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Analysis of DNA polymorphism in Lithuanian populations of *Saxifraga hirculus* L.

Donatas Naugžemys,

Donatas Žvingila,

Edita Meškauskaitė*,

Jonas R. Naujalis

*Department of Botany and Genetics,
Vilnius University, M. K. Čiurlionio 21,
LT-03101 Vilnius, Lithuania*

We used RAPDs (random amplified polymorphic DNAs) to analyze genetic diversity in the arctic-alpine yellow marsh Saxifrage (*Saxifraga hirculus* L.) which is considered to be a glacial relict. Five populations from different regions of Lithuania were studied. A total of 76 individuals were examined in this research. By RAPD analysis 93 loci were detected, of them 71% were polymorphic. All the plants showed different RAPD phenotypes. The percentage of polymorphic bands within populations correlated with population size. UPGMA and PCO analyses showed the genetic specificity of the populations. The genetic distance among individuals in different populations ranged from 0.225 (Juodlė population) to 0.29 (Merkinė population). Some population-specific minor loci were identified in the Merkinė and Juodlė populations.

Key words: *Saxifraga hirculus*, RAPD, genetic diversity

INTRODUCTION

S. hirculus is a perennial cross-pollinated plant propagating in a vegetative way or by seeds. The species is widespread in Arctic regions, in damp and mossy spots of tundra. In Lithuania, as well as in other Central European countries, it is a postglacial relict. Its habitats reduced markedly due to climatic changes in the postglacial period, therefore only small, fragmented populations occur in marshy and springy places. This is the reason why *S. hirculus* is protected by international and national laws in many countries [1]. *S. hirculus* is included into the Bern Convention (1996) Appendix I and Habitat Directive (1992/43/EEC) Annex II lists of species. *S. hirculus* is ascribed to the second (i.e. rapidly becoming extinct) category of Lithuanian Red Book. In Lithuania, not only the warming of the climate, but also agricultural expansion and melioration, especially in the second half of the 20th century, are supposed to contribute to the rapid extinction of this species.

Studies on *S. hirculus* are comparatively recent [2] and were induced by the interest to the genetic processes in vanishing plant populations. In Lithuania, so far only results concerning the demographic structure of *S. hirculus* populations and ecological impacts on them have been published [3]. Relict populations are very interesting objects for studying the demographic and genetic processes that are taking place in them. It is a well known fact that the level of genetic diversity greatly depends on population size [4]. The level of demographic and genetic diversity in vanishing populations is supposed to be not high, because the populations of such plants are not numerous. However, there are some deviations from this rule. There have been studies [5] to show that rare species may have not only a low but also a rather high level of genetic diversity. This fact should be accounted for while planning the preservation strategy for species that may become extinct. Therefore, one of the basic tasks of the current work was a comparative analysis of the genetic diversity of *S. hirculus* populations. Data on the genetic structure of plants allow a better evaluation of the evolutionary potential of a given species and thus a prognosis of the peculiarities of the dynamics of this index.

MATERIALS AND METHODS

To assess the genetic diversity of *S. hirculus* populations, five populations from different regions of Lithuania were examined (Fig. 1; Table 1). In May–October 2005, from each population 10–20 individuals were taken at a distance of no less than 1 m from one another. In total, 76 plants were examined. For DNA extraction, about 100 mg of fresh *S. hirculus* leaves was used. DNA was extracted within two days after sampling.

