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Genetic State of *Pulsatilla Patens* (L.) Mill. Coenopopulations Based on DNA Labeling

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Abstract: In the article, the results of studying the gene pools of the Kazakhstan coenopopulations of a rare plant species *Pulsatilla patens* (L.) Mill. are shown. The biomaterial taken from five coenopopulations located in Northern Kazakhstan has been used as samples. To identify the multilocus DNA polymorphism, the authors have used ISSR (Inter Simple Sequence Repeats, or intermicrosatellite analysis) labeling. The most effective primers have been selected, which provide reproducible results with a length of 17-19 nucleotides. As a result, multiple genomic labeling of five *Pulsatilla patens* coenopopulations has been performed. The main indicators of genetic diversity (P_{95} - 0.965, H_E - 0.162, I - 0.373 for the total sample), the structure and differentiation of the populations (H_T - 0.232, H_S - 0.162, G_{ST} - 0.3 for the total sample) and the type and state of the gene pools of the studied coenopopulations have been determined. The specificity of the gene pools has been characterized by the number of rare alleles and the Coefficient of Genetic Originality (CGO). The maximum value of CGO has been noted at Pp5 (2.964) and the minimum one - at Pp4 (2.666). The gene pools of the Pp1 and Pp4 coenopopulations have been characterized as typical and gene pools of the Pp2, Pp3 and Pp5 - as specific. Based on the obtained data, tree diagrams have been made that reflect the degree of similarity of the studied coenopopulations as to the ISSR spectra. Identification of the state of the gene pools of rare plant species will allow recommending scientifically based measures for preserving them.

Keywords: Gene Pool, Polylocus Marking, ISSR-PCR, Genetic Diversity, Coefficient of Genetic Originality

Introduction

By the middle of this century, an increase from 7 to 60 thousand in the number of endangered plant species is predicted (Pimm *et al*., 2014). The decline in the species and genetic diversity poses a real threat to the biosphere since the sustainability of natural ecosystems reproduction is directly related to their genetically determined potential for adaptation to changes in the environmental conditions (Joppa *et al*., 2011; Durán *et al*., 2020; Reisch *et al*., 2018). Genetic diversity plays an important role in the comprehensive assessment of biodiversity, i.e., keeping the genotypic heterozygosity, polymorphism and other genotypic variabilities, which is caused by the need for adaptation in natural populations (Joppa *et al*., 2011; Durán *et al*., 2020).

Due to the peculiarities of historical development, the flora and vegetation of Northern Kazakhstan are unique. Their gene pool has been formed in the process of the long evolution and now provides the possibility of species population's existence in the modern physical and geographical environment. Denvelopment of economy, modern infrastructure and tourism on the territory of Northern Kazakhstan inevitably entail increased use of natural resources and lead to a gradual degradation of typical coenoses.

One of the approaches to studying the plant genetic diversity is the use of molecular markers, which are polymorphic DNA sequences that can be detected using the methods based on the polymerase chain reaction. The polymorphism of nucleotide sequences between individual DNA samples is detected by the presence or absence of specific DNA fragments in the spectrum during electrophoresis. The absence of a DNA fragment can be the result of point mutations, insertions, deletions, or inversions in the DNA template sequence (Dorogina and Zhmud, 2020).

The number of microsatellite repetitions in plant genomes is very large, which makes this method convenient for genetic analysis. Microsatellite sequences surround many genes and can be used as anchor sequences to these genes. For primers selection, the ISSR method for determining the DNA polymorphism does not require prior cloning and sequencing. Primers for intermicrosatellite analysis, i.e., ISSR primers, contain a fragment of a microsatellite locus (most often di- or trinucleotide) and one or two "anchor" nucleotides on one of the repetition flanks. This method started to be developed in 1994 and has now become widely spread, especially in studying the gene pools of various plant species for mapping genomes and marking the agronomically important traits (Nasim *et al*., 2020; Ul Haq and Ijaz, 2019).

Along with the traditional methods, modern molecular genetic methods are used for preserving the gene pools of rare plant species. Currently, DNA banks of valuable and rare plant species have been created, the intraspecific variability of protected objects is studied, controversial issues of their taxonomy and classification are clarified and the genetic stability of the taxa stored in situ is studied (Jolivet and Degen, 2012; Boronnikova, 2013).

For the studies, the authors selected the rare plant species growing in the territory of Northern Kazakhstan and affiliated to the *Ranunculaceae* Juss. family, the *Pulsatilla* Mill. genus - *Pulsatilla patens* (L.) Mill.

The *Ranunculaceae* Juss. family includes over 2,000 species of plants with various properties-medicinal, poisonous, insecticidal and fungicidal. The family includes many rare plants that need protection (Kubentayev *et al*., 2018).

The *Pulsatilla* Mill. genus includes about 40 species. All species of the *Pulsatilla* Mill. genus are small perennial herbaceous plants with long vertical underground rhizomes.

