Estimation of the Genetic Structure of *Helix pomatia* L. Populations (Mollusca, Pulmonata) by Capillary Electrophoresis of ISSR DNA Fragments

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Abstract—Using the capillary electrophoresis of ISSR DNA fragments, the genetic structure of the adventitious populations of *Helix pomatia* L. (Mollusca, Pulmonata) inhabiting the territory of Eastern Europe was studied. A significant decrease in genetic heterogeneity ($H_e = 0.095 \pm 0.002$) and population differentiation level ($F_{st} = 0.074$) was revealed in comparison with similar data obtained using the ISSR method with electrophoretic detection in agarose gel and allozymes in polyacrylamide gel. Similar to other methods, an effect of spatial isolation ($R_M = -0.476$) was found on the basis of regression analysis between genetic and geographical distances. The effective population size, calculated with the help of M. Slatkin's model, turned out to be the largest of the previously calculated values ($N_e = 41.8$), which indicates the high viability of the studied groups of Roman snails in newly developed territories.

INTRODUCTION

The development of new technologies made it possible to assess the level of genetic variability of organisms in a different way at both the intrapopulation and interpopulation levels. The most convenient method is the analysis of polymorphic variants of inter-microsatellite DNA fragments (ISSR, inter simple sequence repeats) [1], which are flanked by a single, primer-containing tandem repeats of nucleotides. Sequences are scattered throughout the genome, which makes it possible to accurately estimate the structure of the genotype of individuals and identify genetic automatic processes in populations. In addition, ISSR fragments are often associated with selectively significant genes, making it possible to determine natural selection vectors. At the same time, the traditional way of detecting such fragments with the help of electrophoretic detection in plates with agarose (AGE) or polyacrylamide gel (PAGE) often produces distorted results [2]. The reason for this is the weak resolution of such detection methods, which do not allow recognizing fragments that are distinguished by a small number of base pairs, since they are part of the same amplicons on the electrophoregrams. In addition, it is not always possible to diagnose fragments containing a relatively small amount of accumulated DNA. As a result, the number of visible amplicons does not exceed several tens, and most of the information on the structure of the gene pools of the analyzed populations is out of sight. Another result is obtained when PCR results are detected by means of capillary electrophoresis (CE) [2], which makes it possible to increase the number of recognizable fragments to several hundred.

In the present work, the level of variability of the adventitious populations of Roman snails (*Helix pomatia* L., 1758) inhabiting the territory of Eastern Europe was considered as the object of research. Earlier, we analyzed the gene pools of these populations by using isoenzyme markers separated in polyacrylamide gel (PAGE) and also by the ISSR method with standard electrophoretic detection in agarose gel [3–5].

MATERIAL AND METHODOLOGY

The material for the study included samples of *H. pomatia* tissues stored in the cryobank, which was created at the Laboratory of Population Genetics and Genotoxicology of Belgorod National Research University. Samples from the populations were made during the expeditions from 2006 to 2012.

A total of 123 *H. pomatia* specimens from ten populations were examined at DNA loci (Fig. 1).

