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Genetic diversity of populations of the rare species *Cypripedium calceolus* L. in Belarus

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Abstract: Studying the level and structure of the genetic diversity of rare species is important for creating an effective strategy for their conservation. Using iPBS markers in our study, it was found that *C. calceolus* genotypes have a rather low level of genetic diversity. Genetic differentiation was found mainly within coenopopulations, which may be caused by the phenomenon of cross-pollination. The genetic distance between coenopopulations corresponded to their geographic location.

Keywords: iPBS, genetic diversity

Introduction

Loss of habitat that can be caused by human activities is a serious threat to the viability of plant populations, which can lead to their decline and extinction (Minasiewicz *et al*., 2018).

Studying the level and structure of the genetic diversity of rare species is important for creating an effective strategy for their conservation. Genetic diversity is essential for the development of populations, and its loss is often associated with inbred depression, which increases the risk of population extinction (Frankham, 2012). In addition to inbreeding, factors other than inbreeding can affect genetic diversity and population structure. These include the mating system, methods of pollination and seed distribution, life cycle, habitat specifics, landscape, and/or climate change (John *et al*., 2019).

The selection of specific populations for conservation is usually based on their contribution to the overall genetic diversity of the species. For this reason, it is important to know the degree to which populations differ from each other and the level of genetic diversity within populations. High intrapopulation genetic diversity is an important criterion when choosing priority populations for species

conservation. In addition to contributing to overall genetic diversity, sufficiently high intrapopulation genetic variability is critical for the long-term survival of a population and the ability to adapt to environmental changes (Guo *et al.*, 2019). The investigation aims to study the level and structure of the genetic diversity of the coenopopulations of *Cypripedium calceolus* L. growing in Belarus using molecular iPBS markers.

Materials and methods

Ladies' slipper (*Cypripedium calceolus* L.) is a perennial short-rhizome plant with a straight leafy stem 15–50 cm high. The flowers are large, 6–8 cm in diameter, with a plump lip, similar to a shoe. *C. calceolus* reproduces by vegetative and seed methods, like most members of the orchid family, the mycosymbiont. The area covers most of Europe (except for the south). A relict in origin species of the Euro-Siberian subtaiga, located on the territory of Belarus in isolated places of the European fragment of the range. It has III national protection category (according to the IUCN scale: VU – vulnerable species). Included in Appendix II to the CITES Convention, included in Appendix I to the Berne Convention, and Appendix II to the European Union Habitats Directive. Protected in Ukraine, the Russian Federation (including the Smolensk region), Poland, Lithuania, and Latvia (Kachanovsky, 2015).

In 2020, field studies were carried out in the southern part of the Republic of Belarus to determine the vitality status of *C. calceolus* coenopopulations. Three habitats were examined in the "Divin – Velikiy Les" reserve (DV1, DV2, DV3), located in the island calciphyte localities. These places of growth of the coenopopulation were described in detail in the monograph by N. V. Mikhalchuk (Mikhalchuk, 1997). The fourth habitat is also located in an island location at a sufficient distance from "Divin", on the territory of the "Vygonoshchanskoye" (VG) reserve (Figure 1). The islands of the "Divin" nature reserve are distinguished by great floristic diversity and greater anthropogenic disturbance. The coenopopulation of the "Vygonoshchanskoe" reserve is characterized by a close to the natural character of vegetation, the closeness of the tree layer, and high soil moisture. All four coenopopulations are located at the ecological optimum.

To determine the level of genetic diversity of four coenopopulations of *C. calceolus*, we used molecular markers iPBS (Kalendar *et al.*, 2010). The iPBS amplification method relies on the almost universal presence of a complementary tRNA sequence as a reverse transcriptase primer binding site (PBS) in LTR retrotransposons. This method is excellent for assessing population diversity due to its high reliability and reproducibility of results, the ability to detect a large number of polymorphic fragments, and relatively low cost (Kalendar *et al.*, 2010). In addition, since iPBS does

not require any prior knowledge of target sequences, this marker is especially suitable for rare orchids, for which molecular genetic studies have been scarce.

Figure 1. Distribution map of four studied coenopopulations of *C. calceolus* (DV1, DV2, DV3, VG) on the territory of the Republic of Belarus.

Total genomic DNA was isolated from dried leaf tissues by the hexadecyltrimethylammonium bromide (CTAB) method (Devi *et al.*, 2013). The concentration and quality of the isolated DNA were examined and controlled using a NanoPhotometer nucleic acid analyzer (Pearl Implen GmbH, Germany). DNA extracts were diluted to 50 ng/µl for amplification by polymerase chain reaction (PCR).

