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GENETIC DIVERSITY OF BELARUSIAN POPULATIONS OF *LIPARIS LOESELII* (L.) RICH. (ORCHIDACEAE)

Abstract. The article presents the results of a study of the genetic diversity of *Liparis loeselii* (L.) Rich. populations using molecular iPBS markers. Samples for analysis were selected from six populations located at the southern border of the species' range in the Grodno, Minsk, and Vitebsk regions of Belarus. The findings indicate that higher levels of genetic diversity are associated with populations that exhibit larger areas, greater numbers of plants, and potentially older ages of the population itself. The predominance of intrapopulation genetic diversity (74 %), as well as a low level of gene flow, indicate that genetic drift significantly affects the formation of the genetic structure of *L. loeselii*. The obtained data generally indicate a low adaptive potential of *L. loeselii*. Consequently, an unfavorable forecast for its conservation in the wild is provided, taking into account the current climate dynamics, as well as successional changes occurring within the bog biotopes.

Keywords: protected plants, Orchidaceae, molecular iPBS markers, population structure

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ГЕНЕТИЧЕСКОЕ РАЗНООБРАЗИЕ БЕЛОРУССКИХ ПОПУЛЯЦИЙ ЛОСНЯКА ЛЕЗЕЛЯ (*LIPARIS LOESELII* (L.) RICH. (ORCHIDACEAE))

Аннотация. Представлены результаты изучения генетического разнообразия популяций *Liparis loeselii* (L.) Rich. (лосняка Лезеля) с использованием молекулярных iPBS маркеров. Образцы для анализа были собраны в шести популяциях, произрастающих на территории Гродненской, Минской и Витебской областей. В результате проведенных исследований показано, что наибольшим уровнем генетического разнообразия характеризовались популяции с превалирующей численностью растений и, вероятно, возрастом происхождения. Преобладание внутрипопуляционного генетического разнообразия (74 %), а также невысокий уровень потока генов указывают, что генетический дрейф оказывает значительное влияние на формирование генетической структуры *L. loeselii*. В целом полученные данные свидетельствуют о невысоком адаптационном потенциале *L. loeselii*, что позволяет дать неблагоприятный прогноз его сохранения в естественных условиях с учетом климатических изменений, а также сукцессионных процессов, протекающих в болотных биотопах.

Ключевые слова: охраняемые растения, Орхидные, молекулярные iPBS маркеры, структура популяций

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Introduction. The reduction of native phytodiversity due to the disappearance of rare vulnerable plant species remains one of the most important environmental problems in Belarus. Currently, the most vulnerable group of plants are cold-resistant boreal species, which are located at the southern limit of their natural distribution in the country. Due to global climate change towards drying and warming,

the consequences of anthropogenic impacts, these cold-resistant species are in a particularly vulnerable position. Among them is *Liparis loeselii* (L.) Rich. – a rare relict species growing at the southern border of its range and included in the Red Book of the Republic of Belarus, where it is assigned the II category of protection as an endangered species [1]. It is also protected in all countries adjacent to Belarus, is rare throughout its vast range, and therefore is included in various international conservation documents on the conservation of biodiversity (Bern Convention, CITES Convention). Everywhere this species is characterized by a small number of coenopopulations, a sharp change in the number of plants over the years, as well as the absence of data on their structure in the scientific literature, which does not allow an objective assessment of its current state and prospects for preservation in the flora. In this regard, in recent years, the attention of researchers to this species has increased significantly. For example, data on the current distribution and ecological characteristics of the species in the Sverdlovsk [2] and Tver [3] regions of Russia, where *L. loeselii* remains an extremely rare species, have been summarized. The territory of the USA is also characterized by a decrease in the population of the species in more than half of the states, but at the same time, regular finds of the species in new habitats are noted [4], which is a reason to believe that the species can be preserved in the peripheral part of the range within the North American fragment of the range [5]. Confirmation of the above trend in Europe can be found by the find of *L. loeselii* in Bosnia and Herzegovina, where until recently this species was considered extinct [6]. For Belarus, there is also a known case of a large population of the species in the peripheral part of its range in the Gomel region [7].

