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MOLECULAR GENETICS

Analysis of Genetic Variation in Rare Endemic Species *Oxytropis chankaensis* Jurtz. (Fabaceae) Using RAPD Markers

E. V. Artyukova, A. B. Kholina, M. M. Kozyrenko, and Yu. N. Zhuravlev

Institute of Biology and Soil Science, Russian Academy of Sciences, Vladivostok, 690022 Russia; fax: (4232) 31-01-93; e-mail: ibss@eastnet.febras.ru

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Abstract—The method of polymerase chain reaction with random primers (RAPD) was used to assess genetic variation and population differentiation in the rare endemic plant *Oxytropis chankaensis* Jurtz. (Fabaceae). DNA samples from plants of two isolated populations were compared at 133 loci detected by use of ten primers. Both populations examined were characterized by high polymorphism levels (P_{95} = 72.9%; A = 1.92 and P_{95} = 74.4%; A = 1.88, respectively). They were also statistically significantly different in the frequencies of most of the amplicons. For each of the plants, unique multilocus RAPD phenotype was established using 17 to 20 RAPD markers. Diagnostic markers were not revealed. The populations were poorly differentiated. On average, the between-population component accounted for about 8% of the variation, while 92% of the variation was detected within populations. High variation along with the low degree of differentiation characteristic of two most geographically remote populations of *O. chankaensis* can have several explanations, among which a polyploid origin of the species seems to be most important.

INTRODUCTION

The development of a conservation strategy for rare plant species requires maximum available information on the level of genetic variation and population subdivision in these species. All measures aimed at the reintroduction of the species and maintenance of the optimal population number must imply restoration of the allelic composition and genetic structure of each population. If these issues are not taken into consideration, recombinations between the populations of different origin occurring upon seed or plant transplantation, would lead to the so-called outbreeding depression, i.e., a disruption of local adaptations and the decreased viability of the plantations created [1–3].

Oxytropis chankaensis Jurtz. is a herbaceous perennial plant, belonging to the family Fabaceae. It is a rare species with extremely limited range, local endemic of the western shore of Khanka Lake (Primorye). *Oxytropis chankaensis* is entered into the local register of rare species [4] and the Red Book of Primorskii krai [5]. This ornamental plant represents one of the key components of the unique coenoses of Khanka Lake [6]. At present, intensive human activity results in dramatic declination of the species number, to the point of the population extinction in some habitats. Preliminary estimates of genetic variation in *O. chankaensis* derived from isoenzyme studies revealed the polymorphism level, which was relatively high for an endemic species. At the same time, the populations located at the opposite parts of the species range demonstrated low level of differentiation [7]. This is somewhat unusual for a rare species existing in the form of small isolated populations. In this situation, further studies utilizing other methods of the analysis are necessary.

In recent years, genetic variation and the levels of intra- and interspecific differentiation have been successfully studied using the method of DNA analysis by use of polymerase chain reaction (PCR) with random primers, RAPD analysis [2, 8–11]. Despite of the existing limitations (dominant mode of inheritance of RAPD loci), the advantages of the method are quite evident. RAPD analysis detects polymorphic states within the entire genome and provides a substantial number of markers, which is essential for the elucidation of the relationships between the populations and species in plants. The performance of RAPD analysis requires small amounts of DNA, which is extremely important in case of rare species.

The present study was focused on determining the level of genetic variation and the degree of differentiation in two populations of *O. chankaensis* from the most geographically distant sites of the range by use of RAPD analysis.

MATERIALS AND METHODS

This study was based on the analysis of the *O. chankaensis* Jurtz. individual plants from two populations located at the opposite sites of the range, at the distance of 50 km. The samples were obtained from randomly chosen plants located about 100 m apart. The first population was situated in the vicinity of settlement Turii Rog, at the shore of Khanka Lake (sample P1, 15 individuals). The second population belonged to the territory of the Khankaiskii preserve and was located in the

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vicinity of settlement Novonikolaevka, Island Sosnovyi, 900 m from the lake shore (sample P2, 23 individuals).

