Genetic Diversity and Relatedness Among Six *Ranunculus* Species Unraveled by SRAP Markers

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Abstract

The main aim of this study was to investigate the genetic diversity and phylogenetic relationships of six *Ranunculus* species using SRAP markers. The genotyping of 73 *Ranunculus* accessions showed that 12 primer pairs generated a total number of 212 polymorphic fragments with a 99.53 % level of polymorphism. Nei's gene diversity ranged between 0.2237 and 0.3436 with an average of 0.3001. The UPGMA method with Nei72's similarity coefficient ranging from 0.23 to 1.11 separated 73 accessions into two distinct groups (A and B). Cluster-A included *R. acris* members, while cluster-B included the rest of the tested species. Cluster-B was subsequently divided into two sub-groups (I and II). Subgroup-I consisted of *R. carpaticus* and *R. serpens* subsp. *nemorosus* accessions, while subgroup-II included the members of *R. platanifolius*, *R. polyanthemos,* and *R. repens*. *R. polyanthemos* and *R. repens* were founded the most closely related species. These findings provide a brand-new understanding of the evolutionary relationships of above mentioned *Ranunculus* species and may be useful tools for plant breeding and conservation purposes.

Keywords: *Ranunculus*; genetic variation, genetic resources, phylogeny, sequence-related amplified polymorphism marker (SRAP)

Introduction

In the last few years, awareness of the importance of adopting a holistic view of biodiversity conservation and sustainable use the natural resources has been increasing. Plant genetic resources are among the most crucial natural resources of the world, thus their conservation is essential for present and future human well-being (Rao and Hodgkin 2002). Because of the differences in evolutionary rates, plant DNA sequences can be used to examine taxonomy, origin, and the evolution of plant species (Rao and Hodgkin 2002). Moreover, analyses regarding genetic diversity and structure at intra- and inter-specific level are especially important for a better use of genetic resources in breeding and conservation strategies of wild plants (Hamrick and Godt, 1996). Molecular markers have been increasingly used as an important tool to evaluate genetic variability and genetic relationships among various plant species including many of the Ranunculaceae family (Idrees and Irshad, 2014; Li *et al*., 2013; Paun *et al*., 2005).

Ranunculaceae Juss. (buttercup family) is one of the early lineages in flowering plants, being characterized by hypogynous flowers with

numerous stamens, including approximately 59 genera and 2500 species (Heywood, Brummitt, Culham 2007). The most representative genera of the buttercup family are *Aconitum*, *Anemone*, *Clematis*, *Delphinium*, *Ranunculus,* and *Thalictrum* which constitutes approximately 90% of the species (Heywood *et al.*, 2007).

Ranunculus L. (buttercups) represents the largest genus within the *Ranunculaceae* family, including ca. 600 species spread worldwide, but most commonly occurs in the temperate and cold areas of the northern hemisphere (Dochev *et al.*, 2014). Buttercups are established in various terrestrial or aquatic habitats from lowlands to high alpine zones, and terrestrial species are often specialized in extreme, mainly cold/humid, but sometimes also xeric conditions (Hörandl *et al.*, 2005). Various morphological adaptations and different reproductive strategies such as vegetative reproduction, self-compatibility, and agamospermy might be important factors for the ability of the species to colonize habitats at higher altitudes and latitudes (Hörandl *et al.*, 2005). From a genetic point of view, an important character for the genus is the presence of the *Ranunculus* type (R-type) chromosome (c.3–12 μm long). The basic chromosome numbers of *Ranunculus* taxa are x=8 (e.g. *R. cantoniensis*, *R. quelpaertensis,* and *R. ternatus*) or x=7 (e.g. *R. crucilobus*, *R. japonicus*, and *R. sceleratus*) (Chung *et al.,* 2013). Considering the two potential basic chromosome numbers, aneuploidy might have played an important role for lineage diversification in the genus. Moreover, different polyploidy levels together with autopolyploidy and allopolyploidy have been reported for many species of the genus, which might have had a major contribution to lineage speciation (Inceer *et al.*, 2007; Hörandl *et al.*, 2005). In addition, most of the species of the genus are rich in a broad variety of chemical components such as alkaloids and lactones, including anemonin, isoranunculin, protoanemonin, and ranunculin which can also be found in *Ranunculus* species (Hao *et al*. 2015a). Due to the presence of various medicinal phytometabolites, many *Ranunculus* species have been used for centuries as medicines, spices, and vegetables after cautious processing to reduce their toxicity (Hao *et al.*, 2015). Many species of the genus have been characterized as an analgesic, antibacterial, anti-inflammatory, and antitumor agents (Hao *et al.,* 2015)