For the study, 30 markers were used which were proposed by R. Kalendar *et al.* PCR was carried out in 25 µl of a reaction mixture containing 40–50 ng of DNA, 5 µl of a ScreenMix PCR ready mixture (Evrogen), 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; 34 cycles at 95 °C for 15 s, 50–60 °C (depending on

primer) for 60 s and 68 °C for 60 s; final elongation 72 °C for 5 min. Amplification was performed in a C1000 Touch Thermal Cycler programmable thermostat (MJ Research Inc., Bio-Rad Laboratories, USA). Electrophoresis was carried out at a voltage of 65 V for 4.5 hours in a 2% agarose gel. The gel was stained with ethidium bromide for 30 minutes and visualized using the UV Imager Gel Doc XR + system (Bio-Rad, USA).

To create a binary data matrix using the PyElph 1.4 program, the resulting DNA amplicons were recorded as present (1) or absent (0). The binary data matrix was analyzed using POPGEN v. 1.31 to estimate the parameters of genetic diversity, including the percentage of polymorphic bands (P), effective (Ne) and observed number of alleles (Na), Nei genetic diversity (He), Shannon Information Index (I), total gene diversity (Ht), gene flow (Nm), gene diversity in populations (Hs), coefficient of genetic differentiation (Gst = [Ht-Hs] / Ht) and gene flow between populations (Nm). The calculation of the polymorphism information content (PIC), the average genetic distance, and analysis of molecular variance (AMOVA) were performed using the GenALEx 6.5 package. For cluster analysis of UPGMA, the SplitsTree 5 software was used.

Results and discussion

Markers 2375, 2239, 2374, 2076, 2390, and 2270 were selected to study the genetic diversity and genetic differentiation of *C. calceolus*, as they allowed to obtain clear DNA fragments with polymorphic loci.

For the iPBS markers used, such indicators as the number of polymorphic loci, their proportion, and the polymorphism information content (PIC) were established (Table 1).

Table 1. Characteristics of selected iPBS markers

IPBS marker	Number of loci	Number of polymorphic loci	Proportion of polymorphic loci (%)	PIC
2375	8	5	62.5	0.15
2239	18	16	88.9	0.2
2374	12	11	91.7	0.16
2076	11	9	81.8	0.16
2390	14	9	64.3	0.12
2270	25	23	92.0	0.28
Total	14.7	12.2	83.0	0.17

Per primer, from 8 to 25 loci and from 5 to 23 polymorphic loci were obtained. The average number of loci and polymorphic loci for all primers was 14.7 and 12.2, respectively. On average, 83% of all detected loci were polymorphic. The maximum value of the measure of information polymorphism (PIC) was obtained for the marker 2270 (0.28), and the minimum for the marker 2390 (0.12). The PIC value for dominant markers ranges from 0 to 0.5 (Roldán-Ruiz *et al.*, 2000), therefore all selected markers are informative and can be used to assess the genetic diversity of *C. calceolus.*

The obtained results of the analysis of genetic polymorphism in various habitats of *C. calceolus*, such as effective (Ne) and observed number of alleles (Na), Shannon information index (I), and Nei genetic diversity (He) are presented in Figure 2.

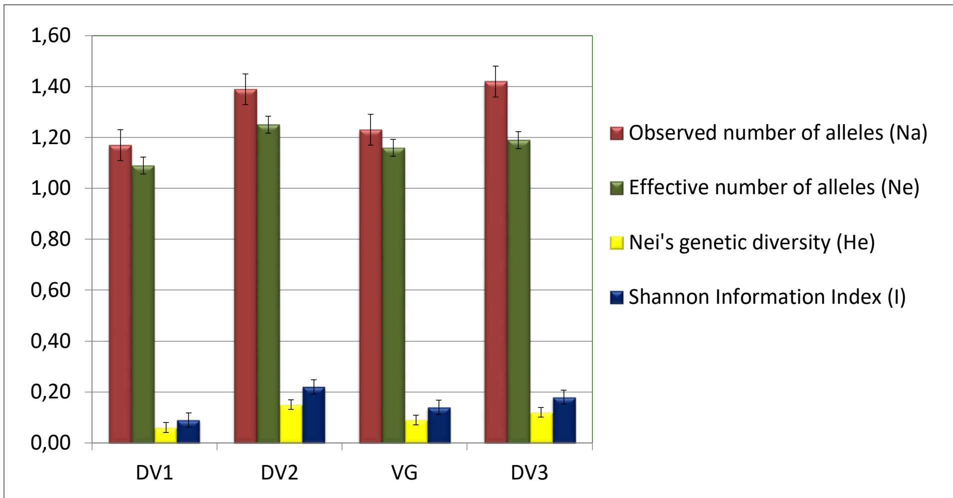

Figure 2. Parameters of genetic polymorphism of the studied coenopopulations (DV1, DV2, DV3, VG) of *C. calceolus*.