Total DNA was extracted from lyophilizied leaves. The extraction buffer contained 100 mM Tris–HCl (pH 8.0); 0.7 M NaCl; 40 mM EDTA; 1% CTAB (hexadecyltrimethylammonium bromide); 10 ml/l β-mercaptoethanol. Extract was incubated at 65°C for 40 min. DNA was deproteinized with the mixture of chloroform–octanol (24 : 1) and precipitated with the equal volume of isopropanol in the presence of 0.3 M sodium acetate. DNA pellets were washed with 75% ethanol and dissolved in a buffer containing 10 mM Tris–HCl (pH 8.0) and 1 mM EDTA. DNA concentration in the samples was determined by comparison with the phage lambda DNA with the known concentration using electrophoresis in 1.4% agarose gel. PCR was conducted in 2 to 4 cycles in a thermal cycler UNO II 48 (Biometra, Germany) with decamer oligonucleotide primers (Operon Technologies, United States). Each sample was run for two to four replications. The reaction mixture and temperature conditions were as described earlier [12]. The amplification products were fractionated by electrophoresis in 1.4% agarose gel in the presence of ethidium bromide and photographed in the UV light. Only distinct fragments reproducible in the recurrent experiments were scored at the electrophoregram analysis. The amplified fragment (amplicon) sizes were determined using *Eco*RI + *Hin*dIII digests of phage lambda DNA (Fermentas, Lithuania) as molecular size markers.

For statistical analysis of the data, binary matrices for each primer were constructed, where the presence or absence of identical fragments in the profile was designated as 1, or 0. Different intensity of the bands corresponding to the amplicons of identical size was not taken into consideration. To estimate genetic diversity, matrix-based calculations of the proportion of polymorphic loci at 95% criterion (P_{95}) were performed. Also, the number of alleles per locus (A) was determined and Fisher's test for population differentiation was conducted using POPGENE [13] and TFPGA [14] software packages. The coefficient of pairwise similarity was calculated according to the formula: $S = 2N_{ab}/(N_a + N_b)$, where N_{ab} is the number of identical fragments in individuals a and b, and N_a and N_b are the numbers of fragments in individuals a and b [15]. Based of the S values between the plants in each sample and between the plants from different populations, the indices of mean within- and between-population similarity were calculated. The matrices of pairwise genetic difference (D_N) were used for the construction of the dendrograms of genetic relationships between the plants examined using the UPGMA and MST (minimum spanning tree) methods within the TREECON software package [16]. Estimates of phenotypic diversity within and between populations were made using Shannon's index, since its application does not require the assumption on the existence of the Hardy–Weinberg equilibrium in the population [8, 17]. Shannon's diversity index was calculated for each population as $H_0 = -\Sigma p_i \log_2 p_i$, where p_i is the frequency of the ith RAPD fragment. Variation for the total sample was determined as $H_{sp} = -\Sigma p_k \log_2 p_k$, where p_k designates the fragment frequencies in the sample. H_{pop} is the mean value of H_0 for two samples. The proportion of within population diversity was determined as H_{pop}/H_{sp}, while the proportion of between population diversity was determined as $(H_{sp} - H_{pop})/H_{sp}$.

RESULTS

Informative primers were selected through typing with the DNA samples obtained from three *O. chankaensis* plants taken from different habitats. A total of 52 primers were tested, including OPE-01–OPE-20, OPH-01–OPH-20, and also OPB-04, OPB-05, OPB-08, OPB-10, OPB-11, OPB-14, OPB-15, OPB-18, OPF-04, OPF-10, OPG-06, and OPG-08, used previously for the RAPD analysis of other representatives of Fabaceae [18, 19]. Of these primers, 16 appeared to be inactive, 11 initiated the synthesis of either one, or two fragments, or the products with the low signal intensities. The remaining primers yielded distinct primer-specific RAPD patterns. Ten of these effective primers, producing RAPD profile differing in the marker plants by three and more fragments, were chosen for further investigation (Table 1). Amplification of the DNA samples from 38 plants from two populations with these primers produced 133 distinct amplicons reproducible in repeated experiments. Of these, 121 amplicons were polymorphic. Depending on the primer used, the number of polymorphic fragments varied from 5 to 20, and their sizes varied from 400 to 2500 bp (Table 1). RAPD patterns of ten representatives from each population of *O. chankaensis* are presented in Fig. 1. Of 133 loci scored, 12 were monomorphic, 3 were found in the RAPD profile of 37 plants, while one fragment was absent in all but one sample. Most of the polymorphic amplicons were present in the RAPD profile of the plants from both populations. Eleven of these fragments were highly frequent (>0.75), while the frequency of 25 was low (<0.25), or they were absent from one of the populations. The unique fragments were represented by 12 fragments in P2 and 4 fragments in P1. Their frequencies, however, were not high: lower than 0.2 for 9 loci, and from 0.2 to 0.4 for seven loci. Fisher's test performed over 133 loci has demonstrated statistically significant differences in the frequencies of most of the fragments between two populations ($\chi^2 = 551.71$; *d.f.* = 266; $P = 0.00$).