In Romania the *Ranunculus* genus is represented by ca. 35 species spread all around the country (Cristea, 2014). Many local species of the genus have economic importance. They have been used as medicinals, insecticides, ornamentals and some are locally consumed as food (eg. *R. ficaria*) (Săvulescu, 2010). In traditional medicine, local species of *Ranunculus* have been used to treat various ailments such as arthritis, bronchitis, cough, fever, gout, neuralgia pains, rheumatism, ulcer and skin diseases (Alexan *et al*., 1991; Tămaş, 2005). However, little is known concerning the genetic variation and phylogeny of *Ranunculus* species from Romania. Previous molecular studies carried out on phylogenetic relationships of various species and accessions of *Ranunculus* has been attempted using isozyme (Suranto, 2002), ITS markers (Emadzade *et al.*, 2010; Paun *et al.*, 2005; Hörandl *et al.*, 2005), microsatellite markers (Florence *et al.*, 2007), RAPD markers (Fischer *et al.* 2000) and rbcL sequences of chloroplast DNA (Cai *et al*., 2010). Besides these, to the best of our knowledge no other reports on genetic variation and phylogenetic analysis of *Ranunculus* species using sequence-related amplified polymorphism (SRAP) markers. Therefore, the main aim of this study was to investigate the genetic diversity and phylogenetic relationships in six *Ranunculus* species using SRAP markers, to establish future breeding and conservation strategies for these plants. SRAP primers proved to be suitable for genetic diversity and phylogenetic analyses among related species and could generate more polymorphic fragments than simple sequence repeats (SSR), inter-simple sequence repeat (ISSR), or RAPD markers (Budak *et al*., 2004; Talebi *et al*., 2012; Zhang *et al*., 2014).

Materials and Methods
Plant material
A total of 75 *Ranunculus* accessions belonging to six species namely *R. acris*, *R. carpaticus*, *R. platanifolius*, *R. polyanthemos, R. repens* and *R. serpens* subsp. *nemorosus* were investigated in this study. Plant samples were collected from their natural habitats in July and August 2016 in two counties from Romania (Braşov and Hunedoara), namely at Mt. Postavaru and Mt. Stamba. The voucher specimens have been stored in the Herbarium collection at the University of Agricultural Sciences and Veterinary Medicine

Table 1. *Ranunculus* accessions used in this study, voucher specimen number and origin of tested species

Plant species	Plant number	HSV*	Site location
R. acris L.	9	CLA30042	46°12´50.8´´N, 22°51´35.4´´E
R. carpaticus Herbich	14	CLA30044	45°34´56.1´´N, 25°33´21.7´´E
R. platanifolius L.	14	CLA30040	45°34´56.1´´N, 25°33´21.7´´E
R. polyanthemos L.	14	CLA30051	46°12´50.8´´N, 22°51´35.4´´E
R. repens L.	14	CLA30045	46°12´50.8´´N, 22°51´35.4´´E
R. serpens subsp. *nemorosus* L.	10	CLA 30043	45°34´56.1´´N, 25°33´21.7´´E
Total number of accessions	**75**	-	-

Note: *Herbarium Specimen number

Table 2. The forward and reverse SRAP primer used in this study

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

of Cluj-Napoca, Romania. The leaves were air dried at room temperature and stored at -20°C until further processing. The list of the accession number used for each species, voucher specimen number and origin of tested samples are provided in Table 1.