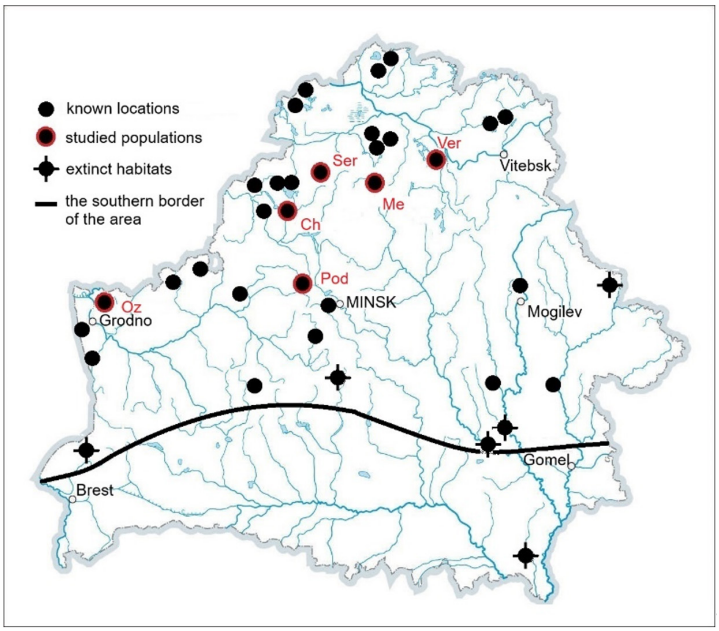
Thus, to assess the current state of *L. loeselii* and develop a strategy for its conservation, a comprehensive approach is needed that takes into account current information on the chorology, ecological and biological characteristics of the species and its population structure. At the same time, one of the important criteria necessary for a better understanding of the state of *L. loeselii* populations is an assessment of their genetic diversity, which determines the ability of species to adapt to changing environmental conditions [8], which is especially important given modern climate change. One of the ways to assess the genetic heterogeneity of plant populations is to use molecular markers, which are considered effective tools for determining genetic diversity, since they are unlimited in number, demonstrate high polymorphism and do not depend on environmental factors [9]. Based on the above, the relevance and purpose of this work is determined – using molecular iPBS markers to assess the genetic diversity of Belarusian populations of *L. loeselii*, which are important in preserving the species in the peripheral part of the European fragment of the range.

Materials and methods of the study. The object of the study is one of the rarest representatives of the Orchidaceae Juss. family in the flora of Belarus – *Liparis loeselii* (L.) Rich. It is a herbaceous perennial up to 20 cm high with an above-ground tuber and two lanceolate leaves close together at the base (Figure). Small yellowish-green flowers in the amount of 2–10 are collected in a sparse raceme. Their bracts are small, membranous, triangular or ovate-lanceolate, not longer than the peduncle. The outer leaflets of the perianth are linear-oblong, three-veined, the inner ones are narrowly linear, single-veined. The lip is almost equal in length to the other leaflets of the perianth. *L. loeselii* blooms in May–July, bears fruit in June–September. The fruit is a dry capsule with numerous small seeds. The plant reproduces by seeds and vegetatively [1; 3; 10].

L. loeselii is a circumpolar boreal species, distributed mainly in the temperate zone of Europe, Siberia and North America. In Belarus, it is located on the southern border of its range, occurring in its northern and central parts, completely absent from the Polesie Lowland (Figure), but reappears in isolated localities in the Ukrainian Polesie. Within Belarus, *L. loeselii* is usually characterized by small populations, including up to 100–200 plants [1]. The species grows in lowland sedge-hypnum and sedge-sphagnum bogs with rich mineral nutrition, as well as on lakeside trembling bogs.

The main threats to *L. loeselii* are economic transformation of lands and drainage of bogs, as well as increased recreational load on trembling bogs. However, there are cases of discovering new habitats of the species in anthropogenically transformed biotopes – abandoned peat extraction sites, overgrown quarries. For example, large populations were discovered in the Lipetsk region of Russia [11], in Belarus *L. loeselii* was recently discovered within the overgrown peat extraction site of the Urochishche Krasnoye nature reserve [12], which may indicate its plasticity.

a



b

Liparis loeselii (L.) Rich. (*a*) and its distribution on the territory of Belarus (*b*)

To study the genetic diversity and population structure of *L. loeselii*, a primer system based on conserved PBS (Primer Binding Site) sequences of LTR retrotransposons was chosen due to its ability to determine genetic diversity within and between populations, which is important for the development of scientifically based recommendations for the conservation of endangered species.

The initial material for assessing the genetic diversity of *L. loeselii* populations was samples of vegetative organs of plants collected during expeditionary research in the 2023 season in the Grodno, Vitebsk and Minsk regions. Using reconnaissance and route methods, populations of the species were found, their vitality was assessed, morphometric parameters were studied, a floristic description of phytocoenoses was made, and plant material from 5 plants in each population was selected for molecular genetic studies. Herbarium material, supplied with voucher labels, was also collected and stored in the herbarium of the Central Botanical Garden of the National Academy of Sciences of Belarus (MSKH). Docu-

mentation of sample collection sites was also carried out using the inaturalist.org information resource, which, if necessary, will allow finding these populations in the future and conducting monitoring studies. Below is a brief description of the studied model populations of *L. loeselii*, which includes a description of the geographic coordinates (a), phytocoenose (b), date and authors of sampling (c), and method of documentation (d).