Pulsatilla patens (L.) Mill. is the plant with a wide Eurasian distribution, however, the systematic position of the species is very uncertain, since modern researchers believe that this is a European species with small disjunctions in the Asian part. The plant is in the Kazakhstan Red Data Book (2014) (Baitulin, 2014). Over the past 50 years, the population of P. patens has decreased significantly. In this regard, measures for protecting this endangered species have been intensified. The development of molecular genetic methods is of particular importance for resolving the main problem in maintaining biodiversity-selecting the most typical representatives of the populations and creating genetically based programs for their preservation, as well as identification of the intraspecific genetic diversity, assessment of the heterozygosity, reconstruction of the phylogenetic relationships between the species and spatial relationships between the populations (Boronnikova, 2013).

The Intermicrosatellite method (ISSR) allows working with the genomes, whose sequences are not sequenced, using primers with the length of 19-24 nucleotides that consist of two, three and four nucleotide repetitions and the so-called "anchor" at the 3' end. Such sequences usually surround unique genes. On the electrophoretogram, one can see many discrete species-specific bands. The number of microsatellites in the genomes of plants and animals is large, therefore, using this method, one can study most of the species genome. ISSR-PCR markers are dominant; therefore, the presence or absence of this sequence in the genome will be determined by the presence or absence of a discrete band on the electropherogram of the studied sample. This method is widely used for identifying the intra- and interspecific differences, determining the genetic diversity indicators, identifying the varieties and lines and labeling the economically valuable traits (Boronnikova, 2013; Olatunji and Afolayan, 2019; Shahabzadeh *et al*., 2020).

Based on the information obtained from monitoring the genetic variability and assessing the genetic diversity, strategies for rare plant species conservation and rational use will be developed.

Materials and Methods

The studies were performed on the material collected in the territory of the Republic of Kazakhstan from April to June 2019. Five coenopopulations of rare plant species *Pulsatilla patens* (L.) Mill. located in the territory of the Pavlodar, the Kostanay and the Akmola regions, were selected as the objects of the study (Table 1).

For studying the structure of the populations, areas with a high density of flowering plants were selected. In each of the populations shown in Table 1, the biomaterial of 30 randomly selected plants was taken. The authors isolated the DNA from 150 samples of dried leaves of *P. patens* following a modified method (Beltyukova *et al*., 2018).

For the genetic analysis of the coenopopulations of *P. patens* using the intermicrosatellite method, ISSR primers were selected by the efficiency of DNA polymorphism detection (Table 2) (Shakirova, 2019b).

Table 1: Characterization of the *P. patens* Coenopopulations (CP)

CP number	CP location
Pp1	The Pavlodar region, outskirts of settl. Bayanaul, N = 50.82009°, E = 75.8000°, h = 407 m above the sea level, Bayanaul mountains, a meadow between granite rocks
Pp2	The Akmola region, outskirts of settl. Ereimentau, N = 51.65717°, E = 73.19056°, h = 320 m above the sea level, Ereimentau mountains, the base of the volcano hill
Pp3	The Akmola region, Burabay district, outskirts of settl. Akylbai, Burabay State National Natural Park, State Institution, Borovskoe forestry, sector 96, N = 53.12304°, E = 70.18010°, h = 343 m above the sea level, feather-grass-forb steppe on the eastern slope of the volcano hill
Pp4	The Kostanay region, Mendygarinsky district, outskirts of settl. Kamensk-Uralsk, N = 53.10970°, E = 70.12750°, h = 359 m above the sea level.
Pp5	The Kostanay region, Mendygarinsky district, outskirts of settl. Borovskoe, 80 km along the Kostanay-Borovskoe highway

Table 2: The efficiency of ISSR primers for PCR with total DNA of *P. patens*

Primers	The nucleotide sequence (5' → 3)	Annealing temperature, °C	Amplified fragments size, bps	Number of amplified fragments
M1	$(AC)_8CG$	56	210-1,115	20
M27	$(GA)_8C$	52	230-1,000	20
X11	$(AGC)_6G$	64	240-1,050	17
ISSR-1	$(AC)_8T$	56	200-1,200	16
ISSR-3	$(TG)_8AA$	56	300-1,200	14

Amplification was performed according to the following program: 94°C - 2 min; (94°C - 20 sec.; annealing t° - 10 sec.; 72°C - 10 sec.) × 5; (94°C - 5 sec.; annealing t - 5 sec.; 72°C - 5 sec.) × 35; 72°C - 2 min. The annealing temperature was chosen depending on the primer used (Table 2). The amplification products were separated by electrophoresis in 1.7% agarose gel. To determine the length of the DNA fragments, a molecular weight marker (100 bp + 1.2+1.5+2+3 Kb DNA Ladder, LLC SibEnzim-M, Moscow) was used; the lengths of fragments were determined using the Quantity One application (Bio-Rad, USA). To check the reliability of the obtained DNA spectra, the experiment was repeated at least three times.

Computer analysis of the obtained data was made using the POPGENE 1.31 application and the specialized GenAlEx6 MS Excel macro with the definition of the share of the polymorphic loci (P_{95}), the absolute number of alleles (n_a), the effective number of alleles (n_e), the expected heterozygosity (H_E) and the Shannon diversity index (I).

Matrices of genetic differences were calculated based on binary data matrices using the TREECON 1.3b and POPGENE 1.31 computer applications (Peakall and Smouse, 2012). Based on the obtained matrix, tree diagrams showing the degree of similarity of the studied coenopopulations by the ISSR spectra were built using the Unweighted Pair Group Method with Arithmetic mean (UPGMA).