DNA extraction

The total genomic DNA was isolated from young leaves by applying the hexadecyltri-methylammonium bromide (CTAB), method as described by Lodhi *et al.* (1994) and improved by Pop *et al.* (2003). The quality and quantity of genomic DNA were estimated by measuring the absorbance at 260 and 280 nm using a NanoDrop, ND100 spectrophotometer. The concentration of DNA samples was adjusted at 50 ng/L and stored at -20 ºC until future analysis.

SRAP reactions

SRAP analysis was carried out according to previously established protocols of Li and Quiros (2001). A total of 64 SRAP primer pairs from eight forward and eight reverse primers were preliminary screened two times on one selected accession of *R. carpaticus* and *R. platanifolius* to test the ability of primer pairs to amplify DNA fragments. The twelve most reliable primers combinations that produced scorable polymorphic bands were used to amplify the rest of the accessions. The sequences of six forward and seven reverse SRAP primers used in this study are listed in Table 2.

Polymerase chain reaction was performed in 15 μL reaction mixture containing 1 × Green Buffer, 1.5 mM MgCl2, 200 μM of dNTPs, 0.3 μM of both forward and reverse primer (Generi Biotech), 1 U of Taq DNA polymerase (Promega) and approximately 50 ng of template DNA. Amplifications were carried out in a Corbette Research PalmCycler (Corbett Research) 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 reproducibility. Amplified products were separated on 1.6 % (w/v) agarose gels in 1.0 × TAE buffer, at 115 V, for approximately 2 hours. The gel was stained with 1μg/mL EtBr for 20 to 30 minutes and the image was taken under UV light with Biospectrum AC for further analysis. A 100 bp DNA ladder was
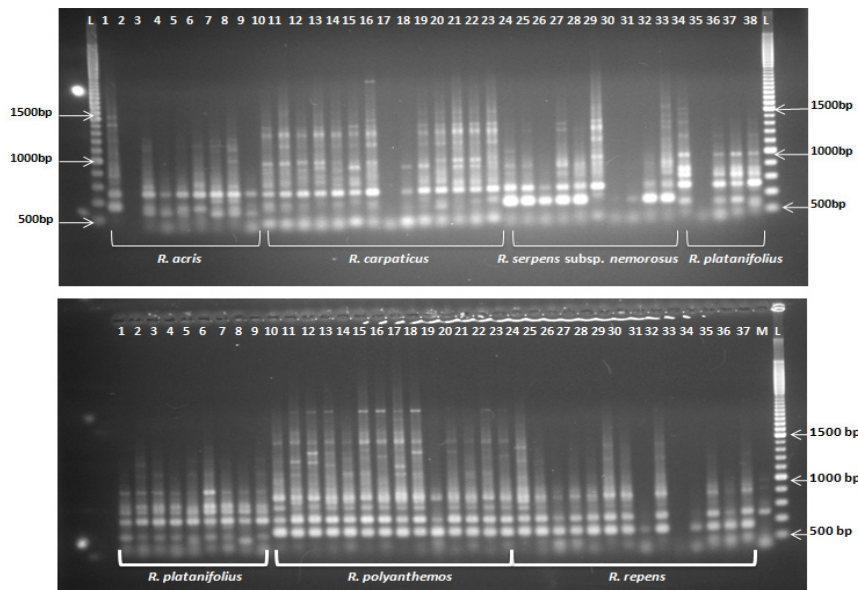
Figure 1. Electrophoresis profile of *Ranunculus* accessions obtained with *Me1/Em3* SRAP primer combination
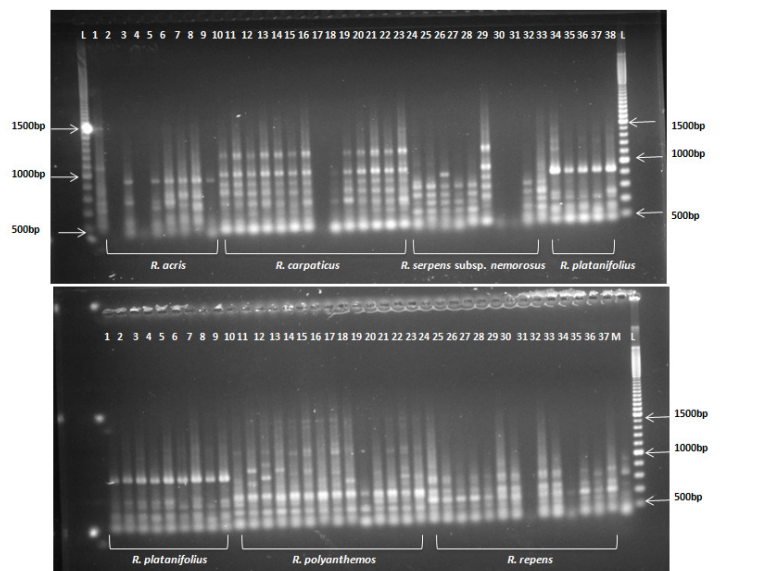(Lane L-100 bp DNA step Ladder; L1-L38/ L1-L37 *Ranunculus* samples; M-negativ control)