Variability of RAPD markers, assessed by the proportion of polymorphic markers at the 95% level (P_{95}) along with the number of alleles per locus (A), was high both in the combined plant sample ($P_{95} = 81.9\%$; $A = 1.9$) and in single populations (P1: $P_{95} = 72.9\%$, $A = 1.92$; and P2: $P_{95} = 74.4\%$, $A = 1.88$).

Table 1. Characteristic of the primers used

Primer	Fragment size, bp	The number of polymorphic fragments in the population		Total number of the fragments	
		P1	P2	scored	polymorphic
OPB-18	410–1690	9 (0.828)	8 (0.727)	11	9 (0.818)
OPE-04	715–2500	16 (0.941)	14 (0.933)	17	16 (0.941)
OPE-07	480–1352	5 (0.714)	6 (0.857)	8	7 (0.875)
OPE-14	660–2070	12 (1.000)	11 (0.917)	13	13 (1.000)
OPE-16	540–2000	15 (1.000)	20 (1.000)	20	20 (1.000)
OPH-05	560–1900	10 (0.833)	11 (0.846)	13	12 (0.923)
OPH-08	575–1150	6 (0.857)	6 (0.857)	7	6 (0.857)
OPH-12	500–1500	11 (0.917)	13 (1.000)	14	14 (1.000)
OPH-13	660–2050	18 (1.000)	17 (0.944)	19	19 (1.000)
OPH-18	625–1770	4 (0.400)	5 (0.455)	11	5 (0.455)
Total		106 (0.876)	111 (0.874)	133	121 (0.910)

Note: P1, population 1; P2, population 2; in brackets is the proportion of polymorphic loci.

Diagnostic markers, present in the RAPD profile of one population and absent in the profile of another one, were not detected. Low frequency of unique amplicons suggested that population differentiation was caused by the differences in the frequencies of most of the fragments, rather than by the fixation of population-specific markers. For more precise identification of population differentiation only polymorphic RAPD loci are usually used [19, 20]. For this reason, monomorphic loci and those, present or absent only in one of the plants examined were excluded from further analysis.

The RAPD profile of each sample in the form of binary matrix represented the phenotype of this particular plant. Depending on the number of polymorphic

