THE ASSESSMENT OF THE GENETIC STRUCTURE OF BUSH SNAIL (FRUTICICOLA FRUTICUM) POPULATIONS BASED ON THE NONSPECIFIC ESTERASES LOCI

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Using the polymorphic esterases loci, the genetic structure of the gastropod mollusk Fruticicola (Bradybaena) fruticum Müll., most of which lives in the south of the Central Russian Upland, was studied. For comparison, the samples were taken from the Romania, the North Caucasus, the Ural and the Vyatka regions. A total of the 1668 individuals were investigated. Of the 28 studied populations in 11 (39.3%), there was significant shortage of the heterozygotes. The level of the expected heterozygosity fluctuated in the range $H_e = 0.116–0.454$. Using the non-parametric statistics (Chao1-bc method and 1st order jackknife method), the populations with potentially high and low diversity of the multilocus genotypes were identified. The indicators of the genetic disunity between populations averaged $\Phi_{st} = 0.276$, $F_{st} = 0.292$. The principal component analysis and the Mantel correlation criterion $R_m = –0.007$ showed the absence of a reliable relationship between the geographical and genetic distance between populations, which indicates a violation of the isolation model by distance and confirms the thesis put forward by us that the urbanized forest-steppe landscape disrupts the natural migration processes, leads to the strong isolation and the genetic drift in the snail populations. At the same time, the phenomenon of increasing the degree of division of the populations against the background of reduced the allelic diversity, noted by us in many groups of bush snails, can be regarded as a shift in genetic equilibrium towards an increase in the interpopulation diversity (according to the Wright model). The revealed absence of the effect of isolation by distance can be a consequence of the action of the stabilizing natural selection. The assumption of the dependence of the esterase alleles frequencies in the bush snail populations on the genetic (biochemical) characteristics of the food objects was proposed. The effective size, calculated using the Slatkin formula turned out to be comparable with the background, adventive and relict species of the terrestrial mollusks living in the study area ($N_e = 2.2–7.6$).

Keywords: terrestrial mollusks; esterases; population structure; multilocus genotypes; effective size.

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INTRODUCTION
Despite the development of DNA technologies in population genetics, the genetic structure of various species populations is still assessed using isoenzyme markers. The reason for this is that unlike DNA loci (for example, ISSR or SSR), the coding part of the genome, which is influenced by natural selection factors, is subjected to analysis.

Esterase-active enzymes responsible for cleavage of esters in the cell have gained significant popularity in population genetics. Unlike other enzymes, they are represented by a large number of polymorphic loci that do not require expensive reagents for detection following electrophoresis, thereby allowing for analysis of representative samples from populations.

This work aimed to assess the genetic structure of the terrestrial mollusk model Fruticicola (Bradybaena) frustricum Müll. (bush snail) in the eastern part of their contemporary habitat using non-specific esterase loci.

Our previous publications present studies on the structure of population gene pools and estimate an effective number of bush snails using polymorphic signs pertaining to shell, allozymes, and DNA markers [1–4]. Further, these studies were continued.

MATERIALS AND METHODS
The materials for this study included tissue samples of Fr. fruticum individuals stored in a cryobank created at the Laboratory of Population Genetics and Genotoxicology, Belgorod State University, Russia. Population samples were collected during expeditions conducted from 2004 to 2017. The bulk of samples from the Central Russian Upland were collected from 2007 to 2012. A total of 1,668 Fr. fruticum individuals from 28 populations were examined (see Table 1 and Fig. 1).

To collect mollusks from a 2 m × 2 m site, mowing was performed using an entomological net. Concurrently, different-aged individuals sitting on grass stems were