The results of the PCR analysis were recorded in the form of a matrix of binary features, in which the presence or absence of fragments of the same size in the ISSR spectra was considered, respectively, as a state of 1 or 0.

To assess the state of the gene pools of the studied rare plant species, *P. patens*, the scale proposed by S. V. Boronnikova (2013) was used.

Results and Discussion

In their work, the authors used the method of identification and certification of rare plant species populations developed by (Boronnikova, 2008) on the example of some species of the buttercup family (spring adonis, Siberian adonis). The method included seven main stages, including the choice of effective methods for determining the level of genetic diversity, material sampling, searching for the most effective molecular markers, analyzing the genetic diversity, identifying the identification DNA markers with further compilation of the molecular genetic formulas, barcodes and genetic profiles.

For the molecular genetic identification, five most effective ISSR markers giving clear reproducible results for the studied rare plant species *P. patens* were selected.

The PCR analysis was made many times for each of the 150 studied plant samples and the five studied coenopopulations of *P. patens*.

For the molecular genetic identification and certification of the five coenopopulations, clear and reproducible in repeated experiments monomorphic (present in all representatives of the population, species and genus) and polymorphic (frequency of occurrence 5 to 95%) ISSR PCR markers were identified.

Following the method, the DNA fragments that are characteristic of a certain genus are called "generic" and those that are characteristic of a certain species are called "specific" (Olatunji and Afolayan, 2019). The generic and

specific DNA fragments are monomorphic and allow identifying the samples at the specific and generic levels. Population fragments can also be monomorphic and be characteristic only for a given population; in this case, they are unique monomorphic and allow identifying the plant affiliation with a certain habitat. A combination of polymorphic fragments in a population can also be unique, which can also be used in compiling molecular genetic formulas and barcodes for each population (Fig. 1).

The molecular genetic formula combines DNA markers of all three types (population, specific and generic) for each population. For each identification DNA fragment, a record is made that contains its type (population, specific, or generic), the molecular length of the fragment on the electrophoretogram and the name of the primer that has initiated its synthesis. An example of such a record is PU_r410_{ISSR1}; in this case, the identification fragment is the generic DNA fragment for genus *Pulsatilla* Mill with the molecular weight of 410 bps obtained as a result of the polymerase chain reaction of the studied plant sample with ISSR1 primers. The generic fragments are shown in the entry by lower suffix "r", the specific fragments - by "v" and the polymorphic fragments - by "p". The name of the primer is also shown in subscript after indicating the molecular length of the fragment (Table 3).

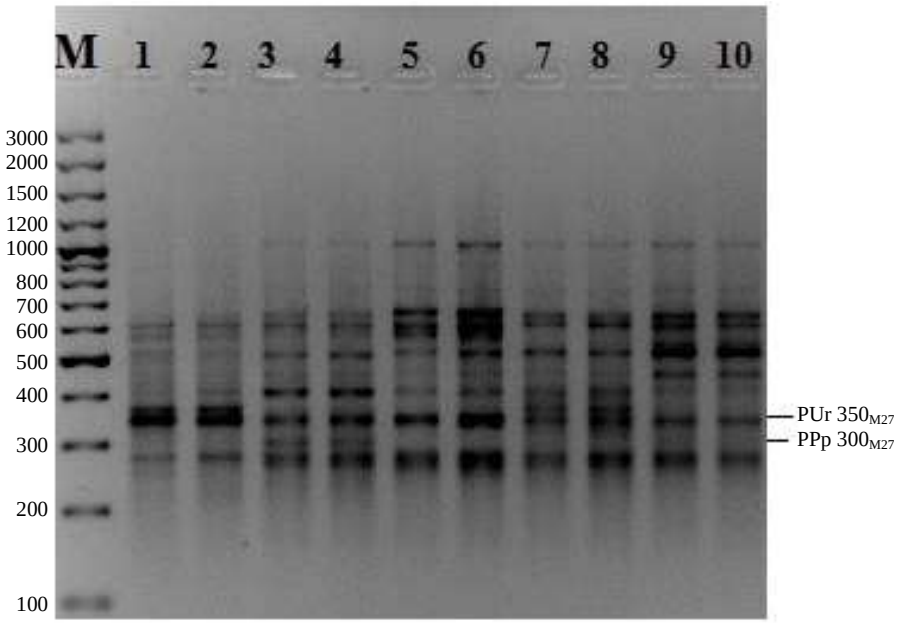


Fig. 1: The ISSR spectrum of the five coenopopulations of *P. patens* with primer M27, where M is a molecular weight marker, the digits denote the sample numbers (1-2 - *Pp1*, 3-4 - *Pp2*, 5-6 - *Pp3*, 7-8 - *Pp4* and 9-10 - *Pp5*), PUr is the generic fragment and PPp is the polymorphic fragment