Figure 2. Electrophoresis profile of *Ranunculus* accessions obtained with *Me6/Em5* SRAP primer combination
(Lane L-100 bp DNA step Ladder; L1-L38/ L1-L37 *Ranunculus* samples; M-negativ control)

used as molecular standard in order to confirm the appropriate SRAP markers.

Data analysis

Each band was scored visually for their presence (1) or absent (0), twice for each accession using Total Lab 120 software. Only reproducible and clear DNA fragments were used for scoring. The data were compiled in a binary data matrix using Microsoft Excel and analyzed using the Numerical Taxonomy Multivariate Analysis System (NTSYS-pc 2.1) software package (Rohlf, 2000). The genetic distances were calculated using Nei72

similarity coefficient (Nei and Li, 1979) and a UPGMA dendrogram was constructed based on a symmetric dissimilarity matrix using the SHAN module in NTSYS pc 2.1. The COPH and MXCOP modules of NTSYS pc 2.1 were used to check the goodness-of-fit between the cluster analysis and dissimilarity matrix. The representativeness of dendrograms was evaluated by estimating cophenetic correlation for the dendrogram and comparing it with the similarity matrix, using Mantel's matrix correspondence test (Mantel, 1967). The result of this test is a cophenetic

Table 3. Polymorphisms detected by SRAP primer pairs among 73 *Ranunculus* accessions

Primer pairs	Total bands effective	Polymorphic alleles	Effective allele number (ne)	Nei's gene diversity (He)	Shannon's information index (I)
Me1/Em3	16	16	15.501	0.3308	0.5030
Me1/Em7	21	21	14.516	0.2888	0.4516
Me3/Em3	13	13	13.498	0.2353	0.3826
Me3/ Em5	14	14	13.331	0.2237	0.3654
Me4/Em7	15	15	15.580	0.3322	0.5023
Me5/Em5	16	16	14.976	0.2976	0.4572
Me6/Em4	15	15	15.725	0.3325	0.4970
Me6/Em6	16	15	14.826	0.2998	0.4596
Me8/Em1	35	35	14.750	0.2997	0.4651
Me8/Em5	19	19	14.542	0.2907	0.4567
Me8/Em6	22	22	15.201	0.3270	0.5020
Me8/Em8	11	11	15.734	0.3436	0.5196
Total	**213**	**212**	-	-	-
Mean	**17.75**	**17.67**	**14.848**	**0.3001**	**0.4635**

correlation coefficient, r, indicating how well dendrogram represents similarity data.

