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Initial Determination of DNA Polymorphism of Some *Primula veris* L. Populations from Kosovo and Austria

Naim BERISHA[1,2] Fadil MILLAKU[1*] Bekim GASHI[1] Elez KRASNIQI[1] Johannes NOVAK[2]

[1] Department of Biology, Faculty of Mathematics and Natural Sciences, University of Prishtina "Hasan Prishtina", Prishtina, Republic of Kosovo
[2] Institute of Animal Nutrition and Functional Plant Compounds, University of Veterinary Medicine, Vienna, Austria

[*]Corresponding author:
e-mail: fadil.millaku@uni-pr.edu

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Abstract

Primula veris L. is a well known pharmaceutical plant, what for it is widely collected in all SE Europe, particularly in Kosovo. Our aim is to determine a molecular polymorphism of its populations from Kosovo. Leaves extracted DNA was investigated in details for presence of polymorphism. RAPD analyses were conducted using 20 different short primers. Genomic DNA amplification profiles were analyzed and processed using data labelling. Comparison between cowslip populations in genetic composition revealed that samples from Bogaj were too distinct on their own. Analyzed molecular variation was observed to have more molecular variation within populations (73%), compared to among populations (27%). Highest genetic distance is observed between Leqinat and M. e Madhe. He values were highest in Bogaj population, while lowest in M. e Madhe population. Results indicated that Bogaj population are more polymorphic. RAPD allows identifying and assessing genetic similarities among plant populations.

Keywords: *Primula veris*, DNA polymorphism, RAPD, populations, Kosovo.

Abbreviations: RAPD – Random Amplification of Polymorphic DNA

INTRODUCTION

Primula veris (Primulaceae) is a small perennial herb, found typically on nutrient-poor grasslands, with a distribution ranging from Spain to eastern Asia [1]. In the early spring, plants produce a rosette of several leaves and one flowering stalk (rarely two or more) that bears 5–15 yellow distylic flowers disposed in an umbel. Flowering usually begins in April and ends 3–4 weeks later in May. Seeds ripen in June–July. Flowers are mainly pollinated by *Hymenoptera* and *Diptera* [2]. Survival rate of seedlings is generally low either though vegetative propagation through side rosettes sometimes occurs [3]. The plant is considered to be a barochorous species, as the seeds of this species have no specieal features facilitating dispersal. Sometimes species leaves, flowers and inflorescences are damaged by insects or molluscs, while often they are destroyed by cattle grazing, affecting this way future potential performance [4]. *Primula* plants have heteromorphic flowers and a sporophytically controlled, diallelic incompatibility system that prevents or reduces both self-fertilization and intramorph fertilization [5]. In this paper we consider the extent of polymorphism of cowslip (*Primula veris* L.) populations in Kosovo. Cowslip as a characteristic species for calcareous grasslands has shown a sharp decline not only in this part of Europe (Kosovo) but in larger parts of Western Europe as well [6]. This particularly comes as a result of overharvesting, as the plant species is widely collected for medicinal and thus commercial needs [7]. These species are particularly at risk as their population numbers are often low and population loss is likely to result in a significant reduction in the number of their remaining populations [8]. Biotic and abiotic processes that operate at the population level also play an important role in governing plant population dynamics [9]. While the effects of environmental and demographic stochasticity on plant population dynamics have been fairly well studied, the effects of genetic stochasticity remain to a large extent unexplored [10]. Due to changed behaviour or isolation and/or abundance of pollinators, reduced gene flow between fragmented populations may lead to inbreeding which, in turn, may cause immediate loss of fitness (the so called *inbreeding-depression*; [11, 12]. In small populations, additionally the random genetic drift may erode their evolutionary potential towards adapting to changing environmental conditions [13, 14]. A number of authors indeed, have shown positive relationships between genetic diversity, population size and other fitness measures, such as reproductive output [15, 16] suggesting that the population dynamics of a plant species may be strongly affected from decreased genetic diversity. Surprisingly though, only a few authors have integrated genetic information into studies on the demography of the plant species [17, 18, 19]. In order to properly understand the relative importance of ecological and genetic factors in determining population viability, this kind of research is crucial [20, 21]. In Kosovo *Primula veris* is mainly distributed in Albanian Alps of Kosovo, though it is found also in other places in lower number of present populations [7]. The Albanian Alps are divided into three parts by a boundary between Kosovo, Albania, and Montenegro. The part of these mountains that is under the administration of Kosovo has a North-East position and is influenced by the continental climate. By collecting plant samples from 3 different Cowslip populations in Albanian Alps, detailed information was gathered in regard to their life cycle, plant associations, habitat types and soil composition. Having needed knowledge about genetic varieties of cowslip populations in Kosovo would be important for having sustainable plant sources in the future. Via this research we have aimed to find out how many existing morphs are present within and between analyzed cowslip populations, which of the observed populations are more closely related