Table 3: Molecular genetic formulas of the coenopopulations of *P. patens*

CP	Type of DNA fragments	Molecular genetic formula
Pp1	Rod	PU_r510_{ISSR1}, PU_r410_{ISSR1}, PU_r350_{M27}, PU_r230_{X11}, PU_r210_{M1}
	Vid	Pp_v615_{ISSR1}, Pp_v450_{X11}
	Polymorph	Pp_p 545_{M1}, Pp_p 420_{M27}, Pp_p 250_{ISSR1}
Pp2	Rod	PU_r510_{ISSR1}, PU_r410_{ISSR1}, PU_r350_{M27}, PU_r230_{X11}, PU_r210_{M1}
	Vid	Pp_v615_{ISSR1}, Pp_v450_{X11}
	Polymorph	Pp_p 470_{ISSR3}, Pp_p780_{M1}, Pp_p300_{M27}
Pp3	Rod	PU_r510_{ISSR1}, PU_r410_{ISSR1}, PU_r350_{M27}, PU_r230_{X11}, PU_r210_{M1}
	Vid	Pp_v615_{ISSR1}, Pp_v450_{X11}
	Polymorph	Pp_p 960 $_{ISSR3}$, Pp_p 660 $_{ISSR3}$, Pp_p 280_{M1}
Pp4	Rod	PU_r510_{ISSR1}, PU_r410_{ISSR1}, PU_r350_{M27}, PU_r230_{X11}, PU_r210_{M1}
	Vid	Pp_v615_{ISSR1}, Pp_v450_{X11}
	Polymorph	Pp_p1140_{ISSR3}, Pp_p750 $_{ISSR1}$, Pp_p530_{ISSR3}
Pp5	Rod	PU_r510_{ISSR1}, PU_r410_{ISSR1}, PU_r350_{M27}, PU_r230_{X11}, PU_r210_{M1}
	Vid	Pp_v615_{ISSR1}, Pp_v450_{X11}
	Polymorph	Pp_p 1350_{X11}, Pp_p 750 $_{ISSR1}$, Pp_p 590_{M27}

Note: PU_r are the generic fragments, Pp_v are the specific fragments and Pp_p are the polymorphic DNA fragments

Five clear monomorphic DNA fragments were selected for genus *Pulsatilla* Mill., which allowed assigning the analyzed sample to this genus – PU_r510_{ISSR1}, PU_r410_{ISSR1}, PU_r350_{M27}, PU_r230_{X11} and $PU_r210_{M1.}$

For the studied species, the following unique specific DNA fragments were selected in comparison with *P. flavescens*: Two specific fragments (Pp_v615_{ISSR1}, Pp_v450_{X11}) for pasque flower and two specific fragments (Pf_v 915_{ISSR3}, Pf_v 680_{M1}) for yellow pasque flower. To assign DNA samples to a specific population, unique monomorphic and polymorphic DNA fragments were selected and unique combinations of polymorphic fragments were developed. The specific and generic DNA fragments were compared with the data available in the literature about molecular genetic identification of *P. patens* and *P. flavescens* (Szczecińska *et al*., 2013; 2016).

Upon combining, the authors obtained unique molecular genetic formulas for the studied rare plant species for each population of generic, specific and polymorphic identification DNA fragments (Table 3).

The next stage of molecular genetic identification and profiling of the five coenopopulations of *P. patens* was the compilation of barcodes. A barcode is an image of the molecular genetic formula in the form of a vertical set of lines of various thicknesses, each representing a separate identification DNA fragment (Fig. 2). Generic fragments on the barcode are shown in thick lines, specific fragments - in lines of medium thickness and polymorphic fragments - in thin lines. A total of 10-12 lines are supposed to be placed in the barcode; the fragments are proposed to be placed from bottom to top in the molecular weight ascending order.

The final stage of molecular genetic identification and profiling is compiling a genetic profile for each of the five studied coenopopulations of *P. patens*.

The genetic profile contains basic information about the spatial distribution of the population, a unique barcode of its identification DNA fragments, as well as main indicators of the genetic diversity and the population parameters (Fig. 3).