The principal coordinate analysis (PCoA) of the original binary data matrix and based on Jaccard coefficient was performed using the DCENTER and EIGEN modules of NTSYS pc 2.1, in order to examine the relationships and differentiations between tested accessions. Effective allele number (ne), Nei's gene diversity (He) and Shannon's information index (I) were used to compute Nei's standard genetic distance coefficients using the Popgene32 program.

Results and Discussion
SRAP analysis
Twelve primer combinations were selected based on their clarity, sharpness and number of bands produced in a pre-screening assay carried out with 64 primer combinations, on two accessions. The most efficient primer combinations were *Me3/Em3*, *Me8/Em1*, *Me8/Em5* and *Me8/Em6*. Two accessions out of the 75 tested (one for *R. carpaticus* and one for *R. serpens* subsp. *nemorosus*) did not amplify with any of the primer combinations employed, therefore these members were excluded from the study.

The selective SRAP primer combinations (12) generated a total number of 213 reproducible fragments, ranging in size from 500 to 2200 bp. The number of bands scored per primer combination ranged from 11 to 35, with a mean of 17.7. The highest number (35) of the amplification products was obtained using the *Me8/Em1* primer pairs and the lowest (11) with *Me8/Em8*. The electrophoresis profile of 75 *Ranunculus* accessions using SRAP primer combinations *Me1/Em3* and *Me6/Em5* is shows in the Figure 1 and 2.

A number of 212 fragments were polymorphic, showing a 99.53% level of polymorphism in average. The effective allele number (ne) ranged from 1.3330 to 1.5734 with an average of 1.4848, Nei's gene diversity (He) ranged between 0.2237 and 0.3436 with an average of 0.3001, and Shannon's information index (I) varied between 0.3654 and 0.5196 with an average of 0.4635 (Table 3).

Phylogenetic analysis
The similarity dendrogram of the tested *Ranunculus* accessions was constructed using UPGMA cluster method based on Nei72's similarity coefficient (Fig. 3). The accessions studied had genetic similarity coefficients ranging from 0.23 to 1.11 with an average of 0.67, indicating a high level of variation. Furthermore, the cophenetic correlation (0.84 value of the r coefficient) indicates a good fit between the original similarity matrix and the built dendrogram.

