Genetic diversity of the endangered endemic species *Hedysarum sangilense* Krasnoborov et Timokhina (Fabaceae)

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ABSTRACT

Hedysarum sangilense Krasnoborov et Timokhina (Fabaceae) is a rare subendemic species of the Fabaceae family. It has been found in a restricted area, only in isolated habitats in Tyva Republic and Northern Mongolia. Two PCR techniques, using RAPD and inter-simple sequence repeat (ISSR) markers, were used to perform a comparative analysis of genetic diversity in this species. When amplifying DNA with three RAPD primers, we produced 51 bands, of which 21 (40.6 %) were polymorphic. Amplification of genomic DNA using ISSR analysis yielded 96 fragments, of which 35 (36.6 %) were polymorphic. Nei,s gene diversity (H) was estimated to be 0.105 within populations (range 0.086–0.150) and 0.191 at the species level. Genetic differentiation among populations (GST) was 0.383. The results indicate that both of the marker systems RAPD and ISSR, individually or in combination, can be effectively used in the determination of the genetic relationship among and within populations of *H. sangilense*.

K e y w o r d s : endangered species, genetic diversity, *Hedysarum sangilense*, ISSR, plant conservation, rare species, RAPD, subendemic

РЕЗЮМЕ

Селютина И.Ю., Кониченко Е.С., Зибзеев Е.В., Кубан И.Н. Генетическая изменчивость эндемичного вида *Hedysarum sangilense* Krasnoborov et Timokhina (Fabaceae). *Hedysarum sangilense* Krasnoborov et Timokhina (Fabaceae) – редкий геми-эндемичный вид семейства Fabaceae. Этот вид встречается на не большой территории, только в изолированных местообитаниях в Туве и Северной Монголии. Для анализа генетического разнообразия у этого вида использовались два метода ПЦР с использованием маркеров RAPD и ISSR. При амплификации ДНК с тремя праймерами RAPD получен 51 фрагмент, из которых 21 (31,04 %) был полиморфны. Амплификация геномной ДНК с использованием шести ISSR праймеров дала 96 фрагментов, из которых 35 (36,6 %) были полиморфными. Генетичекое разнообразие Nei (H) составило 0,105 внутри популяций (диапазон 0,086–0,150) и 0,191 на видовом уровне. Генетическая дифференциация между популяциями (GST) составила 0,383. Результаты показали, что обе маркерные системы RAPD и ISSR, по отдельности или в сочетании, могут быть эффективно использованы для выявления генетической изменчивости в популяциях и определения генетической взаимосвязи между популяциями *H. sangilense*.

Ключевые слова: генетическое разнообразие, охрана растений, редкие виды, эндемик, *Hedysarum sangilense*, ISSR, RAPD

Hedysarum sangilense Krasnob. (Fabaceae) is a rare subendemic plant of Tuva Republic and Northern Mongolia. This species has been found in restricted areas in isolated habitats. *H. sangilense* is a perennial plant that occurs on the Sangilen Plateau in alpine habitats, larch forests and riverbanks. The range of this Central Asian rock endemic is restricted to this plateau, which occupies the southeastern part of the Republic Tuva in Russia (Kurbatskii 1994). According to the herbarium label, *H. sangilense* is also found on the eastern Tannu-Ola Ridge and the surrounding areas of northern Mongolia (Krasnoborov & Timokhina 1975, Ma lyshev & Sobolevskaya 1980, Gunin et al. 1999, Urgamal et al. 2014).

The species *Hedysarum sangilense* Krasnob. et Timoch. was first described by Krasnoborov & Timokhina (1975). This new species was different from all other species of the genus *Hedysarum* common in Siberia by morphological characteristics.

Six populations of *H. sangilense* have been studied from tundras of *Kobresia myosuroides* (Vill.) Fiori and *Dryas*

oxyodonta Juz. on the highlands of the Sangilen Plateau, as well as from dwarf birch thickets with *Betula fruticosa* Pall. in the middle reaches of the Naryn River (Fig. 1).

H. sangilense populations on the Sangilen Plateau are restricted to a small area of few mountainous sites, severely restricting gene flow or seed dispersal among populations; all sites occur within a small geographic area. These factors increase the threat of extinction for the species.