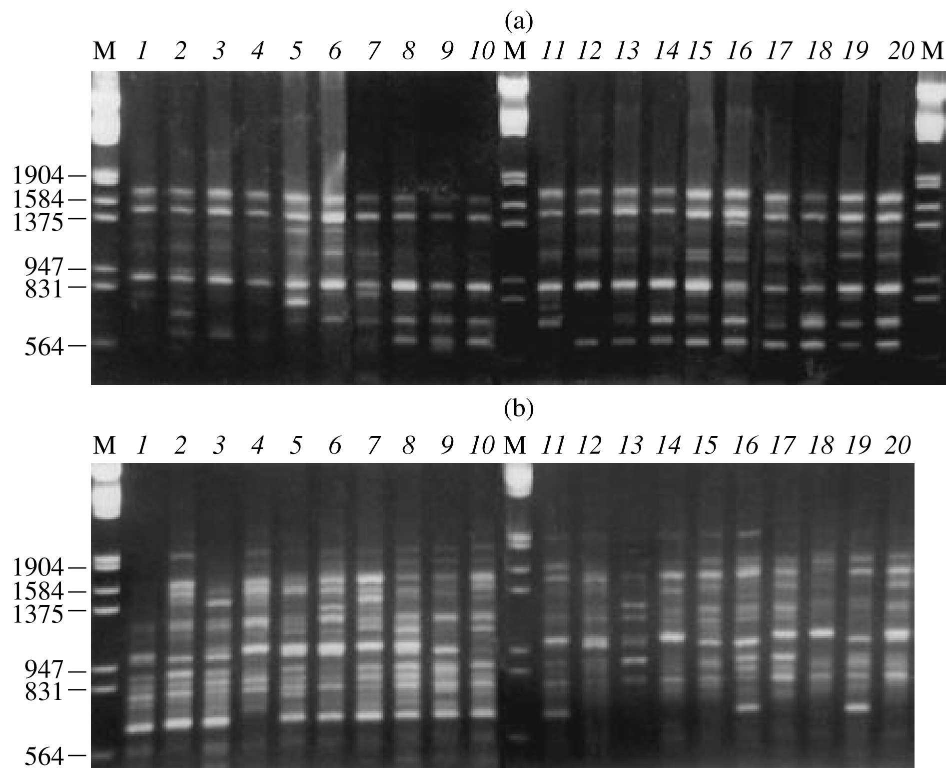

Fig. 1. DNA amplification products from 10 representatives of each population of *O. chankaensis*, obtained with the primers OPH-18 (a), OPH-13 (b). Lanes *1–10*, P1 plants; lanes *11–20*, P2 plants. M, *Eco*RI/*Hin*dIII digests of the phage lambda DNA.

fragments identified, each primer provided distinguishing of different numbers of phenotypes in the populations examined (Table 2). The number of phenotypes shared by both populations varied from 1 (OPH-12) to 5 (OPE-07). With each of the primers, OPH-08, OPH-18, and OPE-07, identifying the lower numbers of polymorphic loci, on average 15 phenotypes were detected. Note that the number of the plants with identical phenotypes in P2 was higher than in P1. Comparison of the DNA samples from 38 plant individuals over 12 to 14 polymorphic loci (primers OPH-05 and OPH-12, or OPE-07 + OPH-08) provided identification of 33 to 35 phenotypes. Further increase of marker's number (18 and more) resulted in identification of the unique multilocus RAPD phenotype for each plant.

High level of within population diversity was also confirmed by the low values of pairwise similarity coefficients, which varied from 0.474 to 0.809 in P1 and from 0.480 to 0.800 in P2. The mean group similarity indices were only slightly different, constituting 0.651 ± 0.083 and 0.639 ± 0.053 for P1 and P2, respectively. The value of the average between-population similarity index (0.580 ± 0.056) was only slightly lower than the average group values, pointing to the low differentiation between the populations of *O. chankaensis* examined.

On the dendrogram of genetic relationships constructed over 117 polymorphic RAPD markers using UPGMA (Fig. 2), all but two P1 plants examined were grouped into two clusters in accordance with the population affiliation. The distance between the clusters was not long (D_N = 0.069). It should be noted that the branching nodes had low support (the bootstrap index lower 20%). This finding could be explained by high within-population variation, which followed from the genetic distances between each pair of plants.

The degree of the population phenotypic variation assessed by use of Shannon's index (Table 3) and the indices of within- and between-population diversity varied depending on the primer used. For instance, primer OPB-18 identified only about 3% of the between-population variation, while primer OPH-05 revealed 21% of the variation. For this reason, the choice and the number of primers used have a substantial effect of the results of the experiment, and hence, on the conclusions on the degree of genetic variation within and between populations. Shannon's indices calculated over all ten primers were high for both populations. The differences between them were insignificant, indicating similar levels of genetic diversity. On average, the between-population component accounted for less than 8% of the diversity, while within-population component accounted for more than 92% of the diversity.

Table 2. The of multilocus RAPD phenotypes in populations of *Oxytropis chankaensis*

Primer	The number of phenotypes observed			Mean number of plants per phenotype	
	combined sample	P1	P2	P1	P2
OPB-18	30	11	21	1.36	1.10
OPE-04	37	15	22	1.00	1.05
OPE-07	16	10	11	1.50	2.09
OPE-14	34	15	20	1.00	1.15
OPE-16	38	15	23	1.00	1.00
OPH-05	33	12	21	1.25	1.10
OPH-08	16	8	11	1.88	2.09
OPH-12	34	15	20	1.00	1.15
OPH-13	38	15	23	1.00	1.00
OPH-18	13	7	10	2.14	2.3
All primers	38	15	23	1.00	1.00