For the extraction of DNA, samples of mantles of molluscs thoroughly purified from mucus were used. DNA was isolated according to the protocol of the Silica uni sorbent-based reagent kit (Biokom, Russia).
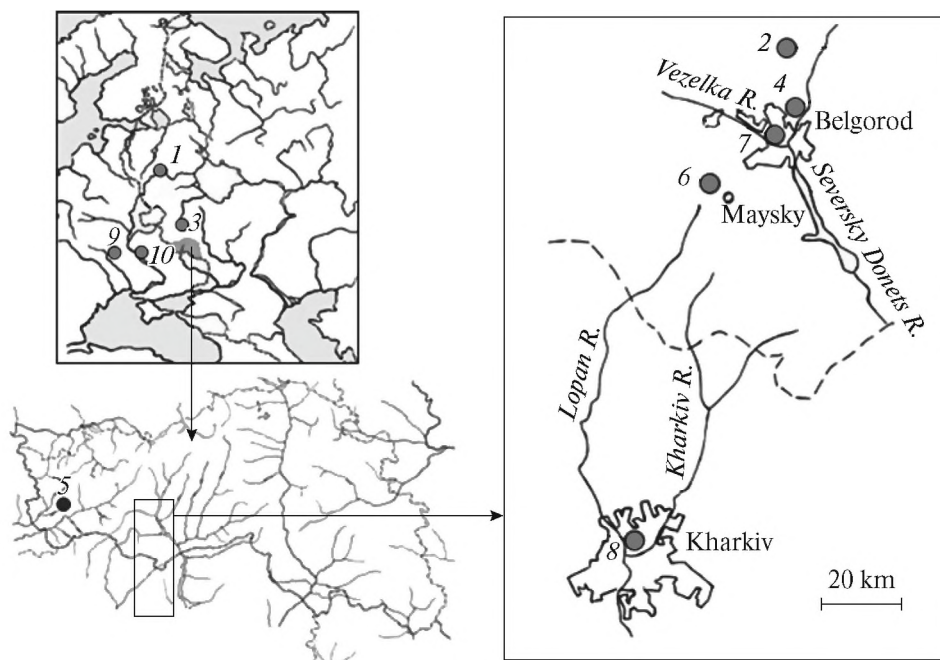
Fig. 1. *Helix pomatia* collection locations: (*1*) Tver, the old park in the area of the Wagon Works in Tver (56°51′41.65″ N 35°54′11.28″ E); (*2*) Shopino, Belgorod oblast, Belgorod raion, pos. Shopino, the bayrak forest near the Belgorod–Moscow highway (50°42′59.37″ N 36°29′29.98″ E); (*3*) Kursk, the territory of the forest adjacent to garages and garbage dump in Kursk (51°45′24.44″N 36°08′28.95″ E); (*4*) Donets, the Seversky Donets River floodplain, surroundings of Belgorod, willow and maple bushes, burdock thickets and nettle (50°36′38.40″ N 36°37′19.19″ E); (*5*) Khotmyzhsk, Belgorod oblast, pos. Khotmyzhsk, the Vorskla River floodplain, the bayrak forest near the Voskresenskaya Church, the recreational area of the Krasivo sanatorium (50°35′26.00″ N 35°54′11.28″ E); (*6*) Maisky, Belgorod oblast, Belgorod raion, pos. Maysky, the bayrak grove (50°30′59.26″ N 36°27′15.98″ E); (*7*) Belgorod, the willow forest in the Vezelka River floodplain in Belgorod, near the complex of buildings of Belgorod National Research University, the underbrush including nettle, burdock, cleaver, and dandelion (50°35′39.17″ N 36°34′04.49″ E); (*8*) Kharkiv, the T.G. Shevchenko city park in Kharkiv, the Lopan River floodplain (50°00′15.72″ N 36°13′31.31″ E); (*9*) Zhitomir, the forest park zone of the Teterev River floodplain in Zhitomir, on the opposite side of the river is the Yu.A. Gagarin city park of culture and recreation (50°14′19.27″ N 28°40′07.79″ E); (*10*) Kyiv, the A.V. Fomin Botanical Garden in Kyiv (50°24′52.38″ N 30°33′29.29″ E).

ISSR-amplicons were used as DNA markers. The PCR reaction was carried out using a Verity Amplifier (Applied Biosystems, United States) in 20 μL of a mixture containing 20 ng of genomic DNA, a PCR buffer (10 mM Tris-HCl pH 8.3, 50 mmol KCl, 2 mM MgCl$_2$), 0.25 mmol dNTP, 0.5 μmol primer, and 1 unit *Taq*-DNA polymerase inhibited for hot start. The primer UBC827 (5'-(AC)$_8$G-3'), labeled with fluorophore 6-FAM (DNK-sintez, Russia), was used for amplification. The reaction proceeded under the following conditions: first cycle 2 min/94°C, 40 cycles (30 s/94°C, 30 s/55°C, 2 min/72°C), and final cycle 10 min/72°C.