Mountain habitats of Central Asia in the most continental areas experience extreme climatic conditions. Effects of habitat isolation should be especially pronounced where mountains rise from dry and cold highlands, such as the Sangilen Plateau. Steep topographic and therefore climatic gradients lead to heavily fragmented habitats, characterised by barriers to migration and genetic exchange. Levels of natural fragmentation are thus generally high, and several studies have demonstrated strong genetic effects and isolation by distance (Bauert et al. 1998, Schonswetter et al. 2002, 2004). Effects of habitat isolation are especially pro-

Figure 1 Map of the six *Hedysarum sangilense* Krasnoborov et Timokhina populations. See Table 1 for abbreviations.

nounced where mountains rise from dry lowlands, such as the Central Asian Gobi.

MATERIAL AND METHODS

Plant material. *Hedysarum sangilense* is a perennial herb 25–35 cm in height with a branched caudex; chromosome numbers are 2n = 16 + 1B, 2n = 14 (Flora Sibiriae 1994, Krasnikov & Schaulo 2004).

Six populations of *H. sangilense* were investigated in this study, and in each of population, 16 to 18 individuals (total 104) were randomly sampled (Fig. 1, Table 1).

Plants were collected in June and July 2014. Population size ranged from 200 to ~600 individuals (Table 1). Distance between population varied from 1 km to up to several tens of km; DNA was isolated from dry leaves. Equal amounts (0.09–0.12 g dry weight) of total genomic DNA were extracted according to the CTAB method described by Doyle & Doyle (1987). The CTAB extraction buffer was used for DNA isolation, and DNA was precipitated by cold isopropanol.

RAPD and ISSR amplification. The DNA amplifications with each RAPD and ISSR primers were repeated at least twice to ensure reproducibility. The PCR amplifications were performed in a total reaction volume of 25 µl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.2 mM each dNTP, 1.15 mM $MgCl_2$, 10 mM primer, 1 U of Tag polymerase and 30–50 ng of genomic DNA. The DNA amplifications were performed using a C 1000 Thermal Cycler (BioRad Laboratories, USA); amplification was conducted for 35 cycles. Each PCR cycle consisted of the following steps: 0.40 min at 94°C, 0.45 min at annealing temperature (Table 2), 1.30 min at 72°C; an initial pre-denaturation step (1.30 min at 94°C) and a final extension step (5 min at 72°C) were added. For each primer, we determined the best annealing temperature by performing a gradient PCR. The PCR products were separated by electrophoresis on 1 % agarose gel in 1 × TBE buffer (50 mM Tris, 50 mM boric acid, 2.5 mM EDTA, pH 8.3) for 3 h 30 min at 100 V and detected by staining with bromphenol blue. Gels were stained with CYBR-Green (Medigen, Russia) and then photographed under UV light. The size of the amplified products was determined by comparison with a 100-bp molecular weight ladder (Medigen, Russia).

For all samples, PCR reactions were carried out using the same thermocycler. The gel images were identified using Bio-Rad GelDoc XR+.

Data analysis. The amplifications were independently repeated three times using the same procedure to ensure that the amplifications obtained with the primers were reproducible and consistent. The amplified bands were scored manually as 1 (present) and 0 (absent). A binary matrix was obtained by visual scoring of the bands in the cases of both RAPD and ISSR.

Data were analysed using the POPGENE software (version 1.31; Yeh et al. 1997) to estimate genetic diversity parameters: PPB (percentage of polymorphic loci), A_E (the effective number of alleles per locus), A_O (observed number of alleles per locus), H_E (Nei's (1973) gene diversity). At the species level, genetic diversity measures (H_T) and the level of gene flow (N_M) were measured using Nei's gene diversity statistics; Nei's genetic distance between populations was computed using the same program (Nei 1972). Genetic diversity was estimated using Shannon's information measure (Lewinton 1972). To examine the genetic relationship at the species level, a dendrogram was constructed using UPGMA of the POPGENE software. We performed AMOVA, which partitions total phenotypic variance into within and among populations, using GENALEX v. 6.51 (Peakall & Smouse 2012). To test the correlation between genetic distance and geographical distance among populations, a Mantel test was performed, computing 999 permutations.