to each other and what are the chances of effective breeding and domestification of the species. Furthermore, by getting to know more about the genetic constitution of cowslip populations, we will enable ourselves to deduce quota for harvesting on wild populations and ensuring sustainable collection.

MATERIALS AND METHODS

Plant Material

This research was conducted in 2010, being split in two main parts. In early spring we have collected plant samples, while the laboratory analyses were conducted during the time period October – December. Plant samples of cowslip (*Primula veris* L.) were collected from a random sample of individuals, growing in populations located in Albanian Alps of Kosovo (Table 1.). From three surveyed populations we took approximately 20 individuals each, sampled from large populations at Bogaj, Maja e Madhe and Leqinat, which contain several thousand individuals. For comparison reasons, in our study we have included also one set of Austrian cowslip samples, collected in suburbs of Vienna (Pötzeleinsdorfer Park). The plant samples were naturally dried at room temperature and voucher specimens of the populations are kept at the Faculty of Natural Sciences herbarium.

Table 1. List of *Primula veris* populations from Kosovo and the Austrian samples

No.	Locality name	Population code
1	Maja e Madhe	L01-XK
2	Bogaj	L02-XK
3	Liqenat	L03-XK
4	Pötzeleinsdorfer Park	L04-AT

Genomc DNA Purification

Genomic DNA was extracted from dried leaves of *Primula veris* using a modified cetyltrimethyl ammonium bromide (CTAB) extraction method [22]. Leaves were ground into a fine powder in liquid nitrogen with pestle and a mortar, re-suspending it in 25 ml CTAB extraction buffer. For 20 min the suspension was incubated at 65 ºC before the addition of 10 ml chloroform-isoamyl alcohol and gentle mixing for 20 min at room temperature. DNA quality was tested into 1.4% gel agarose and its concentration was measured with fluoremeter. DNA quantitation data were generated using reagents and protocol contained in the Total DNA Quantitation Kit [23] . This method is based on the action of a compound known as Hoechst and TEN – commercially known buffer.

RAPD PCR

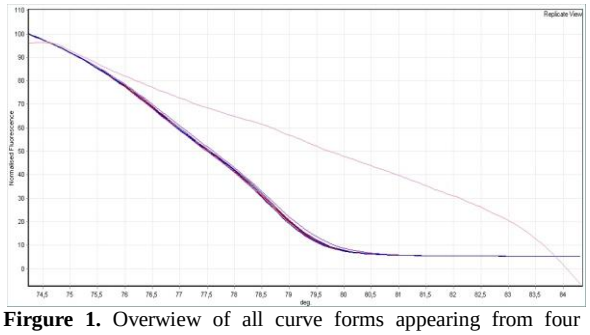
Amplification reactions were performed in a 25 µl volume containing 10 mM TRIS-HCl, pH 8.3, 50 mM KCl, 2.25 mM MgCl2, 0.001% gelatine, 0.2 mM each dNTP and 1.75 units Taq (Super-Taq, HT Biochemicals). Each reaction contained 5–50 ng of genomic DNA template obtained using the CTAB method. The PCR cycling started with an initial phase of 15 min (for the Taq HOT FIREPol® polymerase) at 95°C, then 40 cycles of 10 s at 95°C, 20 s at 60°C and a 20 s elongation step at 72°C. High resolution melting was carried out immediately following PCR from 70°C to 90°C at steps of 0.05°C, each step with a 1 s hold. Gel electrophoresis samples were mixed in 4 µl loading dye and put on 2% agarose. In order to enable measuring of DNA band lengths, in the left and the right side of the agarose gel we have put 1 µl of standard DNA (100 bp ADN-Ladder, 0,5 mg/ml, Peqlab Biotechnologie GmbH, Erlagen, Deutschland). Activity time of electrophoresis was 60 minutes. After this, gel was brought into ethidium bromide solution in order to dye and enable DNA bands be visualized by UV fluorescence. Only clear, distinct and reproducible bands were included in the diversity analysis. Bands of the same size were scored as identical. The reproducible bands were converted into binary data (presence = 1, absence = 0), and the resulting data set was analyzed.