DNA from *S. hirculus* leaves was isolated by the modified CTAB method [6]. Genomic DNA amplification was performed in a Biometra TPersonal thermocycler [7], the number of PCR cycles being reduced to 35. Gel electrophoresis was performed in a GNA-200 chamber (Amersham Pharmacia Biotech) at 4 V/cm. As a standard of DNA fragment size, a GeneRulerTM

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* Corresponding author. E-mail: edita.meskauskaite@gf.vu.lt

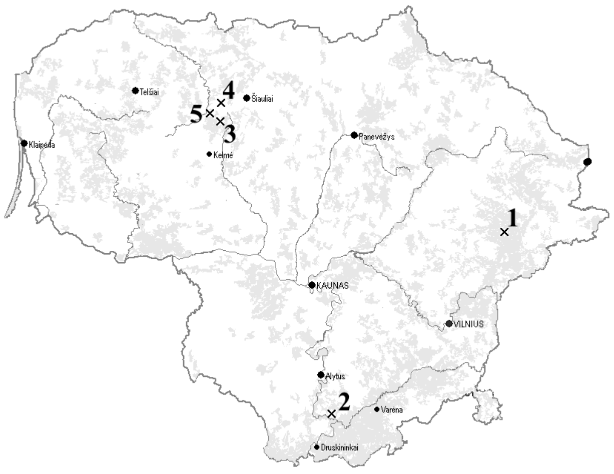

Fig. 1. Locations of five Lithuanian populations of *Saxifraga hirculus* L. studied. Population designations: *1* – Labanoras, *2* – Merkinė, *3* – Galvydiškė, *4* – Jautmalkė, *5* – Juodlė

Table 1. **Sample size and geographical location of *S. hirculus* populations**

Population	Number of plants sampled	Latitude (°N)	Longitude (°E)
Labanoras	10	55°15’20”	25°44’45”
Merkinė	20	54°11’07”	24°08’41”
Galvydiškė	19	55°47’01”	22°58’16”
Jautmalkė	17	55°47’49”	23°01’16”
Juodlė	10	55°48’44”	22°55’39”

DNA Ladder Mix (Fermentas, Vilnius) was used. The BioDocAnalyze documenting system (Biometra) was employed to register electrophoresis results. Only distinct, reliably repeating DNA bands obtained in two replications were analyzed.

DNA bands of equal mobility were treated as identical. Genetic distance between individuals (GD_{xy}) within a population was calculated according to Nei’s and Li’s formula [8]: $GD_{xy} = 1 - 2N_{xy} / N_x + N_y$, where N_{xy} is the number of DNA bands present in both *x* and *y* plants, N_x is the number of DNA bands inherent in the plant *x*, and N_y is the number of DNA bands inherent in the plant *y*. The GD_{xy} values were used to construct a UPGMA dendrogram employing TREECON for Windows [9]. The genetic parameters of populations, such as the number of polymorphic loci (P), the average observable number of alleles per locus (n_a) [10], Nei’s (1973) gene diversity (*h*) [11], the Shannon index of phenotypic diversity (*I*) [12] were estimated with PopGene v.1.31 [13]. Loci with the most common allelic frequency below 95% in all populations were considered polymorphic.

Principal coordinate analysis (PCO) of *S. hirculus* populations data was performed using GenAlEx v.6 (*Genetic Analysis in Excel*) program [14]. STATISTICA v.7.0 software (StatSoft Inc. USA 2004) was used to calculate the dependence of genetic diversity on the size of population sample.

RESULTS AND DISCUSSION

While studying *S. hirculus* DNA polymorphism by the RAPD method, we tested 14 random DNA sequence oligonucleotide primers whose sequence length was 10 nt. RAPD analysis with these primers revealed 93 RAPD loci to reliably repeat in two independent tests. The length of DNA fragments studied varied from 470 bp to 2400 bp. The largest number (15) of RAPD bands suitable for analysis was furnished by primer 380-01 and the least number (8) by primer 270–10 (Table 2).

