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GENETIC DIFFERENTIATION OF TWO PHENOTYPES OF *PLANTAGO MEDIA* L. IN SOUTH TIMAN

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❀ **Background.** The investigation of the genetic nature of plant phenotypic variability is of great importance for understanding biological diversity, distribution and adaptation of plants to environmental conditions. **The aim** of our work was to study the genetic differentiation of two phenotypes of *Plantago media* in South Timan. **Materials and methods.** The genetic differentiation of light and shadow phenotypes of *Plantago media* plants was evaluated using intersimple sequence repeats (ISSR) markers. **Results.** The population-genetic analysis of 210 loci revealed two clusters, which boundaries coincided with the boundaries between plants of light and shadow phenotypes. The results of the discriminatory analysis of the main components and AMOVA ($F_{ST} = 0.07$, $p = 0.001$) confirmed that there are statistically significant genetic differences between these phenotypes even though they possess a high genetic similarity. **Conclusion.** Light and shadow *Plantago media* phenotypes adapted to different ecological conditions are genetically differentiated. The population genetic analysis using ISSR markers is a sensitive tool for identifying the genetic diversity of phenotypic plant variations formed under the influence of environmental factors.

❀ **Keywords:** *Plantago media* L.; genetic analysis of population; microsatellite markers; genetic polymorphism; environmental condition; adaptation; light; phenotype.

ГЕНЕТИЧЕСКАЯ ДИФФЕРЕНЦИАЦИЯ ДВУХ ФЕНОТИПОВ *PLANTAGO MEDIA* L. НА ЮЖНОМ ТИМАНЕ

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❀ Исследован уровень генетической дифференциации двух фенотипических вариаций *Plantago media* L. на Южном Тимане. Популяционно-генетический анализ с использованием межмикросателлитных маркеров (ISSR) по 210 локусам выявил два кластера, границы которых совпадали с границами между растениями светового и теневого фенотипа. Результаты дискриминационного анализа главных компонент и AMOVA ($F_{ST} = 0{,}07$, $p = 0{,}001$) подтвердили, что на фоне высокого генетического сходства существуют статистически значимые генетические различия между этими фенотипами. Полученные результаты свидетельствуют о роли экологических факторов в адаптивной дифференциации и проявлении генетического полиморфизма растений.

❀ **Ключевые слова:** *Plantago media* L.; популяционный генетический анализ; межмикросателлитные маркеры; генетический полиморфизм; экологические условия; адаптация; свет; фенотип.

INTRODUCTION

The plasticity of a genotype, that is, its ability to express various phenotypic states, has great adaptive value and is controlled genetically. Individual and groups of genes may be involved in a species' adaptation to specific environmental conditions. In other words, the limits of modification variability are determined by the norm of an organism's reaction to the external environment, which is predetermined by its genotype. Some adaptive modifications are believed to affect hereditary variability [1].

The genetic and molecular mechanisms of plasticity and the role of ecological heterogeneity of the habitat in the genetic differentiation of plants have attracted increased research interest [2]. Studies on the genetic nature of the modification variability of plants in natural populations are of particular importance for understanding their expansion, colonization of a particular habitat, and maintenance in this environment. However, unlike animals, plant phenotypes are not strictly canalized. This characteristic complicates the identification of the genotypic basis, if any, of phenotypic variations in natural populations.

Plantago media L., also known as hoary plantain, is a perennial herbaceous short-rhizogenous taproot member of the family *Plantaginaceae*. The habitats of this species include Europe, Siberia, and Western and Central Asia. In the northeastern regions of Russia, the plant has been observed in the Arctic (Vorkuta) as an adventitious species [3]. It can be found in floodplain meadows, sparse forests, agricultural lands, and along roadsides and may often be the pioneer plant in shallows and limestone outcrops. *P. media*, like many other species of the genus *Plantago* L., is characterized with high phenotypic plasticity [4]. The habitus, size and shape of leaves, length of leaf petioles and flower shoots, accumulation and distribution of biomass by organs, and seed productivity of the plant depend largely on various environmental factors, such as temperature, light conditions, and chemical composition of the soil.