HRM and Data Processing

PCR and HRM were performed on a RotorGene 6500 (Corbett Research Pty Ltd, Sydney, Australia) with a HRM-module, the results were analyzed using the RotorGene 6000 series software, Version 1.7.65. In order to obtain interrun comparability standard, samples were used in every run. Analyzed genetic parameters - Principal Coordinate Analyses (PCA), Percentage of Molecular Variance, Genetic Distance, Mean and SE for each population, were calculated using a software program 'GenAleX 6' [24].

RESULTS

Using high resolution melting curve analysis (HRM) as a technique for measuring exactly the decreasing fluorescence of intercalating dye in the process of dissociation of double stranded DNA, we have tried to find appropriate molecular markers in search for polymorphism between analysed *Primula veris* populations of Kosovo. For this reason 20 molecular markers were used. Despite of the fact that HRM is a very specific, time-and labour-saving method for identifying DNA sequence variations and is ideally suitable for routine PCR analysis [25], in our study none of the used markers was showing any polymorphism, as we can see in Figure 1., where all the melting curves are the same. After this, we started to test different microsatellite primers, that were previously developed but yet not tested in *Primula veris* samples, always with the aim to verify if any genetic polymorphism between and within the populations from Kosovo can be found. Again, out of 20 different microsatellite markers that were used, no one was polymorphic. In the figure we can see that the lines indicating melting temperature of double stranded DNA from *Primula veris* samples are similar to each other. These HRM graphs show no presence of polymorphism, so all these used molecular markers were not doing their job (Figure 1). After this, we switched the rest part of the work to RAPD (Random Amplification of Polymorphic DNA), that is a PCR reaction type but with the difference that the amplified DNA segments are



Firgure 1. Overwiew of all curve forms appearing from four *Primula veris* population samples.

random. Also, the use of RAPD markers is favourable over other methods (e.g., AFLP, nuclear (SSRs), or allozymes) for their technical simplicity and for providing genome-wide information from many loci [26]. Using RAPD we were able to find and use good primers and this way detect and evaluate the genetic constitution of *Primula veris* populations from Kosovo.

We have successfully tested 15 RAPD primers. As shown in Figure 2, we have formation of DNA bands in different lengths. Afterwards, we continued with data processing. In the Figure 3 is shown the difference in regard to genetic and geographical factors between analyzed populations, explained in three axes. We see here that samples from Bogaj (L02-XK) are too distinct within their own, while the Austrian set of samples (L04-AT) are not so far from the rest of Kosovarian set of *Primula veris* samples. We have had variations between analyzed populations, either though the real genetic distance between populations at site was not so high (with exception of Austrian samples). While analyzing the percentage of molecular variation (Figure 4 & Table 2) of populations we have observed that the variation among populations is lower (27%) when compared with expressed variation within populations, which is surprisingly higher (73%). Within our L04-AT samples we have had also some hybrids of *Primula veris x vulgaris*, which surely have increased values of variance inside populations versus those among populations. The analysis of genetic distance (Table 3) between populations varies between 0.028 (L02-XK and L04-AT) and 0.088 (L01-XK and L03-XK, having so the largest genetic distance). Also, we have variations between populations when it comes to pairwise matrix of genetic identity (Table 4). Pairwise distances between populations vary from 0.972 between L02-XK and L04-AT to 0.916 between L01-XK and L03-XK.

We have measured also the expected level of Heterozigosity (*He*) among other values in studied populations, these values are given in Table 5. *He* is higher in L02-XK when compared with three other populations, while lowest *He* values were recorded in L01-XK. Expected level of heterozygosity is an important value when it comes to genetic variation of natural populations. Higher *He* means higher genetic variability and vice versa.

