TESTING OF SRAP PRIMERS IN FOUR *RANUNCULACEAE* SPECIES FROM ROMANIA

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Abstract

The main aim of this paper was to identify the most reliable primer pairs out of the 64 used in order to investigate the genetic diversity of four medicinal Ranunculaceae species as follow: Aconitum moldavicum, Anemone transsilvanica, Ranunculus carpaticus and R. platanifolius. Amplified products were separated on 1.6% agarose gel and a total number of 886 DNA fragments were visualized by EtBr staining. After primer combinations screening, 27 primer pairs were selected based on clarity, reproducibility of the amplified bands and high rates of polymorphism for further research. The selected SRAP primer pairs resulted in 553 informative fragments with an average number of 20.5 per primer combination. The most efficient primer combinations were Me1/Em1, Me3/Em3, Me7/Em4, Me7/Em5, Me7/Em8, Me8/Em1 and Me8/Em5 which generated a total number of 201 visible DNA fragments. The current research represents a preliminary study for further conservation perspectives regarding the genetic diversity of several medicinal plants belonging to the Ranunculaceae family.

Keywords*: Ranunculaceae, genetic diversity, primer testing, SRAP.*

INTRODUCTION

Ranunculaceae Juss. (buttercup family) is one of the early lineages in flowering plants distributed worldwide, most commonly in the temperate and cold areas of the northern hemisphere (Heywood et al., 2007). The family including 56 genera and approximately 2,500 species used as medicinals, spices, and ornamentals, however, most of them are highly poisonous (Nyirimigabo et al., 2014; Aslam et al., 2012). The most representative genera of the buttercup family are *Aconitum*, *Anemone*, *Clematis*, *Delphinium*, *Ranunculus,* and *Thalictrum* which account approximately 90% of the species (Heywood et al., 2007). From genetic point of view this family has a great chromosomal variation in numbers, sizes and structures, including species with 2n=14 (e.g. *Hepatica asiatica* Nakai, *Ranunculu sceleratus* L.), 2n=16 (e.g. *Ranunculus cantoniensis* DC), 2n=18 (e.g. *Clematis brachyura* Maxim), and 2n=42 (e.g. *Thalictrum aquilegiifolium* var. *sibiricum* Regel & Tiling) (Chung et al., 2013).

In Romania, the *Ranunculaceae* family is represented by 23 genera and approximately 110 species including endemic plants such as *Anemone transsilvanica* Fuss (Săvulescu, 2007). Many local plants belonging to *Ranunculaceae* family (*Aconitum moldavicum* Hacq.*, Anemone transsilvanica*, *Ranunculus carpaticus* Herbich, *R. platanifolius* L.) have been used as human and veterinary medicine to treat various ailments such as bronchitis, cough, diarrhea, fever, hepatitis, gout, rheumatism, and skin diseases (Tămaş, 2005). However, little is known concerning their genetical diversity and conservation.

The main purpose of this work is to assess the genetic diversity at molecular level of *A. moldavicum, A. transsilvanica, R. carpaticus* and *R. platanifolius* using sequence-related amplified polymorphism (SRAP) markers in order to establish some conservation strategies for these species. SRAP primers are considering to be more suitable to revealing genetic diversity among related species than amplified fragment length polymorphism

(AFLP), simple sequence repeats (SSR), inter-simple sequence repeat (ISSR) or even random amplified polymorphic DNA (RAPD) markers (Budak et al., 2004). Therefore, preliminary testing of SRAP primers was necessary to be able to select the appropriate primer combinations on a large-scale use to characterize the genetic diversity of the studied species. Although, previous scientific reports show intra- and interpopulation genetic diversity of various *Ranunculaceae* species [*Ranunculus acris* (Odat et al., 2004), *R. cabrerensis* (Cires et al., 2013), *R. kuepferi* (Cosendai et al., 2013) and *R. reptans* (Fischer et al., 2000; Prati et al., 2016)] employing AFLP or RAPD primers, this is the first report regarding to assessment of genetic variation in this family using SRAP markers.

MATERIALS AND METHODS

Plant samples were collected from their natural habitats in two districts from Romania (Brașov and Hunedoara) namely at Mt. Postăvaru (45°35.215′ N, 25°433.146′ E) and Mt. Stâmba (46°12.508′ N, 22°51.354′ E). The voucher specimens have been stored in the Herbarium collection at the University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca, Romania. The location and the voucher specimen numbers of the species are shown in Table 1.