Our earlier studies revealed that *P. media* plants inhabiting an open, well-lit slope in a sparse cenosis differ from those plants found in shaded habitats in terms of morphological parameters and vital process activities [5–7]. Analysis of differences in leaf anatomic and morphological structures, pigment contents and there ratios, photosynthesis intensity, and several other plant parameters has revealed adaptive modifications in the light and shadow phenotypes of the species [5].

This work aimed to investigate the level of genetic differentiation of two phenotypic variants of *P. media* plants growing under different light conditions.

MATERIALS AND METHODS

The plants were collected and the soil and climatic conditions in the middle reaches of the Soiva River, a tributary of the Pechora River, in Troitsko-Pechora District, Komi Republic, Russia, were assessed in July 2014. The study area is part of the Timan taiga province of the East European taiga zone. Coenopopulations of *P. media* plants were localized in areas with contrasting insolation and thermal conditions: (1) On a talus slope from the watershed to the above-floodplain terrace of the Soiva River (62.74908 °N, 55.82615 °E). The exposure of the slope is southeastern, the steepness is approximately 30°, and the vegetation cover is poorly developed. (2) At a watershed (62.74761 °N, 55.82187 °E) under the canopy of arboreal and herbaceous layers in a spruce forest rich in herbs. The area occupied by each of the coenopopulations was approximately 350 m^2. The distance between the most distant sampling points of two coenopopulations was approximately 400 m, and the distance between the nearest coenopopulations was 200 m. Parallel samples of leaves of *P. major* (greater plantain) growing in an area adjacent to the study area were also collected.

Thirty typical *P. media* plants were selected to study the morphometric characteristics of each coenopopulation at the floral initiation stage (first week of July). The number of leaves and length, width, and area of the leaf from the middle part of the rosette of each plant were determined; the wet weight of the aboveground and underground parts and length of roots (shortened rhizome + main root) were also measured. The specific surface density of the leaves was calculated as the ratio of the weight of the leaf dried at 80 °C to its area.

Microclimatic parameters (e.g., temperature and photosynthetic active radiation intensity (400–700 nm)) were measured using a portable weather station (Data Logger LI-1400, USA), and the supply of ultraviolet (UV) radiation to plants in the wavelength ranges of 400–315 nm (UV–A) and 315–280 nm (UV–B) were assessed using a UV radiometer (TKA-PKM 12, Russia). Soil samples were taken from the root habitable layer (depth, 0–10 cm), and analysis of the physicochemical properties of soils and the elemental composition of plants was performed in accordance with generally accepted methods [8]. Nitrogen levels were estimated by gas chromatography using a specialized elemental CHNS-O analyzer at the Common Use

Center "Chromatography" of the Institute of Biology, Federal Research Center, Komi Scientific Center, Ural Branch of the Russian Academy of Sciences (Syktyvkar).

The genetic differentiation of plants of two *P. media* coenopopulations was assessed using intersample sequence repeats (ISSR). For this analysis, functionally mature leaves of the middle formation (one from each of 30 plants) were selected from each coenopopulation, fixed in liquid nitrogen, and stored at −70 °C. For comparative studies, leaf samples from five *P. major* plants were also selected. A total of 65 leaf samples were collected for further laboratory studies.