Fragment analysis of PCR products was carried out on the automatic capillary DNA sequencer ABI PRISM 3500 (Applied Biosystems, United States) using capillaries 50 cm long and the polymer matrix POP-7TM.

After PCR, the obtained ISSR fragments were purified using the Diatom RDNA Clean-Up sorbent-based kit (Isogen, Russia). After amplification, 9 μL Hi-DiTM formamide and 0.3 μL of a GS 600LIZv2.0 standard size solution were added to 1 μL of the PCR product. The samples thus prepared were analyzed using the DNA sequencer indicated. Fragment size analysis was carried out using GeneMapper Software v. 4.1 (Applied Biosystems, United States). In total, 559 loci were diagnosed in the range from 100 to 600 bp. Based on the obtained data, binary matrices were compiled in which the presence of a peak was designated as "1" (allele *p*) and absence as "0" (allele *q*).

Analysis of molecular variance (AMOVA) [6] and such indicators of the genetic diversity of populations as the expected heterozygosity (H_e), the average number of alleles per locus (A), the effective number of alleles (A_e), the Shannon index (I_{sh}), and the percentage of polymorphic loci (P, %) was calculated in the GenAlEx v. 6.5 program [7]. In addition, the relationship between the level of pairwise intergroup gene flows and pairwise geographical distances was estimated in this program using the Mantel test [8, 9], and the genetic distances according to Nei and Lee were also calculated [10]. Based on the resulting matrix of genetic distances, sample clustering was carried out using the unweighted pairwise grouping method (UPGMA) [11] in the MEGA6 program [12] (Fig. 2).

RESULTS

The indices of genetic heterogeneity of Roman snail populations are presented in Table 1. The results demonstrate low values of genetic heterogeneity, which are significantly ($p < 0.05$) inferior to similar data obtained using ISSR markers with electrophoretic detection in agarose gel and allozymes in PAGE.

Analysis of the molecular variance showed that the interpopulation variability accounted for 7%, while the level of differentiation of the population F_{st} was equal to 0.074, $p = 0.001$, which is significantly lower than similar data obtained by other methods. For example, according to the ratio of allozyme frequencies, the level of interpopulation variance was 29% ($F_{st} = 0.291, p = 0.01$) and that for ISSR loci with AGE was 20% ($F_{st} = 0.203, p = 0.001$). In this case, a complete identity or an unreliable difference in the ISSR loci detected by capillary electrophoresis (for example, between Donets and Belgorod groups) was observed between individual populations (Table 2). No such uniformity of population gene pools was observed for allozymes and ISSR loci with AGE.

Cluster analysis based on genetic distances (Fig. 3), as in the case of allozymes and ISSR markers with AGE, revealed a partial dependence of genetic distances on the geographic location of groups of snails. It is noteworthy that the groups living in large adjacent cities or their surroundings formed one cluster (no. 1), except for the Kursk and Tver locations.

Regression analysis between the logarithms of the pairwise level of the gene flow and the geographical distance between the populations showed that the Mantel correlation coefficient between these two indices ($R_M = -0.476, P = 0.0001$, 9999 permutations) differs insignificantly from similar coefficients calculated
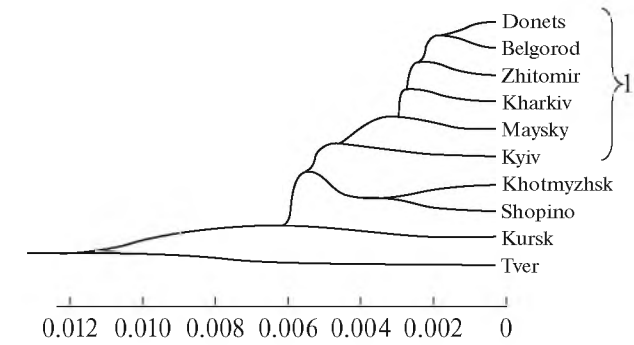