The monomorphic loci were detected with all primers. The number of such loci ranged from 18% (primer 380-07) to 50% (primer 270-10). For instance, a rather low but clearly distinct RAPD band polymorphism was established using primer 270–01 (Fig. 2). A population-specific locus ($270–01_{790}$) was detected in two individuals (Nos. 6 and 7) of the Juodlė population (Fig. 3). Rare specific loci were also found in the Merkinė population. For instance, locus $380–06_{1350}$ was detected in two plants from this population. Moreover, some loci ($270–05_{1100}$, $380–01_{900}$ and $380–08_{650}$) might be genotype-specific. Interestingly, in one *S. hirculus* individual (No.4) we found two rare loci ($270–05_{1100}$ and $380–06_{1350}$). In summary, *S. hirculus* polymorphism

Table 2. **Primers used in RAPD analyses, numbers and sizes of generated bands**

Primer	Primers sequence 5'→3'	No. of DNA bands	Number of monomorphic loci	Number of polymorphic loci	Percentage of polymorphic loci (%)	DNA bands size range (bp)
270–01	GTC TCG TCG G	10	4	6	60	600–2100
270–04	CGG GTC GAT C	9	2	7	77.8	650–2000
270–05	GCC CTC TTC G	10	2	8	80	590–1800
270–10	TGC ACG GAC G	8	4	4	50	810–2300
380–01	ACG CGC CAG G	15	4	11	73.4	590–2400
380–06	CCC GAC TGC C	10	4	6	60	780–2400
380–07	GGC AAG CGG G	11	2	9	81.8	470–1700
380–08	CGC ACC GCA C	10	2	8	80	650–2000
380–09	ACG GCG GCT C	10	3	7	70	700–2000
	Total:	93	23	66	71	470–2400

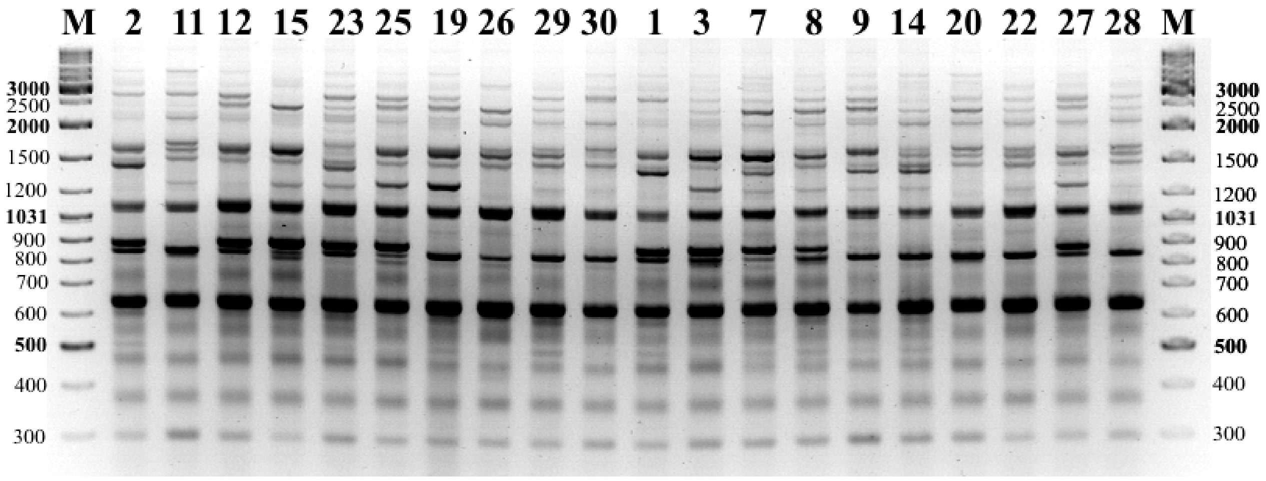

Fig. 2. RAPD analysis with primer 270-01 of *S. hirculus* from Merkinė population

M – standard of DNA fragment size GeneRuler^{TM} DNA Ladder Mix (100–10000bp). Arabic numerals on the top (2, 11, 12…etc.) indicate the code number of the individuals.