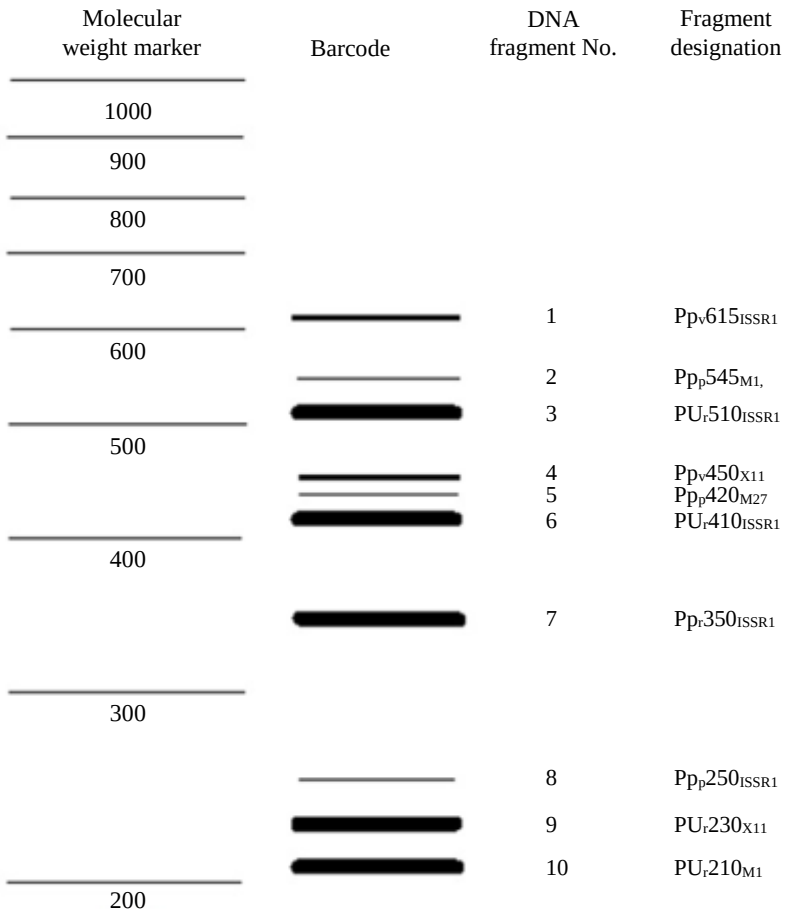
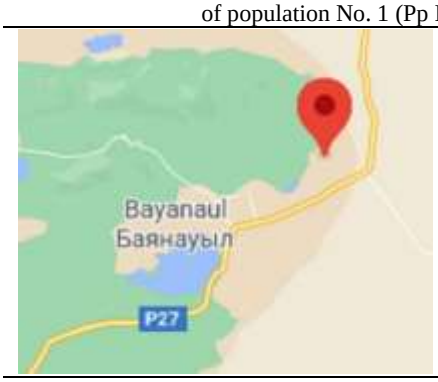
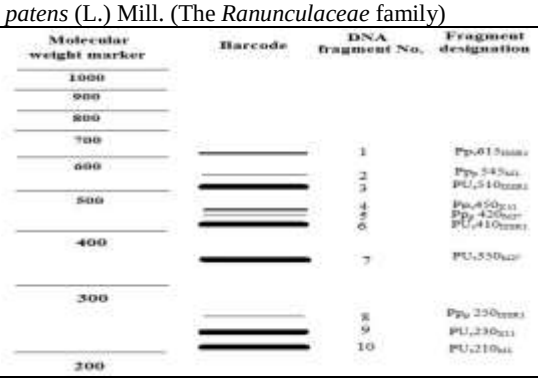


Fig. 2: A genetic barcode of the first coenopopulation of *P. patens* (*Pp1*), PU_r are the generic fragments, Pp_v are the specific fragments and Pp_p are the polymorphic DNA fragments

GENETIC PROFILE
of population No. 1 (Pp No. 1) of *Pulsatilla patens* (L.) Mill. (The *Ranunculaceae* family)





General information	Genetic diversity
Species rarity category: II (rare)	DNA polymorphism percentage (P%): ISSR: 77.2
Source: Kazakhstan Red Data Book, 2014	The number of identified DNA markers: ISSR: 101
Country: Republic of Kazakhstan	Expected Heterozygosity (He): ISSR: 0.131±0.013
Region: Pavlodar region	The absolute number of alleles per locus (n_a): ISSR: 1.597±0.492
Positioning: outskirts of settl. Bayanaul	An effective number of alleles (n_e): ISSR: 1.197±0.266
Coordinates: N 50.82009°, E 75.8000°, h = 407 m above the sea level.	Intrapopulational diversity indicator (μ): ISSR: 1.634±0.006
Habitat: Bayanaul mountains, a meadow between granite rocks	The number of rare and unique alleles (R): ISSR: 1
Total population: $174*10^3$	Molecular genetic formula: Pp_v615_{ISSR1}, PU_r510_{ISSR1}, Pp_p 545_{M1}, Pp_v450_{X11}, Pp_p 420_{M27}, PU_r410_{ISSR1}, PU_r350_{M27}, Pp_p 250_{ISSR1}, PU_r230_{X11}, PU_r210_{M1}
Area: 2,000 m^2	Rare and unique fragments: Pp_u 250_{ISSR1}
Age spectrum: prevalence of mature reproductive plants (41.4%)	Sequenced DNA sequences: –
Population type: normal	Population state: Depauperization
Anthropogenic impact level: -	Population self-renewal: natural
The average life cycle of the species: 20 years	Conclusion: High level of genetic diversity, presence of a rare, unique allele

Fig. 3: Genetic profile of population No. 1 (Pp No. 1) of *Pulsatilla patens* (L.) Mill. (The Ranunculaceae family)

Thus, molecular genetic identification and certification were performed for the five coenopopulations of *P. patens*. For each studied coenopopulation, unique molecular genetic formulas, barcodes and genetic passports were made, based on the data obtained through the ISSR-PCR analysis with five primers. The presented material makes it possible to relatively quickly and cheaply identify the plant samples' affiliation with certain genus, species and population. Genetic profiles make it possible to quickly and clearly describe each population, given the characteristics of the genetic structure, the preservation of which is of primary importance in the development of measures for protecting and restoring rare and endangered plant species.

To describe the genetic structure of the population, the following parameters were used: The expected share of Heterozygous genotypes (H_T) in the entire population, the expected share of Heterozygous genotypes (H_S) in a subpopulation and the population subdivision index (G_{ST}). The level of intrapopulation diversity was assessed through the following indicators: The average number of morphs ((μ) and the shares of rare morphs (h).