Fig. 2. Dendrogram of genetic distances according to Nei and Lee [10] (UPGMA) between *H. pomatia* populations by ISSR markers.
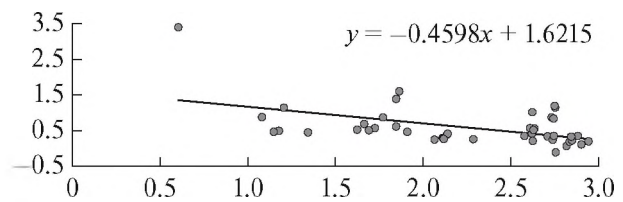

Fig. 3. Dependence of the gene flux level N_m (vertically, $\log N_m$) between pairs of *H. pomatia* populations on their geographical distances D (horizontally, km).

for allozymes ($R_M = -0.525, P = 0.002$, 9999 permutations) and for ISSR markers with AGE ($R_M = -0.591, P = 0.0004$, 9999 permutations). This fact confirms that the population structure of *H. pomatia* reflects the effect of spatial isolation (Fig. 3).

Table 1. Measures of genetic heterogeneity in *H. pomatia* populations averaged over the aggregate of DNA loci

Population	N	P, %	A	A_e	H_e	I_{sh}
Tver	6	44.72	0.900 ± 0.042	1.176 ± 0.011	0.116 ± 0.006	0.187 ± 0.010
Shopino	12	47.41	0.950 ± 0.042	1.129 ± 0.009	0.090 ± 0.005	0.153 ± 0.008
Kursk	12	40.79	0.816 ± 0.042	1.119 ± 0.009	0.081 ± 0.006	0.136 ± 0.008
Donets	14	66.37	1.327 ± 0.040	1.114 ± 0.006	0.091 ± 0.004	0.170 ± 0.006
Khotmyzhsk	13	50.45	1.009 ± 0.042	1.127 ± 0.009	0.090 ± 0.005	0.155 ± 0.008
Maysky	14	61.00	1.220 ± 0.041	1.121 ± 0.007	0.093 ± 0.004	0.169 ± 0.007
Belgorod	14	66.37	1.327 ± 0.040	1.119 ± 0.006	0.094 ± 0.004	0.173 ± 0.007
Kharkiv	12	62.61	1.252 ± 0.041	1.136 ± 0.007	0.103 ± 0.005	0.184 ± 0.007
Zhitomir	13	62.97	1.259 ± 0.041	1.104 ± 0.005	0.085 ± 0.004	0.159 ± 0.006
Kyiv	13	56.71	1.134 ± 0.042	1.144 ± 0.009	0.103 ± 0.005	0.178 ± 0.008
Average		55.94 ± 2.97	1.119 ± 0.013	1.129 ± 0.003	0.095 ± 0.002	0.167 ± 0.002
Average for ISSR with AGE		70.35 ± 4.61	1.703 ± 0.057	1.315 ± 0.046	0.190 ± 0.025	0.295 ± 0.035
Average for allozymes with PAGE		70.00 ± 8.16	1.925 ± 0.338	1.459 ± 0.224	0.248 ± 0.100	0.392 ± 0.151

For comparison, the averaged indices of the genetic heterogeneity of the ten populations under study are presented on the basis of the ISSR analysis with electrophoretic detection in agarose gel (AGE) and allozymes in polyacrylamide gel (PAGE).