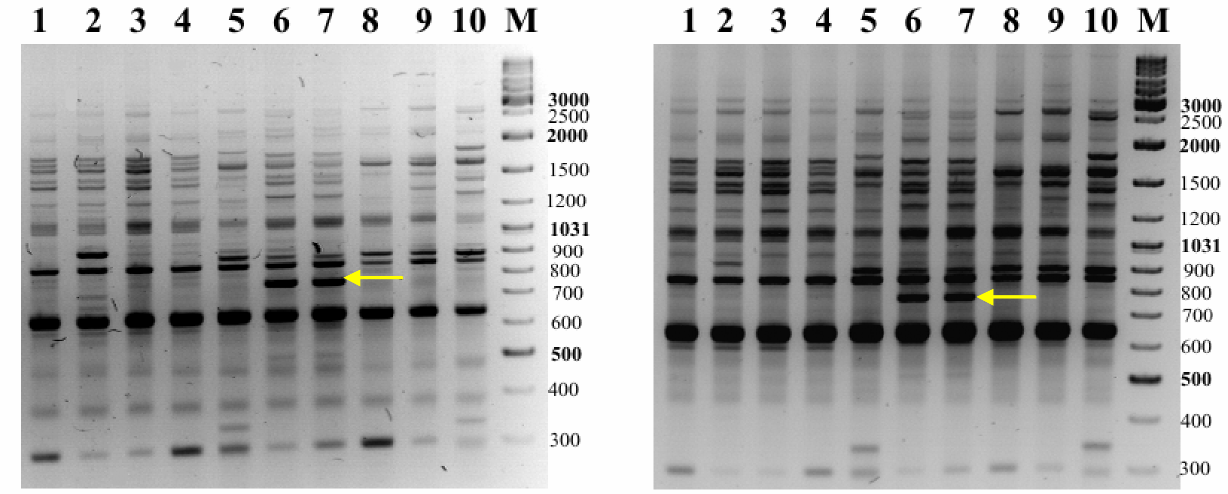

Fig. 3. RAPD analysis with primer 270–01 of *S. hirculus* from Juodlė population performed in two independent experiments

M – standard of DNA fragment size GeneRuler^{TM} DNA Ladder Mix (100–10000bp). Arabic numerals (1–10) indicate the code number of the individuals. The arrow indicates population-specific RAPD bands.

level in our study was 71% (Table 2). Since *S. hirculus* belongs to a category of rare plant species, such level of polymorphism should be regarded as high [5].

To assess the genetic relationship of the 76 *S. hirculus* individuals studied, genetic distances among the individuals were calculated. In total, 2850 pairs of individuals were formed. UPGMA cluster analysis based on GDxy values demonstrated patterns of genetic relatedness among individual plants from different populations (Fig. 4). Firstly, all the *S. hirculus* individuals studied showed to be genetically different. Secondly, it is obvious that *S. hirculus* populations are genetically peculiar, because the dendrogram shows compact groups of plants from a corresponding population. The Jautmalkė and Galvydiškė populations stand out by this feature, because their individuals group only among themselves. Individuals of the Merkinė population also group among themselves; however, they form two neighboring clusters. Only the Labanoras and Juodlė populations are more heterogeneous (Fig. 4). Thus, the genetic relatedness of *S. hirculus* individuals is undoubtedly strongly affected by the habitat. This conclusion has been confirmed also by analysis of RAPD loci distribution within the populations, performed by the method of principal coordinates (PCO) (Fig. 5). The Nei (1973) [11] genetic distances shown in the plane of the coordinates reflect differences among *S. hirculus* individuals within the populations. The three PCO axes describe the major part of *S. hirculus* populations' genetic diversity: the first axe 27.4%, the second axe 22.3% and the third (not shown in Fig. 5) 18.3%. In Fig. 5 one can see that individuals of the same *S. hirculus* population form rather compact groups. This holds in particular for the Jautmalkė, Merkinė and Galvydiškė populations which practically do not overlap with the other populations. Individuals of the Labanoras and Juodlė populations, like in the dendrogram (Fig. 4), are more scattered in the system of two coordinates. Besides, the Labanoras and