Table 1. Voucher specimen numbers of the *Ranunculaceae* species and their geographical origins

Species name	VSN[b)]	Longitude E	Latitude N
Aconitum moldavicum Hacq.	CLA30049	22°51.354′	46°12.508′
Anemone transsilvanica FUSS.	CLA30047	25°433.146	45°35.215′
Ranunculus carpaticus Herbich.	CLA30044	25°433.146	45°35.215′
Ranunculus platanifolius L.	CLA30040	25°433.146	45°35.215′

Note: [b)]Voucher specimen number

The harvesting leaves were air dried at room temperature and stored at -20°C until processing. To find out the most suitable SRAP primer combinations four randomly selected samples were used one of each species.

In order to extract the genomic DNA, its isolation was made by applying the CTAB method as described by Lodhi et al. (1994) and improved by Pop et al. (2003) and Szabo et al. (2015). Concentration and purity of the extracted DNA were determined using NanoDrop1000 spectrophotometer. The SRAP analysis was carried out according to previously established protocols by Li and Quiros (2001). In order to select the most reliable primer combinations for further analyses, 64 different primer combinations were employed in this assay using eight forward and eight reverse primers (Table 2).

Table 2. SRAP primers sequence

Forward primers	
Me1	F: TGA GTC CAA ACC GGA TA
Me2	F: TGA GTC CAA ACC GGA GC
Me3	F: TGA GTC CAA ACC GGA AT
Me4	F: TGA GTC CAA ACC GGA CC
Me5	F: TGA GTC CAA ACC GGA AG
Me6	F: TGA GTC CAA ACC GGA CA
Me7	F: TGA GTC CAA ACC GGA CG
Me8	F: TGA GTC CAA ACC GGA CT
Reverse primers	
Em1	R: GAC TGC GTA CGA ATT AAT
Em2	R: GAC TGC GTA CGA ATT TGC
Em3	R: GAC TGC GTA CGA ATT GAC
Em4	R: GAC TGC GTA CGA ATT TGA
Em5	R: GAC TGC GTA CGA ATT AAC
Em6	R: GAC TGC GTA CGA ATT GCA
Em7	R: GAC TGC GTA CGA ATT CAA
Em8	R: GAC TGC GTA CGA ATT CAC

The polymerase chain reaction was performed in 15 µL reaction mixture containing 1 × Green Buffer, 1.5 mM $MgCl_2$, 200 µM of dNTPs, 0.3 µM of both forward and reverse primers (Generi Biotech), 1 U of Taq DNA polymerase (Promega) and approximately 50 ng of template DNA. Amplifications were made in a Corbette Research PalmCycler with an initial step at 94°C for 5 min and five cycles of 1 min. at 94°C, 1 min. at 35°C, and 1 min. at 75°C. The following 35 cycles consisted of 94°C for 1 min., 50°C for 1 min., and 72°C for 1 min., with a final extension at 72°C for 10 min. The PCR reactions were repeated to ensure the reproducibility of the DNA. Amplified products were separated on 1.6 % (w/v) agarose gels in 1.0 × TAE buffer at 115 V, for approximately 2 hours. A 100-bp DNA ladder was used as molecular weight marker in order to confirm the appropriate SRAP markers. The electrophoretic profiles were stained with 1µg/mL EtBr form 20 to 30 minutes and photographed with Biospectrum AC

The obtained images were processed with Total Lab 120 software (Figure 1).

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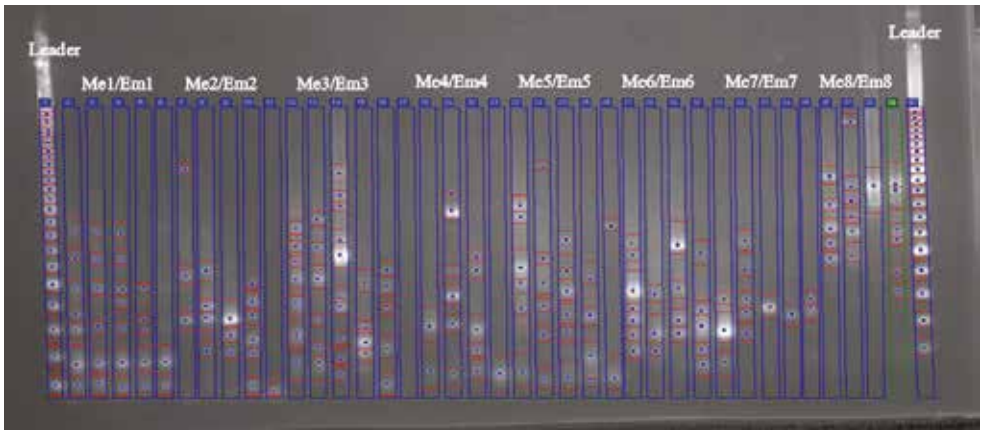

Figure 1. Sequence-related amplified polymorphism (SRAP) profiles obtained from 16 primer combinations of four species

RESULTS AND DISCUSSIONS

The screening of 64 SRAP primer pairs (eight forward and eight reverse) generated a total number of 886 DNA fragments ranging from 1 to 35 bands per primer combination. The average number band per primer set recorded was 13.9. The size of amplification products ranged from 400-3,200 bp.