Total DNA was isolated using FastDNA Spin Kit reagents (QBioGene). The amplification reaction was performed in a volume of 50 μl, which included 20 μl of the fluorescent labeled primer (Applied Biosystems, USA), 10 μl of Screenmix (Applied Biosystems), 18 μl of nuclease-free water (Ambition, USA), and 2 μl of isolated DNA. Two primers, namely, ISSR-1.2 ($(GT)_7$-YG) and ISSR-AG8 ($(AG)_8$YT), labeled with 6FAM™ fluorescent dye were used. Polymerase chain reaction was performed according to the following protocol: initial denaturation for 5 min at 94 °C; 5 cycles of 90 °C (30 s), 45 °C (60 s), and 72 °C (90 s); 27 cycles of 90 °C (30 s), 55 °C (45 s), and 72 °C (60 s); and final elongation at 72 °C for 5 min. The amplification product was denatured using formamide (Applied Biosystems) for 3 min at 95 °C, and the resulting samples were analyzed on a genetic analyzer (ABI PRISM 310; Applied Biosystems) at the Common Use Center "Molecular Biology" of the Institute of Biology, Federal Research Center, Komi Scientific Center, Ural Branch of the Russian Academy of Sciences. All samples were analyzed in three replicates.

Electrophoretograms were analyzed using an internal standard in GeneMapper 5.0 software (Applied Biosystems) to establish the lengths of the obtained fragments. ISSR markers refer to markers of the dominant type of inheritance, the polymorphisms of which were tested for the presence/absence of a fragment. Fragments differing in length by less than one base pair (bp) were combined manually. The analysis included fragments ranging in size from 20 bp to 600 bp, and the presence or absence of fragments was coded as 1 or 0, respectively. Matrices containing the lengths of the fragments obtained and the corresponding peak heights from three analytical replicates were combined using an R language-based program [9] of our own design [10]. Population genetic analysis was performed using loci reproduced in three analytical replicates.

Analysis of molecular variance (AMOVA), including determination of the fixation index F_{ST}, was performed in the *R* environment using the poppr package [11]. F_{ST} is a measure of the genetic differentiation of a subpopulation, interpopulation genetic differences, and the divergence of subpopulations and varies from 0 (panmixia, equal allelic frequencies in subpopulations, no divergence) to 1 (complete isolation, extreme differentiation, subpopulations are fixed for different alleles, clean lines). Discriminant analysis of principal components (DAPC) was also performed in the *R* environment using the adegenet package [12]. Clustering based on the models described in [13, 14] was performed using STRUCTURE version 2.3.4. The results obtained from STRUCTURE, including the most probable number of clusters according to the method described in [15], were processed in the CLUMPAK online service (http://clumpak.tau.ac.il/).

RESULTS

Microclimatic and soil conditions of the two habitats

The soil in site 1 (i.e., the middle part of the talus slope) was underdeveloped, featured a discontinuous humus-accumulative horizon, and formed on a crushed stony stone-free layer with a close underlayment of large fragments of carbonate rocks. The soil in site 2 (forest) formed on carbonate rocks, the thickness of the litter was 3−5 cm, and the humus-accumulative horizon was well developed. The root layer of the soil exhibited a weakly alkaline reaction to the environment (Table 1) and relatively high contents of interchangeable forms of calcium and magnesium, which is due to the close locality of carbonate rocks. The upper soil layer of the watershed area under the spruce forest, which was rich in herbs, had a higher total nitrogen content than that on the slope. The difference in gross contents of phosphorus, potassium, aluminum, and iron between habitats was not especially significant.

Table 1

Physical and chemical properties of the surface soil layers (0–10 cm depth) of the *Plantago media* habitats in South Timan

Habitat	pH (H_2O)	N (total), %	Ca (exchangeable), mmol/100 g	Mg (exchangeable), mmol/100 g	K_2O (available), mg/kg	P_2O_5 (available), mg/kg	Fe (total), g/kg	Al (total), g/kg
Open slope	7.6 ± 0.1*	0.12 ± 0.2	20.9 ± 1.6	13.8 ± 1.0	126 ± 15	17 ± 4	15 ± 4	16 ± 4
Forest	7.2 ± 0.1	0.27 ± 0.4	21.7 ± 1.6	12.3 ± 1.0	81 ± 12	14 ± 3	15 ± 4	15 ± 4

Note. * ±Δ – means the boundaries of the interval of the absolute error of the measurement method at $p = 0{,}95$.