Table 1. Sampling details of populations used in the present study.

№	Population code	Locations	Coordinates	Altitude (m)	N^A	Habitat	Community type
1	NRN1	Plateau Sangilen, left bank of the Naryn River	95°56'25.7"N 50°09'52.9"E	1530	200	river valley	Larch forest after fire
2	NRN 3	Plateau Sangilen, the left bank of the Naryn River	96°08'18.4"N 50°08'42.3"E	1733	300	river valley	Thickets *Betula fruticosa* in place of burned wood
3	NRN 2	Plateau Sangilen, left bank of the Naryn River	96°10'11.2"N 50°10'00.8"E	1740	200	river valley	Thicket of *Betula fruticosa*
4	SNG 4	Plateau Sangilen, the upper Balik-Tig-Khem River	96°18'20.5"N 50°15'05.2"E	2408	200	rocky slope north-west exposition	*Dryas* tundra
5	SNG 3	Plateau Sangilen, the upper Balik-Tig-Khem River	96°19'35.4"N 50°15'28.0"E	2497	200	rocky slope south exposition	*Dryas* tundra
6	SNG 2	Plateau Sangilen, the upper Balik-Tig-Khem River	96°28'12.9"N 50°19'17.6"E	2375	600	rocky slope south-west exposition	Tundra (*Kobresia myosuroides*)

RESULTS

Two independent methods, RAPD and ISSR, were applied to investigate the variability among the genotypes of *Hedysarum sangilense*.

In the case of ISSR, a set of 25 primers was used for screening of all accessions; of these, 6 produced clear and reproducible fragments, which were chosen for further analysis. A total of 96 replicated bands were present, of which 35 bands were found polymorphic, which amounted to a 36.6 % polymorphism. The bands produced ranged from 10 to 18 per primer, with an average of 16; their size varied from 300 to 1500 bp (Table 2).

Percentage polymorphism ranged from 0 to 100, with an average of 35.11 Shannon's information ranged from 0.099 to 0.310, with a mean of 0.176. Mean Nei's (1973) genetic diversity (H) in populations was 0.124 (range from 0.066 to 0.202) and on the special level 0.189 (Table 3). Nei's (1978) genetic distance between populations ranged from 0.061 to 0.142. The lowest genetic distance was between populations SNG2 and SNG3 and the highest between populations SNG3 and NRN3.

A cluster tree of ISSR markers grouped the genotypes into two clusters (Fig. 2). The first cluster included populations NRN1, NRN2 and NRN3 and the second cluster populations SNG2, SNG3 and SNG4. Thus, ISSR primers were able to distinguish *H. sangilense* genotypes from different geographical areas.

Out of six primers screened, only three RAPD primers were selected to detect polymorphism based on reproducible banding patterns. In total, 51 bands were obtained, out of which 21 (40.6 %) were polymorphic. The number of bands per primer ranged from 13 to 20, with an average of 17. The percentage of polymorphism detected with three RAPD primers was 41.2 %. Genetic similarity was calculated from Shannon's information and ranged from 0.065 to 0.268, with a mean of 0.144. Nei's (1973) gene diversity (H) values within populations were 0.114 (range from 0.044 to 0.211) and 0.175 on the species level (Table 3).

Nei's (1978) genetic distance among populations ranged from 0.056 to 0.158 (Table 4). The lowest Nei's genetic distance value was calculated as 0.056 between populations SNG4 and NRN2, and the highest value was noted between populations NRN1 and NRN3 (0.158). The distance matrix was subjected to UPGMA clustering to generate a dendrogram, which separated *H. sangilense* genotypes into three clusters (Fig. 2).

Combining both markers, a total of 147 DNA fragments were produced, of which 51 (34.7 %) were polymorphic, with an average of 5.2 polymorphic fragments per primer. Genetic similarity was calculated from Shannon's information index for all 106 genotypes of the *H. sangilense* complex; combined RAPD and ISSR ranged from 0.099 to 0.336, with a mean of 0.174. Mean Nei's (1973) genetic diversity (H) was 0.132 (range from 0.046 to 0.210); on the species level, it was 0.173. Nei's (1978) genetic distance range was from 0.053 to 0.156. Two clusters were produced (Fig. 2) in patterns that more closely resembled the ISSR analysis compared to the RAPD analysis.