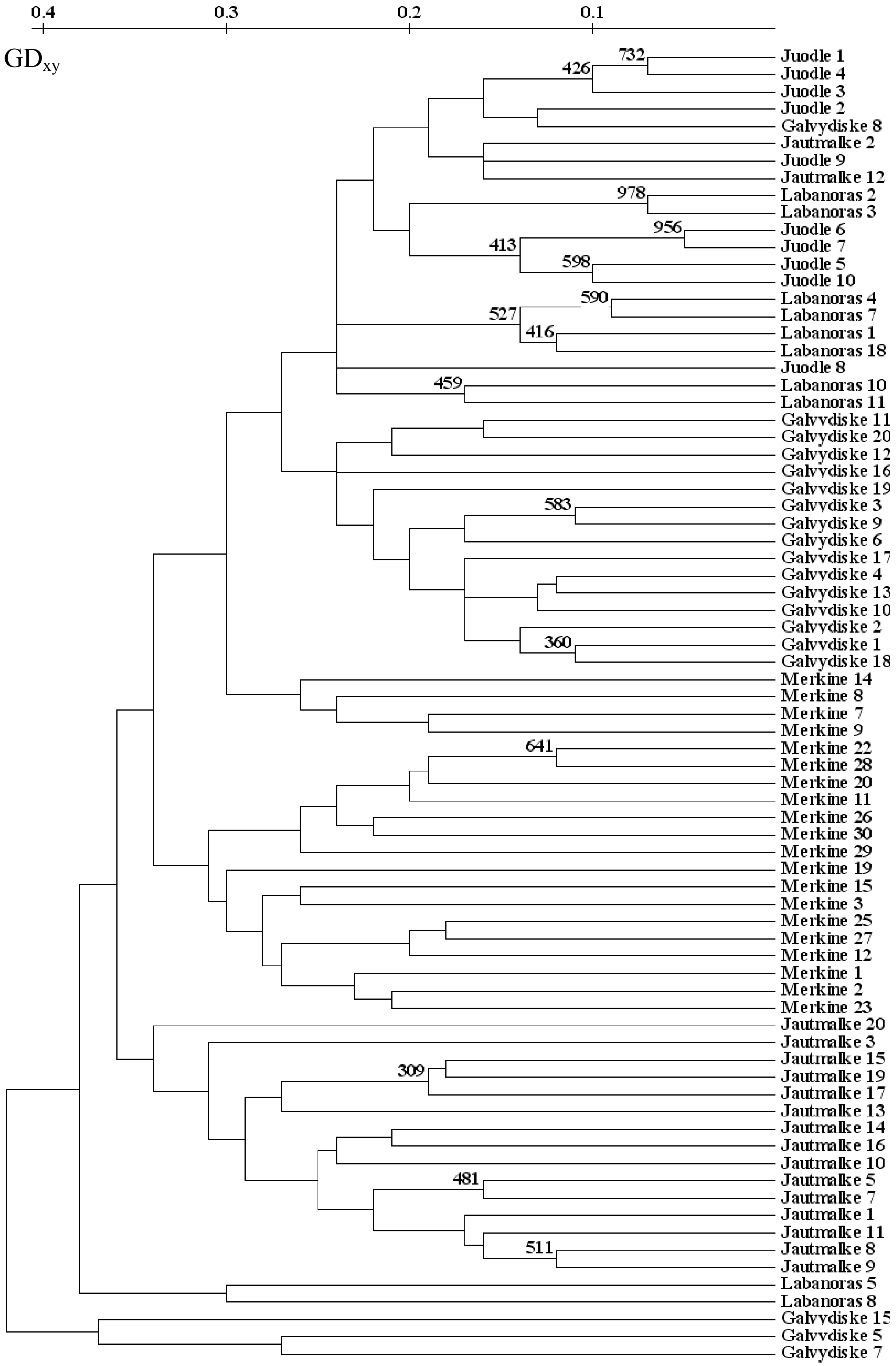

Fig. 4. Bootstrapped (1000 iterations) cluster analysis (UPGMA) of 5 populations based on the genetic distance among 76 *S. hirculus* individuals