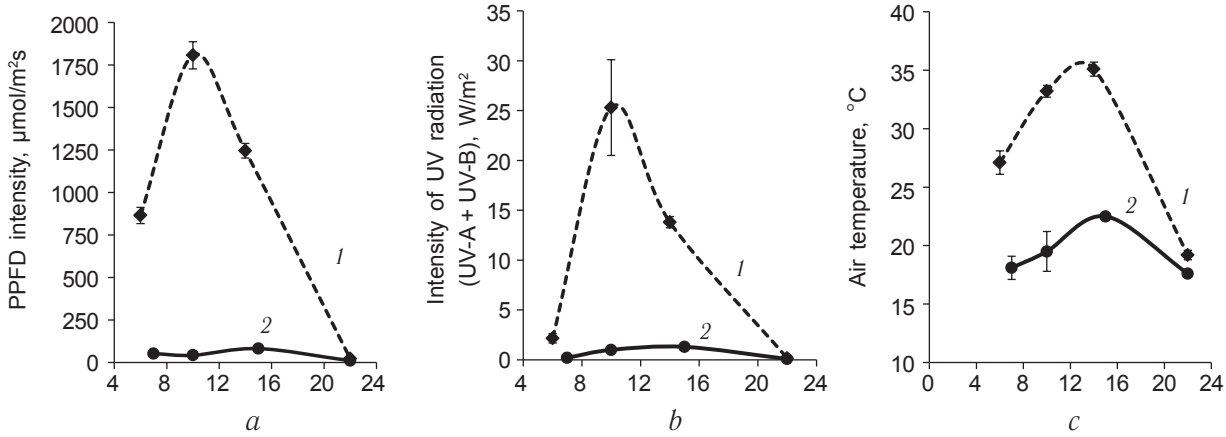

Fig. 1. Diurnal course of photosynthetic photon flux density (PPFD) (*a*), intensity of the ultraviolet radiation (*b*), and the air temperature (*c*) in *Plantago media* habitats. *1* – open slope, *2* – forest. Measurements were made in the first decade of July 2014 on a cloudless sunny day

The habitats of *P. media* differed significantly in terms of microclimatic conditions. On clear sunny days, plants on the slope received photosynthetic active and UV radiation one order of magnitude greater than that received by plants under the forest canopy (Fig. 1). The maximum air temperature at noon in the grass stand under the forest canopy was 10 °C–12 °C lower than in the open slope in direct sunlight.

Morphophysiological parameters of plants

Comparative analysis revealed significant differences in morphometric parameters between the plants of two coenopopulations (Table 2). Plants on the slope had a significantly smaller habitus and smaller, more pubescent leaves and accumulated less biomass compared with plants in the forest ecotope. The leaf laminae of the plants also differed in terms of specific surface density, which indirectly characterizes the photosynthetic activity of leaves. The aboveground/underground mass ratio of plants in the forest ecotope was higher than that of plants on the slope.

The leaves of plants in the forest ecotope contained more potassium and phosphorus, while the leaves of plants on the slope accumulated more iron and aluminum (Table 3). Despite differences in the nitrogen contents of the soils of the plots, differences in the content of this element in plant leaves were not significant.

Genetic differentiation of plants

The number of DNA fragments interpreted as loci in the population genetic analysis differed between three repeat analyses of the same samples of *P. media* and *P. major*. The numbers of polymorphic loci reproduced in one, two, and three analytical replicates were 592, 397, and 210, respectively. AMOVA using 210 loci showed expectedly significant differentiation between the two species of *Plantago*, and the fixation index (F_{ST}) was 0.38 ($p = 0.001$; Table 4). The F_{ST} between *P. media* plants inhabiting the open slope and forest ecotope was 0.07 ($p = 0.001$). Thus, the AMOVA test revealed small but statistically significant differences between the two *P. media* phenotypes.