UPGMA cluster analysis grouped all the 73 *Ranunculus* accessions into two major groups (marked A and B). Cluster-A included nine *R.*
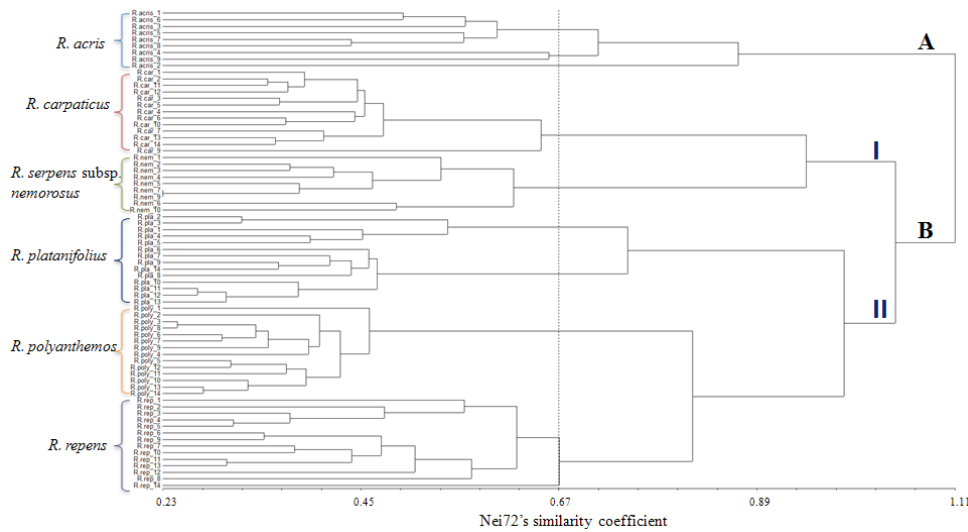
Figure 3. UPGMA dendrogram of 73 *Ranunculus* genotypes based on SRAP-based genetic similarities
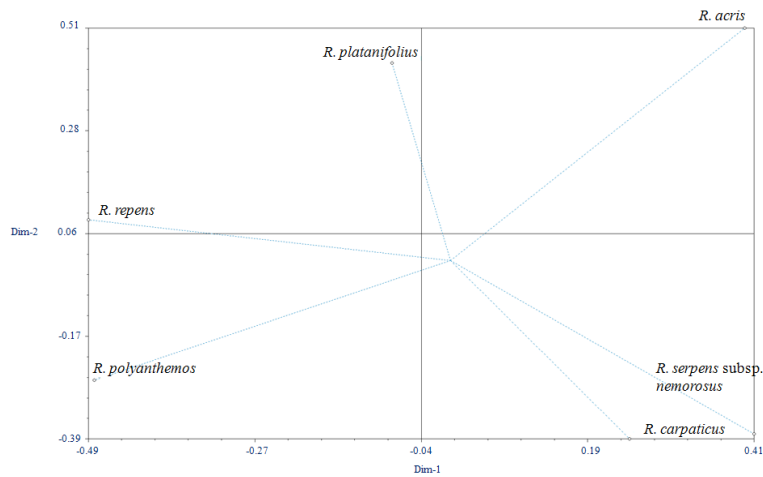

Figure 4. Relationships among *Ranunculus* species visualized by SRAP markers-based PCoA

acris members while cluster-B included the rest of the *Ranunculus* species tested (Fig. 3). This is in accordance with the results obtained by Paun *et al.* (2005) and Hörandl *et al.* (2005), where *R. acris* has been revealed as distinct species and was closely related to *R. lanuginosus*, *R. japonicus,* and *R. grandiflorus*. Moreover, cluster-B could be further divided into two sub-groups (I and II). Subgroup-I consisted of thirteen accessions of *R. carpaticus* and nine of *R. serpens* subsp. *nemorosus*, while subgroup-II included the fourteen accessions of *R. platanifolius*, *R. polyanthemos,* and *R. repens*, respectively. Furthermore, *R. platanifolius* has been revealed as a distinct species and formed an intermediate group. Regarding subgroup II, it is noteworthy that *R. polyanthemos* and *R. repens* were determined to be the most closely related

species with a similarity level of 0.73. In contrast to our findings, Paun *et al.* (2005) reported a great similarity between *R. polyanthemos* and *R. serpens* subsp. *nemorosus* taxa, based on parsimony analyses of the matK/trnK and ITS dataset. Besides, *R. carpaticus* has been revealed as distinct species and was included in the same subgroup with *R. montanus*, *R. villarsii*, *R. pollinensis,* and *R. gouanii*.

The principal coordinate analysis (PCoA) based on the Jaccard dissimilarity coefficient confirmed the number of clusters revealed by UPGMA cluster analysis (Fig. 4). Substantial dispersion of *Ranunculus* accessions in the PCoA plot suggests a high diversity amoung the these species.

Conclusions

To the best of our knowledge, SRAP markers were used for the first time in this study to analyze genetic diversity within the Ranunculaceae family. The results provide deeper understanding of the genetic diversity and evolutionary relationships of the above-mentioned *Ranunculus* species and might be a useful tool for plant breeding and conservation purposes. UPGMA cluster analysis showed that most of the accessions from the same species tended to have high genetic similarity and clustering into the same groups or subgroups. *R. polyanthemos* and *R. repens* were found to be the most closely related species, while *R. acris* has been revealed as a distinct taxon. Finally, the use of more accessions in different species might lead to more insightful results.

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