Combining both markers, genetic differentiation among populations at the species level (GST) was 0.383, and gene flow among populations (N_M) was 0.804. The results

Table 2. ISSR primers in this study and analysis of ISSR-generated banding patterns.

Primers	Sequence 5' to 3'	Annealing temperature (°C)	No. of bands scored	No. of polymorphic bands	P, %
RAPD					
OPA 08	GTGACGTAG – <G>	32	13	5	38.5
OPA 09	GGGTAACGC – <C>	34	20	9	45
OPA 20	GTTGCGATC – <C>	32	18	7	38.9
Average			17	7	40.6
Total			**51**	**21**	
ISSR					
UBS 807	$(AG)_8 G$ – <T>	53	17	5	29.4
UBS 834	$(AG)_7 GY$ – <T>	55	14	5	35.7
UBS 835	$(AG)_7 G$ – <A>	54	18	8	44.4
UBS 855	$(AC)_8 CY$ – <T>	60	14	6	42.8
UBS 857	$(CTC)_3 G$ – <C>	55	17	4	23.5
99B	$(CA)_6 G$ – <G>	42	16	7	43.7
Average			16	5.8	36.6
Total			**96**	**35**	

Table 3. Genetic variability within populations of *Hedysarum sangilense* detected by RAPD and ISSR analyses.

Population	A_O	A_E	I	H	PPB (%)	PrB
RAPD						
NRN1	1.118	1.077	0.065	0.044	11.76	0
NRN3	1.588	1.295	0.286	0.185	58.82	0
NAR2	1.333	1.186	0.168	0.111	33.33	0
SNG4	1.157	1.110	0.091	0.062	15.69	0
SNG3	1.274	1.118	0.116	0.074	27.45	0
SNG2	1.392	1.237	0.141	0.211	39.22	0
Mean	1.310	1.170	0.144	0.114	31.04	0
Species level	1.961	1.277	0.290	0.175	39.22	0
ISSR						
NRN1	1.184	1.112	0.099	0.066	18.45	4
NRN3	1.388	1.238	0.210	0.140	38.83	6
NAR2	1.621	1.326	0.310	0.202	62.14	14
SNG4	1.320	1.176	0.105	0.160	32.04	4
SNG3	1.233	1.138	0.123	0.082	23.30	1
SNG2	1.359	1.149	0.150	0.095	35.92	3
Mean	1.351	1.190	0.176	0.124	35.11	5.33
Species level	1.932	1.297	0.310	0.189	93.20	32

A_O, observed number of alleles per locus; A_E, the effective number of alleles per locus; H, Nei's (1973) gene diversity; I, Shannon's information index; PPB, percentage of polymorphic loci; PrB, private bands for population.

of AMOVA point to higher levels of variation within populations (68 %) than between populations (38 %) (Table 5).

The result of the Mantel test with 999 permutations revealed a significant correlation between matrices of genetic distance and geographic distance ($r = 0.668$, $P = 0.011$).

In the PCA based on Nei's genetic distance matrix of genets, the first (PC1) and second (PC2) principal component axes accounted for 28.60 and 12.54 % of the total variation, respectively (Fig. 3).

Table 4. Nei's (1978) unbiased estimates of genetic identity and genetic distance among populations of *Hedysarum sangilense* by improved RAPD and ISSR.