In order to select the most efficient primer combinations for subsequent analyses, the main criteria taken into consideration were the clarity and reproducibility of the amplified bands together with the rate of polymorphism.

Therefore, after the screening of the amplified products, 37 primer pairs were discarded out of the 64 tested, because they either yielded no amplification or no polymorphic patterns.

The remaining 27 primer combinations produced a total number of 553 DNA fragments with an average number of 20.5 clear bands per primer pairs (Table 3).

Table 3. The remaining 27 primer combinations

Primer combination	Number of amplified fragments				Total number of bands	Average number of bands
	A. moldavicum	*A. transsilvanica*	*R. carpaticus*	*R. platanifolius*		
Me1/Em1	7	6	7	5	25	6.3
Me1/ Em3	3	6	5	3	17	4.3
Me1/ Em4	5	6	6	4	21	5.3
Me1/Em6	4	4	6	1	15	3.8
Me1/ Em7	5	6	6	1	18	4.5
Me2/Em2	3	4	3	6	16	4.0
Me2/ Em3	6	1	4	3	14	3.5
Me3/ Em2	6	2	1	2	11	2.8
Me3/Em3	7	6	10	4	27	6.8
Me3/ Em4	6	6	1	1	14	3.5
Me3/ Em5	3	8	4	4	19	4.8
Me4/ Em1	2	5	8	3	18	4.5
Me4/Em4	0	2	6	5	13	3.3
Me4/ Em6	3	4	7	4	18	4.5
Me4/ Em7	2	5	7	7	21	5.3
Me5/Em5	6	6	6	4	22	5.5
Me6/ Em4	3	6	7	3	19	4.8

Primer combination	Number of amplified fragments				Total number of bands	Average number of bands
	A. moldavicum	*A. transsilvanica*	*R. carpaticus*	*R. platanifolius*		
Me6/ Em5	4	8	6	5	23	5.8
Me6/Em6	7	3	4	5	19	4.8
Me7/ Em4	7	6	6	9	28	7.0
Me7/ Em5	6	4	8	7	25	6.3
Me7/ Em6	5	3	5	8	21	5.3
Me7/ Em8	10	7	6	8	31	7.8
Me8/ Em1	9	4	8	9	30	7.5
Me8/ Em6	3	2	5	10	20	5.0
Me8/ Em5	7	10	10	8	35	8.8
Me8/Em8	6	1	6	0	13	3.3
Total number of bands	**135**	**131**	**158**	**129**	**553**	
Average number of bands	**10.4**	**10.1**	**8.8**	**9.9**	**20.5**	

The most representative SRAP profiles were obtained with the primer combinations *Me1/Em1, Me3/Em3*, *Me7/Em4*, *Me7/Em5*, *Me7/Em8*, *Me8/Em1* and *Me8/Em5* which generated a total number of 201 visible DNA fragments with a mean value of. 28.7 clear bands per primer set (Figure 2).

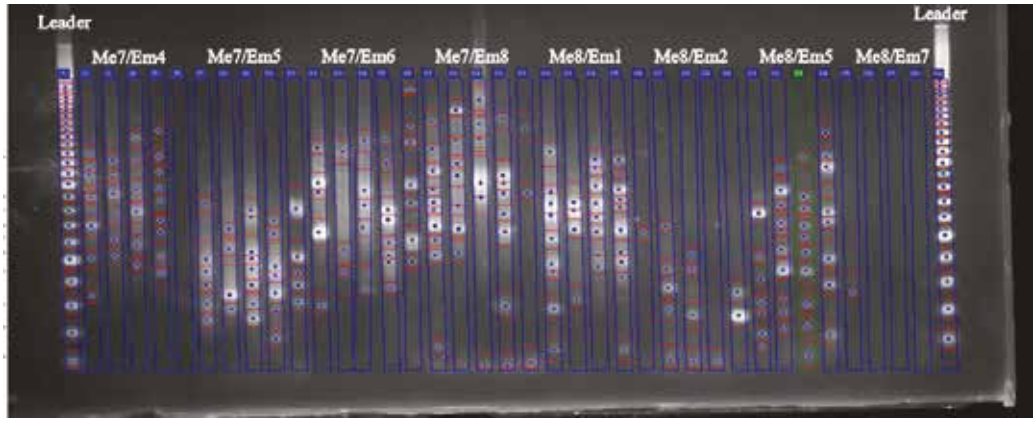

Figure 2. Electrophoretic profiles of four species obtained with eight primer combinations

A. moldavicum samples show good results with the primer combinations: *Me7/Em8, Me8/Em1, Me8/Em5, Me7/Em4*, *Me6/Em6*, while for *A. transsilvanica* the most effective primers pairs were founded to be: *Me3/Em5, Me6/Em5, Me7/Em8, Me8/Em5*. For *R. carpaticus* the following combinations were the most informative: *Me3/Em3, Me4/Em1, Me7/Em5, Me8/Em1, Me8/Em5*. Besides, the most effective primer combinations for *R. platanifolius* were *Me7/Em4, Me7/Em6, Me7/Em8, Me8/Em1, Me8/Em6*.