Table 2

Morphometric parameters of *Plantago media* plants from different habitats

Parameter	Open slope	Forest	*p*-value for *t*-test
Number of leaves per plant	6.2 ± 1.2	6.0 ± 1.1	0.544
Leaf length*, cm	4.6 ± 2.0	9.2 ± 2.4	<0.001
Leaf width*, cm	4.2 ± 1.7	3.0 ± 0.7	<0.001
Leaf area*, cm^2	12.5 ± 4.2	19.0 ± 7.5	<0.001
Leaf mass per area (LMA)*, g/dm^2	0.76 ± 0.02	0.36 ± 0.02	<0.001
Leaf petiole length*, cm	1.5 ± 0.5	4.2 ± 1.9	<0.001
Fresh weight of aboveground part of the plant, g	1.5 ± 1.1	3.1 ± 1.3	<0.001
Root length, cm	13.6 ± 3.4	10.1 ± 3.2	<0.001
Fresh weight of underground part of the plant, g	1.2 ± 0.8	1.5 ± 0.5	0.144
Weight ratio of aboveground and underground parts	1.3 ± 0.7	2.1 ± 0.9	<0.001

Note. * Data are presented for the largest leaf of rosette.

Table 3

Chemical composition of the leaves of *Plantago media* from different habitats, mg/g DW

Habitat	N	Ca	Mg	K	P	Fe	Al
Open slope	10.8 ± 1.9*	35 ± 10	6.6 ± 2	20 ± 8	1.3 ± 0.4	0.38 ± 0.11	0.46 ± 0.12
Forest	13.8 ± 2.5	31 ± 9	6.1 ± 1.8	31 ± 12	2.5 ± 0.8	0.25 ± 0.07	0.29 ± 0.08

Note. * ±Δ – means the boundaries of the interval of the absolute error of the measurement method at $p = 0{,}95$.

Table 4

Results of molecular dispersion analysis between of *Plantago major* and *Plantago media* plants and between of *Plantago media* plants from different habitats (AMOVA)

Variability		Degrees of freedom	Sum of squares	Variance percent
Two groups: *P. media* и *P. major*	Between groups Inside each group $F_{ST} = 0.38$ ($p = 0.001$)	1 63	95.0 909.9	37.7 62.3
Two groups: *P. media* – open slope, *P. media* – forest	Between groups Inside each group $F_{ST} = 0.07$ ($p = 0.001$)	1 58	44.7 809.2	6.8 93.2

The genetic structure of the studied plants was assessed using two clustering methods. One method is based on a theoretical model of the distribution of allelic frequencies in a population of amphimictic diploid organisms [13]. The clusters (populations) identified during the analysis are assumed to be in Hardy–Weinberg equilibrium. The loci studied should be inherited independently of each other and not exhibit linkage disequilibrium of genes. This method of analysis was performed using the STRUCTURE computer program [13].

Another method to check one of the *a priori* specified clustering options involves reduction of the dimensions of the data by the principal component method, followed by the use of discriminant analysis to select those principal components that best delimit the clusters specified by the researcher. This method does not impose restrictions on the properties of the initial data [12]. The analysis was performed in the *R* environment using the adegenet package [16].

Clustering of the ISSR analysis results of plant samples of *Plantago* at 210 loci in the STRUCTURE program revealed two clearly separated clusters coinciding with the division of samples into *P. media* and *P. major* (Fig. 2). Repeated analysis of the samples, including only samples of *P. media* plants, revealed two clusters with boundaries coinciding largely with the boundaries between plants of different coenopopulations.

Discriminatory analysis of the main components (DAPC) indicated that all three groups (*P. media* plants on the slope and forest ecotope and *P. major* plants) are reliably separated (Fig. 3). Plants of two *P. media* coenopopulations were found to be much closer to each other than to *P. major* plants. The quality of clustering of individuals determined by the DAPC method directly depends on the number of principal components used for discriminant analysis. An increase in the number of main components (up to the number of loci analyzed) usually increases the quality of cluster separation. Classification success can be assessed by random selection of 10% of the individuals, information about which will not be used when constructing clusters. These individuals will be further classified into clusters using the values of the discriminant functions calculated for 90% of the sample. This procedure is repeated many times to evaluate the average values of the test results. In the present case, differences between the clusters persisted when the minimum number of principal components was equal to 5. Optimal results were obtained when the number of principal components was 20; in this case, the proportion of successfully classified individuals was 91% with a mean square error of classification of 11%.