Population	NRN1	NRN3	NRN2	SNG4	SNG3	SNG2
RAPD						
NRN1		0.854	0.866	0.909	0.924	0.941
NRN3	0.158		0.908	0.882	0.875	0.907
NAR2	0.144	0.097		0.945	0.933	0.882
SNG4	0.095	0.125	0.056		0.981	0.899
SNG3	0.079	0.133	0.069	0.019		0.903
SNG2	0.061	0.098	0.125	0.106	0.102	
ISSR						
NRN1		0.918	0.880	0.897	0.883	0.922
NRN3	0.086		0.904	0.886	0.868	0.927
NAR2	0.128	0.100		0.904	0.875	0.894
SNG4	0.109	0.121	0.101		0.923	0.924
SNG3	0.124	0.142	0.133	0.080		0.941
SNG2	0.081	0.075	0.112	0.079	0.061	

Genetic similarity is listed above the diagonal and genetic distance is listed below the diagonal.
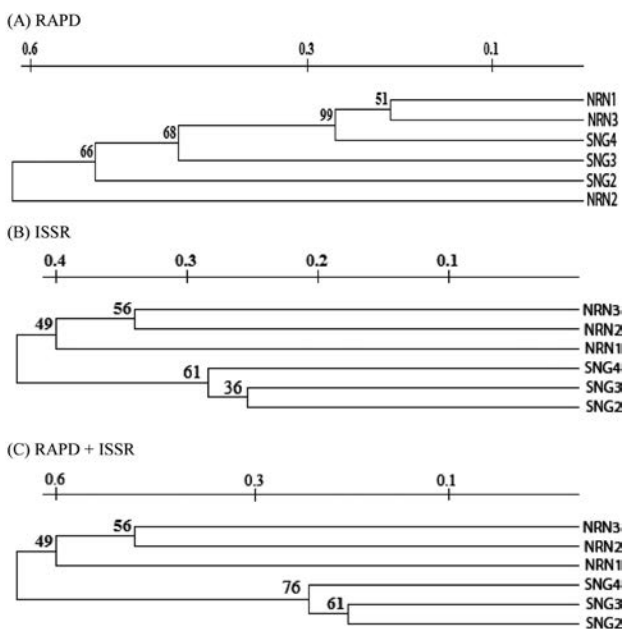

Figure 2 UPGMA dendrogram based on Nei's (1972) genetic distance using (a) RAPD (b) ISSR and (c) ISSR + RAPD data

DISCUSSION

Under changing environmental conditions, genetic diversity is crucial to effectively manage and conserve valuable, rare endemic species, such as *H. sangilense*. To understand the extent of genetic diversity, genetic structure and differentiation among *H. sangilense* populations occurring in different geographic regions, two PCR-based molecular markers, namely RAPD and ISSR, were used. The combined use of several markers types enables coverage of different parts of the genome and provides more informative data than the use of individual markers alone (Josiah et al. 2008).

In the present study, we show that these markers revealed a genetic variation among geographically separated sub-populations of *H. sangilense*. Inter-simple sequence repeats and RAPDs also revealed diversity within each population. The obtained results based on heterozygosity data for both the markers are in accordance with various studies in other wild plant species (Hien & Phong 2012, Selyutina et al. 2014, 2016).

Endemic and narrowly distributed plants usually show lower levels of genetic diversity and higher levels of genetic structure compared with their relatives with wider distribution areas. This is probably caused by the more accentuated effects of genetic drift and restricted gene flow in the rarer plants (Hamrick & Godt 1989, Nybom 2004). For the endemic *H. sangilense*, we revealed that whatever the mountain range or the population within a mountain range, most of the genetic diversity was found within populations. The same trend has commonly been reported for outcrossing and/or perennial species (Hamrick et al. 1992).

Hedysarum sangilense exhibits low levels of genetic diversity within populations (expected heterozygosity, $H_E = 0.115$), which was lower than that of the average plant species with restricted distribution ($H_E = 0.191$), using RAPD markers (Nybom & Bartish 2000). This conforms to the value ($H_E = 0.20$) found by Nybom (2004) in a literature survey for endemic species, using dominant markers.
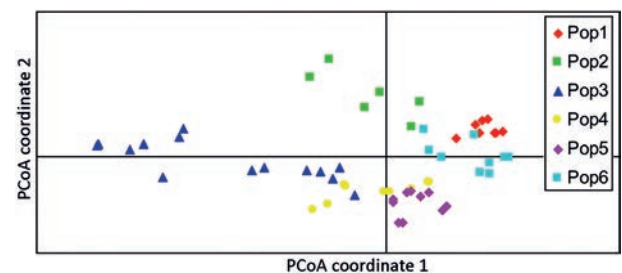

Figure 3 A two-dimensional plot of the PCA combine of RAPD and ISSR DNA markers data showing the clustering of populations of *Hedysarum sangilense* Krasnoborov et Timokhina The first and second principal coordinates account for 28.60% and 12.54% of total variation, respectively

Table 5. Analysis of molecular variance (AMOVA) within and among *Hedysarum sangilense* populations.