Table 2. Pairwise estimates of genetic differentiation (F_{st}) between the studied *H. pomatia* populations by DNA markers

Population	Tver	Shopino	Kursk	Donets	Khotmyzhsk	Maysky	Belgorod	Kharkiv	Zhitomir	Kyiv
Tver		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Shopino	0.138		0.001	0.001	0.008	0.001	0.001	0.001	0.001	0.001
Kursk	0.241	0.123		0.001	0.001	0.001	0.001	0.001	0.001	0.001
Donets	0.104	0.073	0.114		0.006	0.074	0.419	0.252	0.035	0.001
Khotmyzhsk	0.175	0.048	0.111	0.063		0.001	0.001	0.006	0.001	0.001
Maysky	0.130	0.081	0.088	0.017	0.069		0.008	0.022	0.009	0.067
Belgorod	0.104	0.078	0.118	0.000	0.071	0.032		0.191	0.069	0.003
Kharkiv	0.099	0.078	0.121	0.006	0.057	0.032	0.010		0.015	0.002
Zhitomir	0.135	0.099	0.123	0.017	0.102	0.035	0.016	0.032		0.001
Kyiv	0.160	0.132	0.087	0.064	0.099	0.023	0.070	0.063	0.094	

Under the diagonal are F_{st} estimates, above the diagonal is the level of their significance (based on 999 permutations).

In addition, we calculated the effective number of the studied *H. pomatia* colonies on the basis of the model proposed by Slatkin [13], which is based on the use of the rectilinear regression equation and the coefficients of the linear function between pairwise estimates of the gene flow (*Nm*) and geographical distance (*D*) between the populations $\log N_m = a + b \log D$.

In this case, the effective population size (for all studied populations as a whole) is calculated as $N_e = 10^a$, where *a* is the coefficient obtained in the equation (Fig. 3):

Allozymes with PAGE	N_e	9.8
	95% Δ	3.9–25.1
ISSR with AGE	N_e	3.1
	95%	2.0–4.9
ISSR with PAGE	N_e	41.8
	95%	11.5–152.3

On the basis of capillary electrophoresis data, the largest average effective population size was obtained, which, judging by confidence intervals, significantly differs from the effective size calculated by ISSR markers with AGE.

DISCUSSION

The method of capillary electrophoresis revealed a decrease in the level of genetic heterogeneity and a decrease in the level of subdivision of *H. pomatia* populations in comparison with the analysis of isoenzymes with PAGE and ISSR markers with AGE. This can be explained by the relatively greater resolving power of the first method. Thus, it was possible to more accurately determine the size of intermicrosatellite fragments, most of which turned out to be similar in different populations. This may be a manifestation of family ties and the unity of origin of the adventitious colonies of *H. pomatia* in the southeastern part of the present range. Previously, we suggested that the so-called "bridgeheads" for settlement were most likely the colonies of Roman snails known since the beginning of the 20th century (for example, from the Kharkiv location) [5, 14]. In addition, the decrease in genetic distances between the populations under study may be caused by similar reactions of population gene pools caused by gene drift (the founder effect) and the effect of genetic revolution in isolated conditions [15].

According to Mayr [15], the transformation of the population from open to closed, which we observe in the case of adventitious groups of individuals, can significantly change the selective value of genes. In a more favorable position in such a situation are the so-called "soloist" genes, which are most viable in the homozygous state, in contrast to "well-mingled genes" that dominate in open groups. As a result, it is possible to observe the trend of the increasing level of homozygosity of populations at the same loci.

CONCLUSIONS

Studies of the genetic structure of the adventitious populations of *H. pomatia* in Eastern Europe using the ISSR method based on capillary electrophoresis make it possible to more accurately estimate the level of genetic diversity and the subdivision of groups of snails. The values of the effective number of Roman snail populations determined using the Slatkin model significantly exceed the similar indices we obtained earlier in populations of mollusc species aboriginal for the area under study [5, 16, 17], which indicates their high viability. The latter is likely to contribute to successful colonization by Roman snails of the areas outside their native range.

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