DISCUSSION

The data obtained pointed to the high level of polymorphism in *O. chankaensis* (P_{95} = 81.9%; A = 1.9). Rare, endemic, and narrowly distributed species are usually characterized by low variation, compared to the related species with wide natural ranges [21, 22]. In some cases, however, plants with small fragmented range display relatively high polymorphism [22, 23]. For this reason, the range size can be considered as

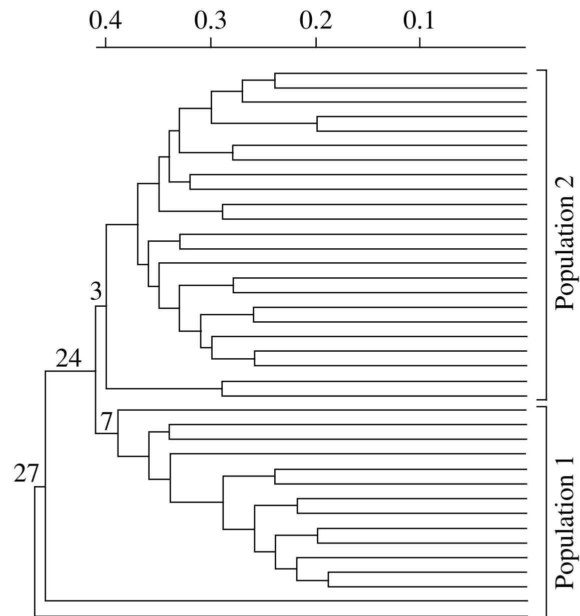

Fig. 2. UPGMA dendrogram of the genetic relationships between the plants from two populations of *O. chankaensis*, constructed using 117 polymorphic RAPD markers based on the D_N matrices. Figures indicate the clusterization robustness (bootstrap index).

Table 3. The indices of gene diversity among and between populations of *Oxytropis chankaensis*

Primer	H_0		H_{sp}	H_{pop}	H_{pop}/H_{sp}	$(H_{sp} - H_{pop})/H_{sp}$
	P1	P2				
OPB-18	0.5333	0.4030	0.4942	0.4813	0.9738	0.0262
OPE-04	0.4754	0.4730	0.5128	0.4875	0.9506	0.0494
OPE-07	0.3391	0.4149	0.4283	0.3876	0.9048	0.0952
OPE-14	0.5253	0.3517	0.4925	0.4508	0.9153	0.0847
OPE-16	0.4017	0.4992	0.4818	0.4631	0.9614	0.0386
OPH-05	0.3478	0.4268	0.5022	0.3981	0.7928	0.2072
OPH-08	0.5888	0.5546	0.6380	0.5877	0.9212	0.0788
OPH-12	0.3684	0.4494	0.4448	0.4203	0.9451	0.0549
OPH-13	0.5743	0.5162	0.5807	0.5605	0.9652	0.0348
OPH-18	0.4763	0.4374	0.5100	0.4696	0.9209	0.0791
Mean	0.4630	0.4526	0.5085	0.4706	0.9255	0.0745

Note: H_0, Shannon's diversity index for each population; H_{pop}, mean value of H_0; H_{sp}, Shannon's diversity index for total sample; H_{pop}/H_{sp}, proportion of within-population diversity; $(H_{sp} - H_{pop})/H_{sp}$, proportion of between-population diversity.

important, but not an absolute indicator of the intraspecific diversity. In addition to the range size, a definite contribution to the variation level is made by the species biology and life strategies [21], among which the reproduction system plays the most important role. It was suggested that high variation indices revealed in rare endemic species, *Aconitum noveboracense* (P = 89.7%; A = 1.5) [10], *Wyethia reticulate* (P = 75%; A = 2.25) [24], and *Leucopogon obtectus* (P = 91.5%) [25], by use of RAPD markers, resulted from the cross-pollination in these species.

The species of genus *Oxytropis* are generally obligate insect cross-pollinated plants [26]. Experiments on raceme isolation have demonstrated the possibility of reserve self-pollination in *O. chankaensis* [27], pointing to the absence of well-defined mechanism of self-incompatibility in this species. At the background of the high frequency of self-pollination in small populations the number of highly frequent phenotypes observed in the samples is usually low [18]. In case of *O. chankaensis* the use of 17 to 20 RAPD markers resulted in the identification of the unique multilocus RAPD phenotypes for each plant in the two samples examined. The presence of unique phenotypes is considered to be a specific feature of obligate cross-pollinating plants [20, 28], and can serve as an evidence of the rare cases of self-pollination in the *O. chankaensis* populations studied.