Ozery (Oz): a) Grodno region, Grodno district, Novaya Ruda village, 0.7 km to the southwest, the Solomyanka river valley, WGS: 53.817401, 24.233517, b) swampy floodplain of the river, c) Mialik A. N., Dubar D. A., 01.08.2023, d) https://www.inaturalist.org/observations/176123219, MSKH Herbarium;

Podsady (Pod): a) Minsk region, Minsk district, Podsady village, 1.4 km to the north-northwest, WGS: 53.940666, 27.143562, b) short-grass mineral bog, c) Mialik A. N., Vabishchevich M. M., 21.08.2023, d) https://www.inaturalist.org/observations /179547433, MSKH Herbarium;

Chistets (Ch): a) Minsk region, Myadel district, Stakhovtsy village, 2.2 km to the southeast, Chistets bog, WGS: 54.761641, 26.800755, b) transitional sedge bog, c) Mialik A. N., Vabishchevich M. M., 21.08.2023, d) https://www.inaturalist.org/ob servations/179548016, MSKH Herbarium;

Servech (Ser): a) Vitebsk region, Glubokoe district, Derkovshchina village, 2.5 km to the southwest, Servech bog, WGS: 55.043302, 27.544799, b) transitional sedge-sphagnum bog, c) Mialik A. N., Dubar D. A., 23.08.2023, d) https://www.inaturalist.org/observations/179862791, MSKH Herbarium;

Mezhuzhol (Me): a) Vitebsk region, Dokshitsy district, Trostyanitsa village, 2.1 km to the northwest, Lake Mezhuzhol, WGS: 54.987533, 28.077744, b) lake trembling bogs, c) Mialik A. N., Dubar D. A., 23.08.2023, d) https://www.inaturalist. org/observations/179861877, MSKH Herbarium;

Verkudy (Ver): a) Vitebsk region, Ushachi district, Verkudy village, 3.1 km to the southwest, Lake Leshevo, WGS: 55.182747, 28.886998, b) lake trembling bogs, c) Mialik A. N., Dubar D. A., Kozlova O. N., 25.08.2023, d) https://www.inaturalist.org/observations/180224884, MSKH Herbarium.

When selecting markers for assessing the genetic structure of the studied *L. loeselii* populations, DNA was isolated from silica gel-dried leaves collected from 6 studied populations (5 samples from each of the Ozery, Podsady, Chistets, Servech, Mezhuzhol populations and 4 samples from the Verkudy population). The isolation was performed using the DNA-Extran-3 reagent kit (Synthol, Russia). The quality and quantity of the isolated DNA were checked using a NanoPhotometer Pearl Implen GmbH (Munich, Germany). A total of 30 iPBS primers were used in the study [13]. PCR was performed in 25 μl of the reaction mixture containing 25–50 ng of DNA, 5 μl of the ready-made ScreenMix PCR mixture (Eurogen, Russia), 1 mM primer for 12–13 bp primers or 0.6 mM for 18 bp primers, and water.

The PCR program consisted of: 1 cycle at 95 °C for 5 min; 38 cycles at 95 °C for 15 s, annealing was carried out at a temperature of 50.0–65.2 °C (depending on the primer) for 60 s, elongation at 68 °C for 90 s. The final elongation was carried out at 72 °C for 8 min. Amplification was carried out in a programmable thermostat C1000 Touch Thermal Cycler (MJ Research Inc., Bio-Rad Laboratories, USA). Electrophoresis was carried out at a voltage of 65 V for 5 hours in 2 % agarose gel. Ethidium bromide was used to stain the gel for 30 minutes and visualized using the UV Imager Gel Doc XR+ system (Bio-Rad, USA).