To preserve the gene pools of rare plant species, (Boronnikova, 2008) proposed a technology for assessing the state of gene pools. In developing this technology at the population level, the parameters of abundance and genetic diversity established during molecular genetic analysis were divided into three groups by the author of the method. It was shown that the population size had an

effect on the parameters of genetic diversity. In this regard, the first group of the parameters recommended for assessing the state of gene pools included assessing the share of Polymorphic loci (P_{95}) and the expected Heterozygosity (H_E). The second group, "Genetic structure and differentiation of populations", included the intrapopulation diversity (μ), the share of rare DNA fragments (h) and the Shannon diversity index (I). The specificity of gene pools (group III) in the proposed technology was characterized by the number of rare alleles (R) and the Coefficient of Genetic Originality (CGO).

High CGO values indicated an increased presence of rare alleles, while populations with minimal CGO values had minimal frequencies of occurrence and number of rare alleles. Such populations were characterized by the most typical or basic gene pool. Populations with the maximum CGO values containing the largest number of rare alleles that were atypical for the region of studies had specific gene pools (Boronnikova, 2013).

In analyzing the DNA fragments amplified by PCR in the five studied coenopopulations of *P. patens*, 144 amplified DNA fragments were identified, of which 139 (P_{95} = 0.965) were polymorphic ones. The number of amplified DNA fragments in the total sample of plants varied, depending on the primer, from 23 (M1) to 28 (M27). On average, in the ISSR analysis, a single primer initiated the synthesis of 28.8 DNA fragments. The number of polymorphic DNA fragments in the total sample of plants varied from 9 to 28 and their sizes - from 200 to 1,420 bps (Table 4).

The share of polymorphic loci in the total sample of *P. patens* was high and, depending on the ISSR primer, ranged from 0.920 (M1) to 1.000 (M27, ISSR3); on average, it was 0.965. The number of polymorphic DNA fragments varied from 78 in Pp1 to 103 in Pp5.

The proportion of polymorphic loci (P_{95}) in the coenopopulations varied from 0.728 (Pp3) to 0.851 (Pp5). The expected heterozygosity (H_E) by loci in the total sample of *P. patens* was 0.162. In the coenopopulations, this value varied from 0.131 in Pp1 to 0.209 in Pp5. The absolute number of alleles per locus (n_a) for the entire population was 1.965. This parameter was the highest in coenopopulation Pp5 (n_a = 1.743), while it was the smallest in coenopopulation Pp1 (n_a = 1.597). The effective number of alleles per locus (ne) for the total sample was 1.361. The greatest value of *ne* was in coenopopulation Pp5 (ne = 1.330) and the smallest value - in coenopopulation Pp1 (ne = 1.197). In the studied coenopopulations of *P. patens*, only two rare DNA fragments were found: one for each in Pp1 and Pp2 (Table 5).

In all studied coenopopulations of *P. patens*, *h* had values less than 0.3. The most balanced structure of diversity was characteristic of coenopopulation Pp5 (h = 0.139) and the least balanced structure of diversity was characteristic of coenopopulation Pp4 (h = 0.188). The Shannon diversity index revealed the greatest diversity in coenopopulation Pp5 (I = 0.327) and the least diversity - in coenopopulation Pp1 (I = 0.215) (Table 5).

In analyzing the intrapopulation diversity of *P. patens* using the μ index, it was found that of the five studied coenopopulations, coenopopulation Pp5 (μ = 1.723) was characterized by a more uniform distribution of allele frequencies and coenopopulation Pp4 - by the least uniform one (μ = 1.625).

Table 4: Characteristics of ISSR-PCR markers of *P. patens*

	Number of polymorphic ISSR-PCR markers in the coenopopulations (their occurrence frequency)											
	Pp1		Pp2		Pp3		Pp4		Pp5		For the total sample	
ISSR primers	N	P	N	P	N	P	N	P	N	P	N	P
M1	19	13 (0.684)	22	18 (0.818)	22	18 (0.818)	14	9 (0.643)	18	15 (0.833)	25	23 (0.920)
ISSR3	19	13 (0.684)	33	28 (0.848)	25	19 (0.760)	25	16 (0.640)	29	25 (0.862)	36	36 (1.000)
X11	22	19 (0.864)	20	17 (0.850)	22	14 (0.636)	20	17 (0.850)	21	20 (0.952)	28	26 (0.928)
ISSR1	18	15 (0.833)	17	15 (0.882)	19	13 (0.684)	26	19(0.731)	27	20 (0.741)	27	26 (0.963)
M27	23	18 (0.783)	25	20 (0.882)	26	19 (0.731)	23	19 (0.826)	26	23 (0.885)	28	28 (1.000)
Total	101	78 (0.772)	117	98 (0.838)	114	83 (0.728)	108	80 (0.741)	121	103 (0.851)	144	139 (0.965)

Note: N - total markers, P - number of polymorphic markers, the share of polymorphic loci is shown in brackets