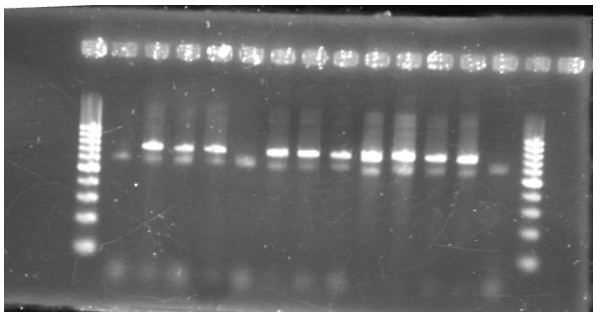


Figure 2. Example of PCR-RAPD amplification profile, generated from genomic DNA of *Primula veris* populations, resolved on 2.0% gel agarose.

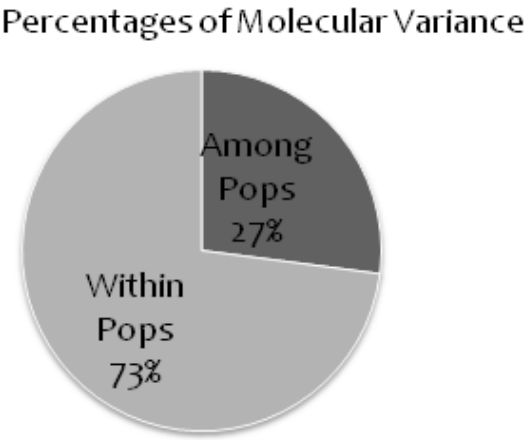


Figure 4. Scatter plot for relationship in terms of molecular variation within and between *Primula veris* populations.

Percentage of variation explained by the first 3 axes

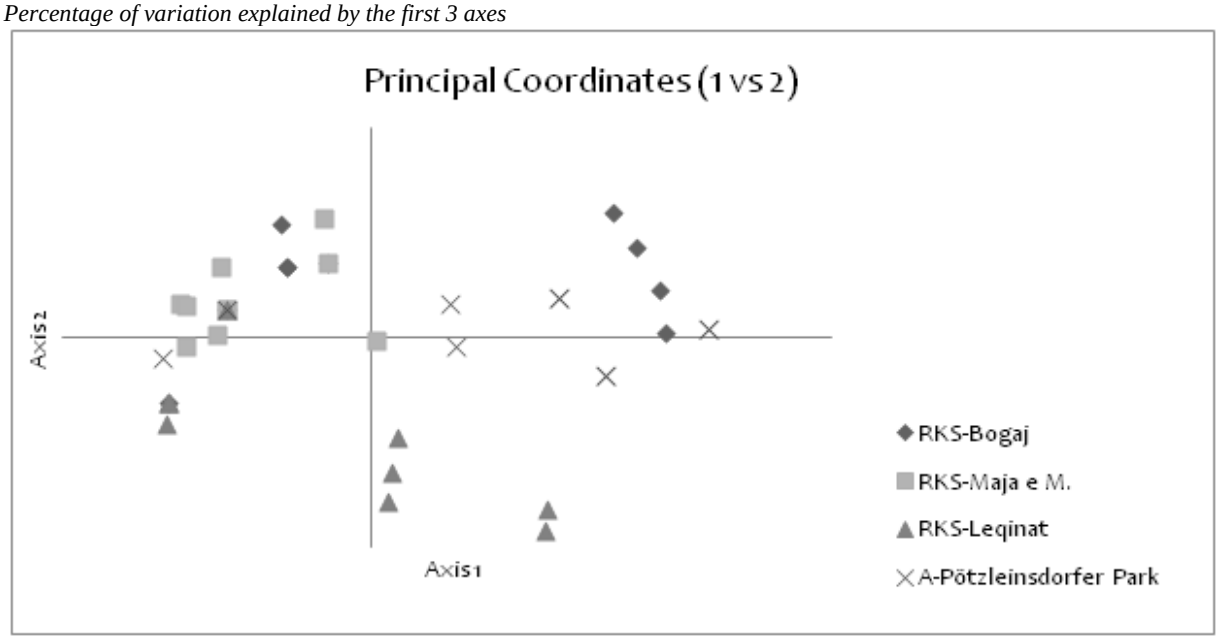


Figure 3. Principal Coordinate Analysis (PCA), showing the difference of genetic composition between analysed populations.