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance component	Total variance (%)	P - value
Among population	6	369.91	61.65	4.81	38	< 0.001
Within population	72	557.96	7.75	7.75	62	< 0.001

The genetic structure of plant populations reflects the interactions of various evolutionary processes, including the long-term evolutionary history, such as shifts in distribution, habitat fragmentation, population isolation, mutation, genetic drift, breeding system, gene flow and selection (Schaal et al. 1998). Factors such as isolation, small populations and gene flow may have a major influence on the levels of genetic diversity within and among populations (Hamrick et al. 1992). Comparisons with other studies are difficult since genetic diversity depends on numerous factors, such as life history, breeding system, growth life forms, geographical range and even the type of molecular method used (Powell et al. 1996, Nybom 2004). In spite of these complications, if we compare the results of studies using dominant markers, it appears that the genetic diversity of *H. sangilense* is lower than that of some other alpine species: *Eryngium alpinum* L. (H_E = 0.20; Gaudeul et al. 2000), *Trollius europaeus* L. (H_E = 0.22 in the Alps and 0.197 in the Pyrenees; Despres et al. 2002), *Epilobium fleischeri* Hochst., *Geum reptans* L. and *Campanula thyrsoides* L. (H_E = 0.19, 0.21 and 0.20, respectively; Kuss et al. 2008) and *Senecio boissieri* DC. (H_E = 0.19 in the Cantabrian Mountains; Peredo et al. 2009).

Our analyses of genetic structure revealed a moderate differentiation among populations (GST = 0.383). In a meta-analysis of RADP-based estimates of ΦST values, Nybom & Bartish (2000) and Nybom (2004) demonstrated that ΦST values are significantly related to life form, with long-lived perennials showing the lowest figures (mean 0.25) and species with a mixed-breeding system showing intermediate levels (means 0.25–0.4; Nybom & Bartish 2000, Nybom 2004). Endemics do not differ from more widespread species in this respect.

The comparatively low levels of population differentiation imply that in *H. sangilense*, isolation may be less severe or has occurred much more recently. This is indicated by the overlap of populations in the PCA (Fig. 2) and by the AMOVA results. Most of the genetic variance remains within populations (68 %), a pattern often described for mountain plants (Gugerli et al. 1999, Wesche et al. 2006).

Pollen dispersal is a primary mechanism of gene flow over long distances in most entomophilous plants (Levin 1987). Because the foraging flights of bumblebees are often longer than 5 km (Heinrich 1978), of the low geographical differentiation and the weak effects on genetic distance observed in *H. sangilense* may be caused by frequent long-distance pollen flow. Pollen flow is likely to be responsible for gene flow among mountain ranges (Pluess & Stocklin 2004), and cross-pollination and long-distance movement of pollen by pollinators could reduce genetic differentiation among populations (Ismail et al. 2012). Available out-crossing assures a minimum genetic flow among populations to weaken or even offset the effects of genetic drift (Bacchetta et al. 2011). As seeds are large (millimeters) and likely to be dispersed by gravity in this species, frequent gene flow via seed dispersal is considered unlikely.

The primary objective in nature conservation is to preserve as much as possible of the evolutionary potential of species through maintaining the main part of genetic diversity.

Knowledge of the genetic variation between and within populations of rare and endangered species plays a significant role in the formulation of appropriate management strategies directed towards their conservation (Milligan et al. 1994, Wallace 2002).

CONCLUSIONS

Our findings provide important genetic information for developing conservation strategies for *H. sangilense*. There is an urgent need to take effective measures to protect this species against further loss of genetic diversity; more attention should be placed on the restoration of suitable habitats and on the effective population size.

ACKNOWLEDGEMENTS

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