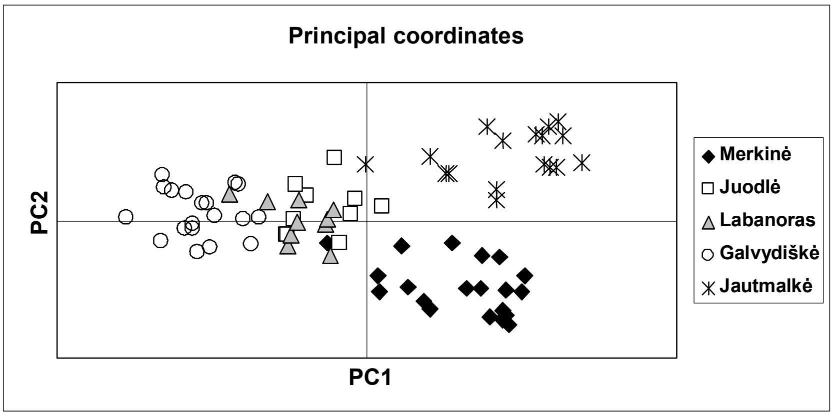

Fig. 5. Two-dimensional representation of principal coordinate analysis of *S. hirculus* populations

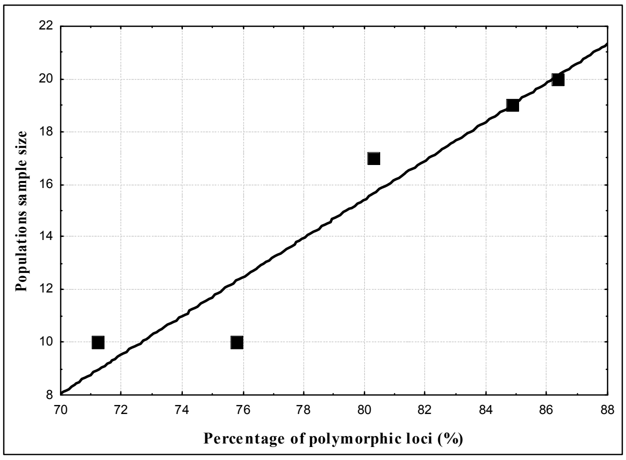

Fig. 6. Correlation between the number of *S. hirculus* individuals sampled in one population and the level of DNA polymorphism

Juodlė populations in part overlap. The PCO results confirm the genetic specificity of the *S. hirculus* populations studied.

The average DNA polymorphism level in *S. hirculus* populations is 79.7 ± 6.3% (Table 3). The Merkinė population is characterized by the highest (86.4%) and the Juodlė population by the lowest (71.2%) DNA polymorphism. A similar diversity was shown also by the other indices that characterize genetic diversity within populations. The lowest values of the Nei genetic diversity (h), Shannon's index (I), the average number of alleles per locus (n_a) are characteristic of the Juodlė population, whereas the highest values of some of these indices were found in the Galvydiškė and Merkinė populations. The mean values of n_a, h and I for the *S. hirculus* populations studied are 1.7970 ± 0.063, 0.3129 ± 0.018 and 0.4580 ± 0.027 (Table 3).

From larger *S. hirculus* populations (Galvydiškė, Merkinė) more plant samples were taken (Table 1), and the level of genetic diversity in them was also higher, implying this level to depend on the number of individuals tested (Fig. 6). Genetic diversity is known to correlate rather often with population size [4]. Our analysis revealed a strong correlation between the number of individuals tested and the level of DNA polymorphism in a corresponding population ($R = 0.955$; $p < 0.011$), indicating that the differences of values characterizing the genetic diversity among *S. hirculus* populations strongly depend on population size.