Table 2. Summary of AMOVA - molecular variation in %

Source	df	SS	MS	Est. Var.	%
Among Pops	3	16.250	5.417	0.427	27%
Within Pops	36	41.400	1.150	1.150	73%
Total	39	57.650		1.577	100%

Table 3. Matrix of populations genetic distance according to Nei

Primula veris Populations				
L02-XK	L01-XK	L03-XK	L04-AT	
0.000				L02-XK
0.032	0.000			L01-XK
0.060	0.088	0.000		L03-XK
0.028	0.040	0.049	0.000	L04-AT

Table 4. Pairwise population Matrix of Nei Genetic Identity

Primula veris Populations				
L02-XK	L01-XK	L03-XK	L04-AT	
1.000				L02-XK
0.968	1.000			L01-XK
0.942	0.916	1.000		L03-XK
0.972	0.961	0.952	1.000	L04-AT

DISCUSSION

This study is the first one to use molecular markers such as DNA sequencing, to determine the polymorphism of *Primula veris* populations in Kosovo. The levels of genetic variation in plant populations are highly variable [27]. In this work, the very low level of genetic polymorphism found in natural populations of *Primula veris* in Kosovo, confirms the similar results reported by other authors [28, 12, 29]. Plant species exhibit great morphological and functional variation, much of which is thought to be adaptive. In order to understand the process of adaption, it is required to synthesize several disparate levels of information. These levels include information from knowledge about measured genetic diversity and exploring the relationship between genetic variants and the environment [30].

Analysed *Primula veris* populations showed that the genetic distance between them is not so high. Our results do indicate that the *Primula veris* samples from L02-XK are with higher genetic polymorphism, being so much more suitable and far more resistant to environment changes. This is an important factor for sustainability of natural *Primula veris* populations and harvesting of these plants in wild. Additionally, we have observed that the variation between populations is lower when compared to molecular variance within populations, what makes us believe that the plant populations are composed also of hybrids, most probably of *P. veris* x *P. vulgaris*, as both species do not differ significantly also in sexual organ positions [31]. Especially at some populations, we have occurred mixed populations of *Primula veris* x *Primula vulgaris*, occurrence phenomenon reported also from other studies [32].

The results show that the *Primula veris* populations in this part of Kosovo are not so genetically diverse, though observed diversity suggests that populations are dynamic and it is possible to increase population size with adequate management [33]. This should encourage medicinal plant cultivators to expand *Primula veris* propagation with the aim of conserving natural populations. Monitoring of reproduction of *Primula veris* populations may therefore provide important indicators of future population trends.

Even though *Primula veris* is not listed as a rare nor endangered species in Kosovo [34], its occurrence in Kosovo was formerly more common than it is today. In many areas of its original distribution, the abundance and occurrence of *Primula veris* declined mainly as a result of changed land-use practices, ongoing habitat fragmentation, harvesting as a medicinal plant, etc. Combining intense grazing, in order to allow seedling establishment and relatively relaxed grazing, allowing seed production, might be the optimal management strategy to maintain viable *Primula veris* populations in long-term scale [35, 32]. This sort of management regime could be achieved through applying a year-round variation in grazing combination.

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Table 5. Mean values of population genetic variation for 4 *Primula veris* populations.

Mean and SE over Loci for each Pop.

Pop		N	Na	Ne	I	He	UHe
L02-XK	Mean	10.000	1.000	1.241	0.234	0.151	0.159
	SE	0.000	0.277	0.087	0.072	0.049	0.051
L01-XK	Mean	10.000	0.929	1.102	0.141	0.080	0.084
	SE	0.000	0.267	0.038	0.049	0.029	0.030
L03-XK	Mean	10.000	1.000	1.143	0.176	0.103	0.109
	SE	0.000	0.277	0.055	0.055	0.035	0.037
L04-AT	Mean	10.000	0.714	1.218	0.194	0.130	0.136
	SE	0.000	0.266	0.091	0.075	0.051	0.054

SE – *Standard error*, N – *Number of individuals*, Na – *Number of alleles*, Ne – *Number of effective alleles*, I – *Shannon Index (or Diversity Index)*, He – *Expected heterozigosity*, Uhe – *Expected neutral heterozigosity*.

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