As a result, it was found that 7 of the 30 markers used were suitable (2271, 2081, 2242, 2076, 2079, 2080, 2270). Similar studies by Latvian colleagues allowed us to select only 3 markers, of which 2079 and 2270 are common [14]. Gradient PCR was performed with 7 selected markers to establish optimal annealing temperatures. The obtained values of optimal annealing temperatures are shown in Table 1. PyElph 1.4 was used to construct a binary matrix based on electrophoresis data. All electrophoresis bands that could be accurately recognized were assessed as present (1) or absent (0) and were considered as single dominant loci. The obtained data were recorded as a binary matrix, which was then processed using the PopGene 1.31 program to calculate the following parameters: the proportion of polymorphic loci (P), the effective (Ne) and observed numbers of alleles (Na), the Shannon information index (I), Nei's gene diversity (He), total gene diversity (Ht), gene diversity in populations (Hs), the genetic differentiation coefficient Gst = (Ht – Hs) / Ht and gene flow among populations (Nm). These parameters were chosen as the most suitable for analyzing the results obtained using dominant molecular markers. Calculation of the polymorphism information coefficient (PIC) and average genetic distance, analysis

of molecular variance (AMOVA) and principal coordinate analysis (PCoA) were performed using the GenALEx 6.5 software package. For the iPBS markers used, such indicators as the number of polymorphic loci, their proportion and the measure of polymorphism information coefficient (PIC) were established (Table 1).

T a b l e 1. **Characteristics of selected iPBS markers**

iPBS marker	Recommended annealing temperature Ta (°C)	Sequence (5′–3′)	Number of loci	Number of polymorphic loci	Proportion of polymorphic loci (P), %	Polymorphism Information Complexity (PIC)
2242	58.1	GCCCCATGGTGGGCGCCA	26	24	92.31	0.26
2076	58.4	GCTCCGATGCCA	31	29	93.55	0.25
2271	59.4	GGCTCGGATGCCA	26	21	80.77	0.21
2080	58.4	CAGACGGCGCCA	29	23	79.31	0.24
2081	57.6	GCAACGGCGCCA	27	22	81.48	0.23
2270	61.6	ACCTGGCGTGCCA	26	21	84.62	0.24
2079	61.4	AGGTGGGCGCCA	23	22	95.7	0.36
Average			26.86	23.14	86.82	0.26

For each primer, 23 to 31 loci and 21 to 29 polymorphic loci were obtained. The average number of loci and polymorphic loci for all primers was 26.86 and 23.14, respectively. The proportion of polymorphic loci averaged 86.82 %. The minimum polymorphism information coefficient (PIC) was obtained for marker 2271 (0.21), and the maximum for marker 2079 (0.36). All selected markers have a sufficiently high PIC value for their further use in studying the genetic diversity of *L. loeselii* populations.

Results and discussion. Based on the data obtained using molecular iPBS markers 2271, 2081, 2242, 2076, 2079, 2080, 2270, the following were calculated: the proportion of polymorphic amplicons, the effective and observed number of alleles, the Shannon information index, and Nei's gene diversity for each population of *L. loeselii*. The above parameters, as well as the total genetic diversity, genetic diversity in populations, the level of subdivision of populations, and gene flow between populations were calculated for all loci of the six studied populations of *L. loselii* (Table 2).

T a b l e 2. **Parameters of genetic diversity of the studied populations of *Liparis loeselii* (L.) Rich.**

Parameter	Model population						Common to populations
	Oz	Pod	Ch	Ser	Me	Ver	
Proportion of polymorphic loci (%)	45.21	31.91	46.28	42.02	34.04	45.74	87.23
Na	1.45 ± 0.04	1.32 ± 0.03	1.46 ± 0.04	1.42 ± 0.04	1.34 ± 0.03	1.46 ± 0.04	1.87 ± 0.02
Ne	1.30 ± 0.03	1.19 ± 0.02	1.27 ± 0.03	1.26 ± 0.03	1.21 ± 0.02	1.32 ± 0.03	1.37 ± 0.02
He	0.17 ± 0.01	0.11 ± 0.01	0.16 ± 0.01	0.15 ± 0.01	0.12 ± 0.01	0.18 ± 0.02	0.23 ± 0.01
I	0.25 ± 0.02	0.17 ± 0.02	0.24 ± 0.02	0.22 ± 0.02	0.18± 0.02	0.26 ± 0.02	0.37 ± 0.02
Ht							0.24 ± 0.002
Hs							0.15 ± 0.001
Gst							0.37
Nm							0.85

The analysis of the obtained data shows that the lowest proportion of polymorphic loci was found in the Podsady population – 31.91 %. Also for this population the lowest values of the effective and observed number of alleles, Shannon information index and Nei's gene diversity were found, which were 1.19, 1.32, 0.17 and 0.11 respectively. Thus, it can be concluded that the population under consideration is characterized by the lowest genetic diversity among all those studied, which is consistent with its small area and the low number of plants identified here during the survey. The Mezhuzhol population, which is characterized by a small area and a low number of plants, also stands out for its relatively low genetic diversity (for example, the Nei's gene diversity index is only 0.12), which is characterized by a small area and a low number of plants.