As regard genetic distances among *S. hirculus* individuals in each population, the Merkinė population was most heterogeneous ($GD_{xy} = 0.2899$). *S. hirculus* individuals from the Juodlė population were genetically closest to one another ($GD_{xy} = 0.2253$) (Table 3).

The lowered genetic diversity has been for a long time regarded as a common trait of all rare species. However, recent studies show that this opinion cannot be accepted as an axiom. There are reports [5] to show that the genetic diversity of a rare species may be even higher than that of widely distributed species of the same genus. *S. hirculus* has not been given sufficient studies (in Lithuania this aspect has never been investigated), therefore it is necessary not only to assess the genetic diversity of this species, but also to analyze some mechanisms of the formation and maintenance of genetic diversity. Our work seems to be the first to employ the RAPD method for studying *S. hirculus*, although this method has been more than once successfully used for investigating other species from the genus *Saxifraga* [15–17]. Our study revealed a high level of genetic diversity in Lithuanian populations of the relict species *S. hirculus*, their DNA polymorphism reaching

Table 3. **Calculated values of genetic diversity indices in *S. hirculus* populations**

Populations	Polymorphism %	Number of alleles, n_a	Nei's gene diversity, h	Shannon's information index, I	GD_{xy}*
Labanoras	75.8	1.7576	0.3090	0.4476	0.2809
Merkinė	86.4	1.8636	0.3253	0.4812	0.2899
Galvydiškė	84.9	1.8485	0.3376	0.4922	0.2442
Jautmalkė	80.3	1.8030	0.2974	0.4413	0.2673
Juodlė	71.2	1.7121	0.2950	0.4276	0.2253
Mean ± SD	79.70 ± 6.3	1.7970 ± 0.063	0.3129 ± 0.018	0.4580 ± 0.027	0.2615 ± 0.026

* GD_{xy} – mean genetic distance among individual plants in population.

71%. A similarly high level of polymorphism has been found also in some other endemic species of the genus *Saxifraga*. For instance, DNA polymorphism in the Central European endemic species *S. paniculata*, also studied by the RAPD method, reached 91.2% [15]. In another relict arctic-alpine species, *S. azoides*, 72.8% of allozyme loci were polymorphic [18]. The high polymorphism of *S. hirculus* RAPD loci could be predetermined by the biological peculiarities of this plant: the practically unlimited duration of life, the prevailing vegetative way of propagation, a potential for seed propagation and allogamy. The results obtained in the present study allow to conclude that, in spite of the high genetic diversity in *S. hirculus* populations, in not abundant populations of this species a tendency to a decrease of the values of this indicator is possible. On the other hand, in the Merkinė and Juodlė *S. hirculus* populations unique RAPD loci have been found. Combined with the UPGMA and PCO data, these results indicate that *S. hirculus* populations are characterized by a genetic uniqueness which should be preserved both in nature and in gene banks.

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Donatas Naugžemys, Donatas Žvingila,
Edita Meškauskaitė, Jonas R. Naujalis

SAXIFRAGA HIRCULUS L. DNR POLIMORFIZMO ANALIZĖ LIETUVOJE

Santrauka

RAPD metodu buvo tirta pelkinės uolaskėlės (*Saxifraga hirculus* L.) genetinė įvairovė. Ištirtos penkios populiacijos iš skirtingų Lietuvos rajonų. Šiuo metodu išaiškinti 93 lokusai, iš kurių 71% buvo polimorfiškas. Nustatytas ryšys tarp tirtų individų skaičiaus ir DNR polimorfizmo populiacijoje. Didžiausias DNR polimorfizmas rastas Merkinės (86,4%), o mažiausias – Juodlės populiacijoje (71,2%). UPGMA ir PCO metodai išryškino tirtų populiacijų genetinį savitumą. Genetinis atstumas tarp individų populiacijose svyravo nuo 0,225 (Juodlės populiacija) iki 0,29 (Merkinės populiacija). Merkinės ir Juodlės populiacijose buvo nustatyta keletas populiacijoms specifinių lokusų.