Regarding the number of amplified bands per primer pair, 13 primer combinations were selected as the most promising for *A. moldavicum*, *A. transsilvanica* and *R. platanifolius,* respectively, while for *R. carpaticus* 18 combinations were the most representative (Figure 3).

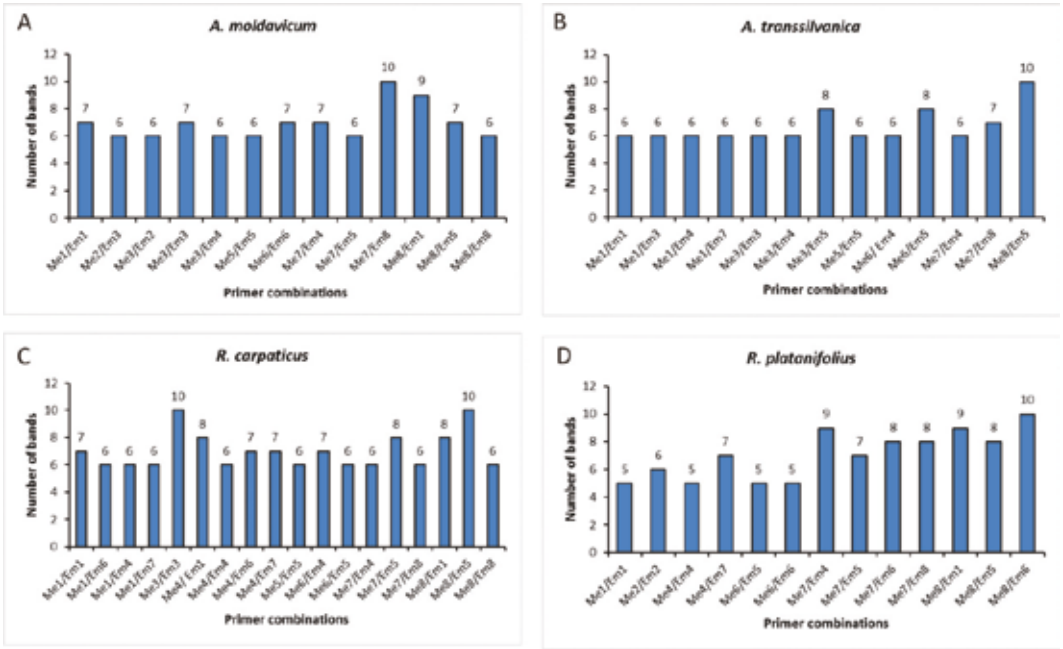

Figure 3. The representative combinations of *A. moldavicum*, *A. transsilvanica, R. carpaticus* and *R. platanifolius*

Similar studies were carried out on *Carthamus* species as reported by Mokhtari et al., 2013, who found that the most informative SRAP primer combinations were *Me4/Em1* and *Me5/Em2*.

Another report published on *Citrus* species show, that the most effective primers pairs that worked, were *Me3/Em2* and *Me6/Em4* (Uzun et al, 2009).

Moreover, based on the number of polymorphic fragments the most representative SRAP primer combinations reported for 76 *Vitis* species were *Me1/Em7, Me3/Em2, Me5/Em6* (Guo et al., 2012). Likewise, Huang et al, 2017 reported that for *Stylosanthes* species the best working SRAP primer pairs for genetic variation analysis were *Me1/Em2, Me6/Em7, Me8/Em1* and *Me9/Em2* (Huang et al., 2017).

Therefore, it can be concluded that the screening of SRAP primer combinations is needed to ensure a high polymorphic content for further analyses.

CONCLUSIONS

As a conclusion, these results provide useful information of the most efficient SRAP marker combinations of four medicinal species namely *A. moldavicum*, *A. transsilvanica*, *R. carpaticus* and *R. platanifolius* also for *in situ* and *ex situ* conservation perspective. Furthermore, in this study have been shown that the selected SRAP markers combinations represent a powerful tool and highly contribute to perform future analyze intra/interspecies and intra/interpopulation genetic diversity.

To the best of our knowledge, SRAP markers were used for the first time to analyze genetic diversity of species belonging to the *Ranunculaceae* family.

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MISCELLANEOUS


EX·TERRA·AVRVM