Genetic variation of *O. chankaensis* is mostly distributed within populations with the low degree of differentiation between the populations despite of their spatial isolation. This distribution pattern of within and between population variation is typical of perennial cross-pollinating plants [21], both widely distributed [17, 20], and rare endemic [10, 25]. The similarity of two geographically distant populations of *O. chankaensis* can be caused by a number of factors, including unidirectional selection pressure, which maintains individual diversity in the population, gene flow between the populations, and their common origin.

Consider some of the most probable reasons for low divergence of the populations of *O. chankaensis*. Despite the ability of bumblebees, the pollinators of this species, to cross long distances, direct crossing and exchange of the alleles between the members of populations studied is impossible due to the presence of physicogeographic isolating barriers. However, it cannot be excluded that the gene exchange can result from the transportation of the pollen by bumblebees among the system of the populations located one after another along the lake shore, or from the distribution of seeds by water or wind. According to the data of allozyme studies, the gene flow between the populations studied constituted 5.08 [34]. It is suggested that from one to ten migrants per generation is sufficient to prevent genetic differentiation between the populations caused by gene flow [29].

On the other hand, fragmentation of the species range under the action of anthropogenic factors is mostly observed during the last three decades. If this narrow-ranged species was originally characterized by a common gene pool, this time interval was not long enough to cause the decrease of genetic diversity accompanied by the increase of the population divergence due to the decrease of the population number and isolation. In addition to cross-pollination, the maintenance of certain polymorphism level is associated with the biology of this species, a perennial plant with the long reproductive period in combination with the long individual lifetime, early transformation to a generative state, and high seed production [30].

However, it seems likely that in *O. chankaensis* the most important factor that prevents the decrease of the polymorphism level and genetic depletion of the population is the increased ploidy level. The species is a tetraploid with $2n = 32$ [31]. Genetic segregation, however, is strongly affected by the type of polyploidy, auto- or alloploidy. Allopolyploids are characterized by disomic type of inheritance and the presence of fixed heterozygosity. Autotetraploids display tetrasomic inheritance, upon which all polymorphic loci exhibit both balanced and unbalanced heterozygotes with expressed gene dose effect, and also with some individuals with the presence of more than two alleles of a single locus [32, 33]. Allozymic analysis of *O. chankaensis* showed the presence of the heterozygote types typical of autotetraploids along with the simultaneous existence of three to four alleles of a singe locus. These findings suggest that *O. chankaensis* is a tetraploid species [34]. Autotetraploids are generally characterized by the increased variation levels, the presence of a great number of polymorphic loci, the increased number of alleles per locus, and higher heterozygosity levels, compared to their diploid progenitors [32, 33, 35–37]. The multiplicity of the enzymes, increased heterozygosity, and allelic diversity in tetraploids result from tetrasomic type of inheritance. Tetrasomic genetic structure is characterized by substantial rigidity, since many loci for a long time exist in the heterozygous state. Due to the low homozygote frequency, segregation of recessive alleles under the action of selection or gene flow is a seldom event [32]. This is extremely important for the species existing in small isolated populations, where the effect of gene flow is compensated by "additional" heterozygosity [38]. Polyploidy also has an effect on population differentiation. Low degree of population divergence, probably caused by weak gene flow, is typical of autopolyploid species [19, 35].

Thus, high variation level along with the low degree of differentiation characteristic of two most geographically remote populations of *O. chankaensis* can be explained by a number of reasons, among which the polyploid origin of the species, probably, plays the key role.

It should be noted that the experiments were carried out using adult plants. Some perennial herbs can live for several dozen years [39]. Aging of senile *O. chankaensis* plants based on the number of annual shoot scars [40] showed that certain individuals could live for more than 50 years. It cannot be excluded that the data obtained reflect the genetic structure of these populations in the past, before the enhancement of the range fragmentation. In some plant species the existence of genetic differentiation of populations of seeds and young plants, compared to the adult plants was demonstrated [41]. Genetic analysis of the juvenile plants, seedlings and seeds of *O. chankaensis* will possibly reveal the changes in the genetic structure after fragmentation.

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