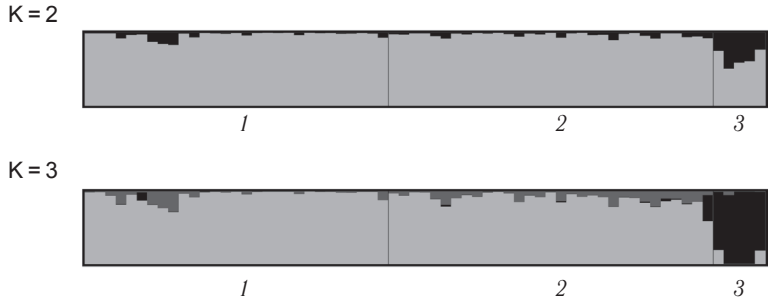

Fig. 2. Results of cluster analysis of the composition of 210 ISSR loci of *Plantago media* and *Plantago major* identified using STRUCTURE [13]. In the horizontal direction: each individual column corresponds to one sample; *1* and *2* – two groups of *P. media* plants on open slope and forest; *3* – *P. major* plants. In the vertical direction: the probability of assigning each sample (individual) to one of the clusters. Variants of the number of clusters are indicated above the graphs: K = 2 – the most probable number of clusters determined by the Evanno method [15]; K = 3 – the number of clusters corresponding to the number of studied plant groups

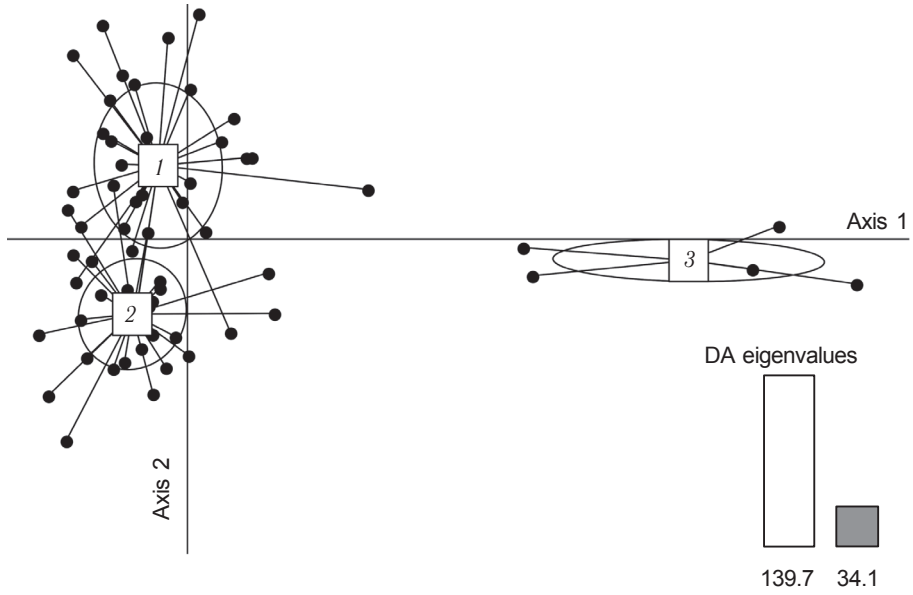

Fig. 3. Scatter plot obtained on the basis of the results of the discriminant analysis of the principal components (DAPC) of the matrix of 210 ISSR loci of *Plantago media* and *Plantago major* plants. *1* and *2* – *P. media* plants from the forest and on the open slope, *3* – *P. major* plants. Axis *1* – values of discriminant function 1, axis 2 – values of discriminant function 2. In the lower right corner of the graph are the values of the DAPC eigenvalues

DISCUSSION

Our studies have demonstrated that the adaptation of *P. media* to different ecological and coenotic conditions is manifested in the formation of phenotypes, the properties of which indicate the selective effect of the light factor. The distribution of plants in ecotopes with different light regimes is believed to depend on the genotypically determined variability of the leaf structure [17]. The anatomorphological and functional characteristics of the leaves of shadow- and light-type plants have been studied extensively [18–22].