Table 5: Genetic diversity of the coenopopulations of *P. patens*

Coenopopulations/values	Pp1	Pp2	Pp3	Pp4	Pp5	For the total sample
Рисунок H_E	0.131 (0.013)	0.166 (0.013)	0.148 (0.013)	0.157 (0.014)	0.209 (0.014)	0. 162 (0.013)
Рисунок n_a	1.597 (0.492)	1.715 (0.453)	1.660 (0.475)	1.632 (0.484)	1.743 (0.438)	1.965 (0.184)
Рисунок n_e	1.197 (0.266)	1.255 (0.296)	1.224 (0.280)	1.247 (0.311)	1.330 (0.317)	1.361 (0.306)
P_{95}	0.772	0.838	0.728	0.741	0.851	0.965
R	1	1	0	0	0	2
μ	1.634 (0.006)	1.668 (0.005)	1.629 (0.006)	1.625 (0.006)	1.723 (0.005)	1.656 (0.006)
Рисунок h	0.183 (0.003)	0.166 (0.003)	0.185 (0.003)	0.188 (0.003)	0.139 (0.002)	0.172 (0.003)
I	0.215 (0.226)	0.268 (0.232)	0.242 (0.229)	0.249 (0.246)	0.327 (0.244)	0.373 (0.196)

Note: Рисунок H_E is the expected heterozygosity; Рисунок n_a is the absolute number of alleles per locus; Рисунок n_e is the effective number of alleles per locus; for all above parameters, standard deviations are shown in brackets, R is the rare fragments

The analysis of the genetic structure of the coenopopulations of *P. patens* showed that the expected share of heterozygous genotypes in the total sample (H_T) of *P. patens* was 0.232 and the expected share of heterozygous genotypes (H_S) in the coenopopulations of *P. patens* was 0.162 (Table 6). Thus, the expected share of heterozygous genotypes (H_S) in the coenopopulations of *P. patens* was lower than for the total sample.

The lowest shares of heterozygous genotypes (H_S) were noted in primer M1, the expected heterozygosity determined by it was 0.121; and the highest values of this indicator were noted in *P. patens* during PCR with primer M27 (H_S = 0.219).

The greatest part of all observed genetic diversity of *P. patens* is also concentrated within populations (G_{ST} = 0.300) and the share of interpopulation variability is 70.0% (Table 6).

In 2016, a study of the genetic diversity of European populations of *P. patens* was made (Szczecińska *et al*., 2016). A total of 29 populations of *P. patens* were studied with the use of microsatellite primers. The results of the study showed that the analyzed populations were characterized by low heterozygosity (H_o = 0.005, H_e = 0.561) and very high levels of inbreeding (F_{IS} = 0.90). At the same time, the results indicated a higher level of variability within the populations (77%) than between the populations (23%) (Szczecińska *et al*., 2016). The data obtained from the use of intermicrosatellite analysis of the genetic polymorphism in the five coenopopulations of *P. patens* in Northern Kazakhstan showed even lower values of the expected heterozygosity (H_e = 0.162), the subdivision of the studied coenopopulations had similar values (G_{ST} = 0.300).

The main indicators of the genetic diversity of *P. patens* were also determined for two coenopopulations in the Perm region. It should be noted that in the coenopopulations of *P. patens* in the Perm Territory, the expected heterozygosity, the share of polymorphic DNA fragments and the Shannon diversity index were lower for both studied rare species, compared to the indices of the coenopopulations of Northern Kazakhstan (for *Pp1* P_{95} - 0.603, H_E - 0.141 *and I - 0.230;* for *Pp2* P_{95} - 0.628, H_E - 0.149, *I* - 0.217) (Shakirova, 2019a) (Table 7).

Based on the data obtained for the ISSR analysis of DNA polymorphism of *P. patens*, genetic relationships between the studied coenopopulations were determined, a matrix of binary traits was composed and matrices of genetic differences were calculated. Based on the obtained matrix, a cluster analysis was made using the Unweighted Pair-Group Method (UPGMA) and a tree diagram that reflected the degree of similarity of the studied coenopopulations of *P. patens* by the ISSR spectra was made (Fig. 4).

To build the tree diagrams, the authors used the Treecon 3.1 computer application with 100 bootstrap replicas. The smallest genetic distance was noted between the coenopopulations of *P. patens* in the Kostanay region Pp4 and Pp5 (D = 0.087) and the largest one - between the coenopopulations located in the Akmola region Pp2 and Pp3 (D = 0.131) (Table 8).

On the tree diagram, the fourth and the fifth coenopopulations *of P. patens* (Pp4, Pp5) formed a cluster from which the rest of the coenopopulations departed according to their geographical distance between the Kostanay and Pavlodar regions. The branching nodes were highly supported (bootstrap index > 50%).