The highest proportion of polymorphic loci was found in the Chistets population – 46.28 %, for the Ozery and Verkudy populations the similar indicator was 45.21 and 45.74 %, respectively. It is important to note that the similar indicator for the Latvian populations of *L. loselii* is significantly higher and varies from 48 to 84 % [15], which indicates their higher heterogeneity. The indicator of the observed number of alleles is the highest in two populations – Chistets and Verkudy – 1.46. Accordingly, the Verkudy population has the highest level of genetic diversity, since it has the highest values of the Shannon information index and Nei's gene diversity, which amounted to 0.26 and 0.18, respectively. According to the considered indicators, it is slightly inferior to the model populations Chistets and Servech, which are also characterized by a fairly high area and number of plants. Thus, it can be concluded that the Belarusian populations of *L. loselii* are characterized by a direct relationship between the level of genetic diversity, the area of the population and the number of plants in it, which must be taken into account when planning nature conservation measures. Perhaps this feature is also due to the greater age of the noted populations, which are located in large bog areas. Populations with the lowest genetic diversity (Podsady and Mezhuzhol), on the contrary, are noted in small swamps and lake trembling bogs, and therefore are younger. A similar pattern is noted for Latvian populations of *L. loselii* [15].

To analyze the genetic variability of all six studied populations, the effective and observed number of alleles, Nei's gene diversity, Shannon information index and the proportion of polymorphic loci were calculated for all loci, which amounted to 1.87, 1.37, 0.23, 0.37 and 87.23 %, respectively. It is noteworthy that the same indicator for a similar boreal species *Goodyera repens* (L.) R. Br. is significantly lower (67.09 %), although it is characterized by a wider distribution and frequency of occurrence within Belarus [16]. The obtained data indicate a relatively high polymorphism of the Belarusian populations of *L. loselii*, their higher variability and adaptive potential.

The gene flow value was 0.85, indicating a significant influence of genetic drift in the formation of the genetic structure of *L. loeselii* populations. Since this indicator is less than one, this indicates a disruption in gene exchange between populations of the species under study. Among other representatives of the *Orchidaceae* family, similar values were noted for *Goodyera repens* (L.) R. Br. (0.86) [16], while for the rarer species *Cephalanthera longifolia* (L.) Fritsch it is significantly lower and equals only 0.52 [17].

Analysis of the interpopulation and intrapopulation genetic structure showed that a smaller part, namely 37 % of the total genetic variability, is interpopulation (the genetic differentiation coefficient is 0.37), which indicates the occurrence of most genetic variations within populations.

Similar results are demonstrated by the AMOVA analysis (Table 3), which shows that most of the genetic diversity (74 %) is intrapopulation. Only 26 % of the genetic variation is observed between *L. loselii* populations. The estimates of variance were based on 999 permutations. The difference between individuals in the populations was statistically significant with a p value of <0.001. The obtained value of pairwise population differentiation (PhiPT) is 0.212, which indicates a high level of genetic differentiation among populations, which is associated with the need to protect as many habitats as possible.

T a b l e 3. **AMOVA results for *Liparis loeselii* (L.) Rich. populations**

Source of variability	Number of degrees of freedom (df)	Sum of squares (SS)	Mean square (MS)	Dispersion	PhiPT	Share in variation
General	28	692.2	–	25.65	0.212*	–
Between populations	5	256.71	51.341	6.71		0.26
Within populations	23	435.5	18.94	18.94		0.74

N o t e. * – Differences are significant at a significance level of $p < 0.01$.

In general, the obtained data, demonstrating low genetic diversity of the studied populations of *L. loselii*, are consistent with the trends of decreasing numbers of this species in Belarus. The genetic isolation of populations and their low adaptive potential are one of the factors of high vulnerability of this species, which is confirmed by the disappearance of a number of habitats at the southern border of the range (Figure).

Conclusion. As a result of the conducted studies using molecular iPBS markers, the genetic diversity of *L. loeselii* populations located in Belarus near the southern border of the range was assessed. A low level of overall genetic diversity similar to other representatives of the Orchidaceae family was revealed for this species. Analysis of the population genetic structure demonstrates that *L. loeselii* is dominated by intrapopulation genetic variability (74 %), which, along with a low level of gene flow between populations, indicates their isolation.

In general, the analysis of the obtained data shows that *L. loeselii* is characterized by low genetic diversity of individual populations, which is associated with the low adaptive potential of this species, as well as an unfavorable prognosis for conservation in the wild, taking into account modern climate change, as well as successional changes occurring within marsh biotopes.

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