The different morphological and physiological characteristics of *P. media* plants inhabiting an open slope and forest canopy indicate a pronounced response to the light regime (Table 1) and confirm our earlier data on the high plasticity of this species with respect to light [5, 6]. Plants on the slope received an order of magnitude more sunlight than those in the forest (Fig. 1). Moreover, the habitats differed not only in terms of the light intensity but also in the spectral composition of the light supplied to the plants. Plants on the open slope received direct sunlight enriched with blue rays (400–500 nm) and were exposed to UV radiation (280–400 nm) for most of the day. Direct measurements showed that the intensity of UV-B radiation (280–315 nm) on the open slope could reach 1.5 W/m^2 whereas that under the forest canopy is negligible (<0.1 W/m^2). These contrasting conditions of insolation were combined with differences in thermal regime. Our data showed that the heat supply of plants on the slope is distinctly higher than that of plants under the forest canopy. Plants on the slope are also subject to greater temperature fluctuations between day and night than those under the forest canopy. The high phenotypic plasticity of the leaves of *P. media* growing under different illumination conditions provides an optimal environment for the functions of the photosynthetic apparatus.

According to Table 1, differences in the supply of plants with the main biogenic elements (i. e., nitrogen, phosphorus, potassium, calcium, and magnesium) were not as pronounced as those for air nutrition. We did not observe significant differences in the levels of microelements, namely, copper, zinc, manganese, boron, and molybdenum, in the root layer of the soil (data not presented). The elemental compositions of the aboveground biomass of plants inhabiting the slope and forest did not differ markedly (Table 3), which, most likely, indicates a similar regime of mineral nutrition.

The literature presents information on the phenotypic lability and physiological reactions of some species of *Plantago* in relation to illumination [23], mineral nutrition conditions [24], and soil moisture content [25]. In a comprehensive study of four species of *Plantago* performed by ecologists, physiologists, and population genetics specialists in the Netherlands [4], the high phenotypic plasticity inherent in representatives of this genus were noted to be controlled genetically and have a genetic basis. For example, genetic diversity may be one of the factors providing the ability of *Plantago lanceolata* to exist in different communities [6].

Our population genetic analysis using ISSR markers for 210 loci revealed two clusters with boundaries coincident with the boundaries between *P. media* plants of the light and shadow phenotypes. The results of discriminant analysis of the main components confirmed that, in the presence of high genetic similarity, statistically significant genetic differences between these phenotypes, which reflects the genetic differentiation of the two *P. media* phenotypes growing in different environmental conditions, may be noted. Differences in ecological conditions, which are manifested primarily by the light and temperature regimes of the habitats, have a certain selective effect. However, despite the proximity of the localization of coenopopulations, the presence of reproductive barriers reducing the exchange of genetic information cannot be completely ruled out. *P. media* is a wind-pollinated herbaceous perennial, the seeds of which do not have the ability to spread over long distances. In addition, the transport of pollen by air currents to plants inhabiting the forest ecotope could be limited by the weak penetration of wind under the forest canopy. Thus, identifying the causes of genetic differentiation in closely located coenopopulations is challenging.

CONCLUSION

Habitation in different ecological and coenotic conditions leads to the formation of adaptive morphological and physiological reactions. The light and shade phenotypes of *P. media* in South Timan show

both high genetic similarity and statistically significant genetic differentiation, as evidenced by the polymorphisms of their genetic markers. Therefore, phenotypic variations could be assumed to be at least partially genetically determined. Maintaining a certain level of genetic diversity in populations is important to conserve the species and maximize its potential. Population genetic analysis using ISSR markers is a sensitive tool for identifying genetic diversity and differentiation in plant populations depending on environmental factors.

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