The results showed that in the four coenopopulation studied the observed number of alleles (Na) ranged from 1.17 in the DV1 coenopopulation to 1.42 in the DV3 coenopopulation. The maximum effective number of alleles (1.25) was found in the DV2 coenopopulation, and the smallest number (1.09) was found in the DV1 coenopopulation. Based on the (He) and (I) values the DV1 coenopopulation showed the lowest genetic diversity while the DV2 coenopopulation showed the highest genetic diversity.

Analysis of the interpopulation and intrapopulation genetic structure showed that the total genetic diversity (Ht = 0.19 ± 0.04) differs from the intrapopulation genetic diversity (Hs = 0.1 ± 0.02). The level of genetic differentiation among

the studied coenopopulations of *C. calceolus* (Gst) was 0.45 with effective gene flow observed between coenopopulations (Nm = 0.61). According to Nei (Nei, 1978), Gst is classified as low if Gst < 0.05, medium when 0.05 < Gst < 0.15, and high when Gst > 0.15. Thus, the Gst coefficient for *C. calceolus* (Gst = 0.45) would be considered high. The Nm value of gene flow is less than 1 (Nm = 0.61), which indicates that our selected coenopopulations are subject to genetic drift since it is believed that when Nm > 1, gene flow can prevent genetic differentiation between coenopopulations caused by genetic drift (Yan *et al*., 2019).

Pairwise matrix and Nei genetic distance (Table 2) also show genetic differences between four coenopopulations from different regions analyzed in our study.

Table 2. Pairwise matrix of genetic similarities PhiPT (above the diagonal) and Nei genetic distance (below the diagonal)

Coenopopulation	DV1	DV2	VG	DV3
DV1	–	0.89	0.85	0.95
DV2	0.14	–	0.9	0.91
VG	0.19	0.14	–	0.85
DV3	0.06	0.11	0.18	–

This matrix showed that the largest value of Nei's genetic distance (0.19) was observed among the DV1 and VG coenopopulations, and the smallest between the DV1 and DV3 coenopopulations. This was also confirmed by the UPGMA results (Figure 3), based on Ney's distance matrix data, which demonstrate the genetic remoteness of the VG coenopopulation from the rest, which corresponds to its geographic location. The DV1 and DV3 coenopopulations are closest to each other and Nei's genetic distance between them is the smallest.

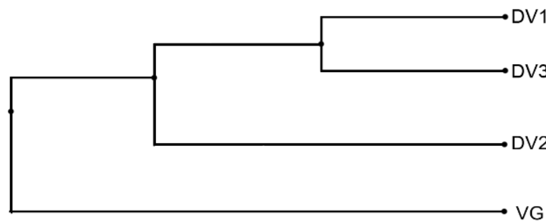

Figure 3. UPGMA dendogram (based on Ney's genetic distance) showing the relationship between the four coenopopulations studied (DV1, DV2, DV3, VG).

Genetic differentiation between coenopopulations in AMOVA is determined using the PhiPT value (an analogue of the F-test in ANOVA), which calculates coenopopulation differentiation based on the genotypic variance of binary data (Meirmans, 2012). Phi > 0.2 means that the coenopopulations differ significantly (Resmi *et al*., 2016).

AMOVA showed that most genetic diversity occurs within coenopopulations (71%), while genetic diversity between coenopopulations is 29%. The difference between individuals in the coenopopulations was statistically significant with a P-value < 0.001. The resulting PhiPT value = 0.29 indicates a high level of genetic differentiation among populations (Table 3). Intrapopulation genetic diversity was expected to be higher than interpopulation genetic diversity, as this distribution is a common feature of cross-pollinated species (Paschoa *et al*., 2018).

Table 3. AMOVA results for coenopopulations of *C. calceolus*

Source	df	SS	MS	Est. Var.	PhiPT	%
Total	18	164.53	–	5.26	0.29**	–
Between coenopopulations	3	55.31	18.44	2.92		0.29
Within coenopopulations	15	109.21	7.28	7.28		0.71

Note. ** – differences are significant at a significance level of $P < 0.01$.

In general, the results obtained demonstrate that IPBS markers are suitable for studying the genetic diversity of *C. calceolus*, since they efficiently detected polymorphism even in individuals belonging to the same coenopopulation. The results obtained showed that the genotypes of *C. calceolus* studied in this work have a rather low level of genetic diversity. Genetic differentiation was found mainly within coenopopulations, which may be caused by the phenomenon of cross-pollination. Taking into account the obtained data on the similarities and differences between coenopopulations, it can be concluded that the coenopopulations DV1, DV2, and DV3 are a single population. Future studies should focus on more coenopopulations and samples collected from more geographic regions, and use other types of molecular markers in addition to iPBS markers to have better understand the genetic diversity of *C. calceolus*.

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