Table 6: Assessing the reliability of the difference in comparing indicators P_{95} and H_E according to Fisher's criterion between the five coenopopulations of *P. patens*

CP	Pp1	Pp 2	Pp 3	Pp 4	Pp 5
Pp 1	-	0.647	0.394	0.280	0.786
Pp 2	0.382	-	1.041	0.927	0.139
Pp 3	0.190	0.192	-	0.141	1.461
Pp 4	0.287	0.095	0.097	-	1.066
Pp 5	0.809	0.427	0.619	0.522	-

Note: the differences in the polymorphic loci shares (P_{95}) are above the veraciousness diagonal, (H_E) is below the diagonal of the expected heterozygosity; with $F_{experiment}$ greater than 1.96, the result is veracious

Table 7: Genetic structure and differentiation of the coenopopulations of *P. patens*

ISSR primer	H_T	H_S	G_{ST}
M1	0.149 (0.013)	0.121 (0.005)	0.185
ISSR1	0.256 (0.023)	0.181 (0.009)	0.292
X11	0.182 (0.019)	0.148 (0.012)	0.190
ISSR3	0.238 (0.025)	0.143 (0.009)	0.397
M27	0.324 (0.019)	0.219 (0.009)	0.326
For the total sample	0.232 (0.023)	0.162 (0.010)	0.300

Note: H_T is the expected share of heterozygous genotypes as a measure of the total gene diversity in the entire population; H_S is the expected share of heterozygous genotypes in a separate population as a measure of its intrapopulation diversity or the average selective gene diversity for all loci; G_{ST} is the share of interpopulation genetic diversity in total diversity or an indicator of the population's subdivision; the standard deviations are shown in brackets

Table 8: Genetic distance between the coenopopulations of *P. patens*

	Pp1	Pp 2	Pp 3	Pp 4	Pp 5
Pp 1	-				
Pp 2	0.109	-			
Pp 3	0.127	0.131	-		
Pp 4	0.111	0.095	0.116	-	
Pp 5	0.120	0.106	0.091	0.087	-

Table 9: Assessment of the state of population gene pools of *P. patens*

	I. The main indicators of the genetic diversity		II. Genetic structure and differentiation of the populations			III. Specificity of the gene pools		Assessment of the state of the gene pools	
Population	Рисунок P_{95}	Рисунок H_E	μ	Рисунок h	I	Рисунок R	CGO	Type	State
Pp 1	0.772	0.131	1.634	0.183	0.215	1	2.744	T	Gene pool depauperization
Pp 2	0.838	0.166	1.668	0.166	0.268	1	2.863	C	Satisfactory
Pp 3	0.728	0.148	1.629	0.185	0.242	0	2.915	C	Satisfactory
Pp 4	0.741	0.157	1.625	0.188	0.249	0	2.666	T	Satisfactory
Pp 5	0.851	0.209	1.723	0.139	0.327	0	2.964	C	Satisfactory

Note: Рисунок P_{95} is the share of polymorphic loci; Рисунок H_E is the expected heterozygosity, μ is the average number of morphs, Рисунок h is the share of rare morphs, I is the Shannon diversity index, Рисунок R is the number of unique fragments and CGO is the coefficient of genetic originality; the types of gene pools are the following: T is the typical gene pool, C is the specific gene pool

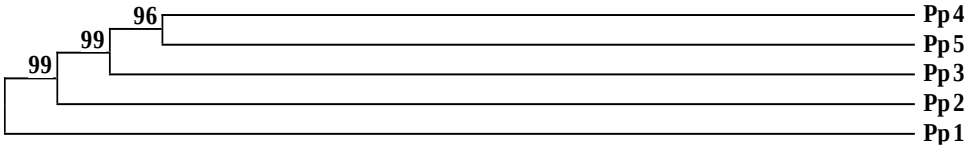


Fig. 4: UPGMA tree diagram of the genetic similarity of the five coenopopulations of *P. patens* built based on ISSR marker polymorphism; scale above is the genetic distance; bootstrap values are specified (in %)

In determining the CGO of the coenopopulations of *P. patens*, it was found that the maximum value of this genetic indicator was noted in Pp5 and the minimum one - in Pp4. The gene pools of coenopopulations Pp1 and Pp4 were characterized as typical and of Pp2, Pp3 and Pp5 - as specific (Table 9).

In analyzing the genetic indicators of the five coenopopulations of *P. patens*, it was found that the lowest expected heterozygosity, the Shannon diversity index and other values were noted in the first coenopopulation of *P. patens* located in the vicinity of settlement Bayanaul in the Pavlodar region. The state of this coenopopulation, according to the used technology, was characterized by gene pool depauperization. The state of the gene pools of the other studied coenopopulations of *P. patens* was satisfactory.

Conclusion

Thus, based on the results of the ISSR analysis of the genetic polymorphism in the five coenopopulations of *P. patens* in Northern Kazakhstan, molecular genetic formulas, barcodes and genetic passports have been made. The presented material allows to relatively quickly and cheaply identify the plant samples' affiliation with certain genus, species and population. Genetic profiles make it possible to quickly and clearly describe each population, given the characteristics of the genetic structure, the preservation of which is of primary importance in the development of measures for protecting and restoring the environment. All of the studied coenopopulations are characterized by high rates of genetic diversity, except for the expected heterozygosity. In two of the five studied coenopopulations of *P. patens* rare DNA fragments have been found. *Pp*5 has the highest genetic diversity (P_{95} = 0.851, H_e = 0.209, I = 0.327), compared to the other coenopopulations studied in the territory of Northern Kazakhstan.

The scientific results of the research can be used for preserving the gene pools of rare and endangered plant species, optimizing the genetic diversity conservation at the population level, monitoring the state of coenopopulations of rare plant species; during introduction for preserving unique coenopopulations of rare plant species; when identifying specially protected natural areas, as well as when regulating human economic activity.

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Authors Contributions

All authors equally contributed in this work.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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