Table 1

| Site No. | Site name                  | Biotope description                                                                 | Coordinates               |
|---------|----------------------------|--------------------------------------------------------------------------------------|---------------------------|
| 1       | Stoylo                     | Oskolets floodplain, Stoylo village. Osiery, undergrowth with burdock and nettles. Territory of the Stoilensky mining and processing enterprise. | 51°17′24.75″ N, 37°44′05.57″ E |
| 2       | Yamskaya step'             | Yamskaya Step' Reserve. Mixed forest, overgrowth of nettles. Territory of influence for Stoilensky and Lebedinsky mining and processing enterprises. | 51°11′04.66″ N, 37°39′31.97″ E |
| 3       | Dybenka                    | Dubenki floodplain (Belgorod region). Floodplain oak grove, undergrowth of nettles, burdock, and hops. Territory of influence for Stoilensky and Lebedinsky mining and processing enterprises. | 51°03′26.75″ N, 37°50′00.50″ E |
| 4       | Olshanka                   | Olshanki floodplain. Osiery, undergrowth of nettles, hops, and hogweed. Territory of influence for Stoilensky and Lebedinsky mining and processing enterprises. | 50°59′11.69″ N, 37°46′33.69″ E |
| 5       | Krasnyi Ostrov             | Halan floodplain near the village of Krasnyi Ostrov. Osiery. Burdock, nettle, and hops. | 50°56′34.06″ N, 37°46′54.17″ E |
| 6       | Dmitriyevka                | Korochi floodplain near the village of Dmitriyevka. Floodplain forest of willow and maple, overgrowth of nettles. Surroundings of the “Long-living oak” nature monument. | 50°30′12.47″ N, 36°59′39.62″ E |
| 7       | Lisya Gora                 | “Lisya Gora” natural monument near the village of Yablokovo. Oskol floodplain. Edge of an oak forest. Burdock, nettle, and hops. | 50°13′24.38″ N, 38°00′34.61″ E |
| 8       | Borki                      | “Borki” natural monument. Kozhinki floodplain, willow forest, overgrowth of burdock, nettle, and hops. | 50°08′16.39″ N, 37°53′02.28″ E |
| 9       | Stenki Izgorya             | Stenki Izgorya reserve area. Wetland biotope, alder overgrowth, undergrowth with burdock and nettle. | 50°41′23.25″ N, 37°49′12.22″ E |
| Site No. | Site name          | Site description                                                                 | Coordinates                   |
|---------|--------------------|----------------------------------------------------------------------------------|-------------------------------|
| 10      | Rovenki            | Rovenki natural park. Aidar floodplain, Rovenki village surroundings. Moderately moistened outdoor area. Overgrowth of burdock and hogweed mixed with nettle. | 49°54'33.31'' N, 38°52'55.29'' E |
| 11      | Borisovka         | Vorska floodplain, territory of the Borisovka village, beneath the motorway bridge. | 50°36'35.86'' N, 36°00'25.06'' E |
| 12      | Hotmyzhsk         | Vorska floodplain near the village of Hotmyzhsk. Burdock overgrowth with nettles. | 50°35'05.99'' N, 35°52'24.83'' E |
| 13      | Yakovlevo         | Vorska floodplain in the Yakovlevo village territory. Willow forest, nettle, and hops. | 50°52'05.12'' N, 36°26'49.92'' E |
| 14      | Syrtsevo          | Pena floodplain in the Syrtsevo village surroundings (Ivynansky District). Osier and maple. | 50°53'48.79'' N, 36°15'34.34'' E |
| 15      | Yasnyy Kolodets   | “Yasnyy Kolodets” natural monument, Koroch floodplain, in the Korocha town surroundings. Edge of sticky alder forest. | 50°49'34.23'' N, 37°12'34.24'' E |
| 16      | Koren              | Koren floodplain, in the Alekseevka village surroundings (Korochansky District). Osier. | 50°45'19.01'' N, 37°01'30.91'' E |
| 17      | Seversky Donets   | Seversky Donets floodplain in the Belgorod surroundings. Osier and maple.          | 50°36'38.40'' N, 36°37'19.19'' E |
| 18      | Nezhegol           | Nezhegol floodplain, territory of the town Shebekino. Willow forest.              | 50°24'32.93'' N, 36°52'38.38'' E |
| 19      | Kupyanska          | Oskol floodplain near the city of Kupyansky (Kharkov region, Ukraine). Floodplain and willow forest. | 49°42'37.60'' N, 37°37'26.18'' E |
| 20      | Divnogorye         | “Divnogorye” natural monument (Voronezh region). Foot of rocky outlets of chalk layers. Tikhaya Sosna floodplain. Burdock, nettle, and hops. | 50°57'48.99'' N, 39°17'40.3'' E |
| 21      | Galichya Gora      | Galichya Gora reserve site (Lipetsk region). Don floodplain. Overgrowth of nettle, hogweed, burdock, and hops. | 52°36'07.54'' N, 38°55'03.95'' E |
| 22      | Vorgol             | Vorgolskoye reserve site (Lipetsk region). Rocky outlets of Devonian limestone; in the Vorgol River floodplain. | 52°34'25.3'' N, 38°21'05.3'' E |
| 23      | Pluschan           | Pluschan reserve site (Lipetsk region). Natural forest landmark on the right bank of the Don River. Upland birch and oak forest. Overgrowth of nettle and burdock. | 52°50'00.1'' N, 38°59'26.66'' E |
| 24      | Kirov              | Vyatka floodplain. Territory of the city park of Kirov. Overgrowth of nettle and meadowsweet. | 58°34'57.11'' N, 49°41'50.75'' E |
| 25      | Olenyi ruchyi      | Olenyi Ruchyi Natural Park (Sverdlovsk Region, Nizhnesserginsky District); pine and fir forest with birch and larch, meadow with overgrowth of meadowsweet and raspberry. | 56°31'01.00'' N, 59°14'49.00'' E |
| 26      | Avrig              | Valley of the Olt River, foothills of the Transylvanian Alps near Avrig village (Romania). Floodplain is willow and maple forest, rocky soil, with high moisture content, overgrowth of nettle, burdock, and hops. | 45°43'36.87'' N, 24°20'30.12'' E |
| 27      | Kudymkar           | North Perm Region. Komi-Permyak autonomous area. Kudymkar city, wasteland on the Gagarin street. Valley of the Yinwa River. Elderberry and nettle overgrowth. | 59°00'59.7'' N, 54°39'58.0'' E |
| 28      | Kislovodsk         | North Caucasus, Kislovodsk surroundings. Kislovodsk National Park. Floodplain of the Olikhovka River. Overgrowth of burdock and nettle. | 43°53'37.1'' N, 42°43'15.6'' E |
Fig. 1. Collection points of the *Fr. fruticum*

| EST2 | 11 | 12 | 22 | 23 | 13 |
| EST3 | 12 | 23 | 22 | 22 | 33 |
| EST4 | 11 | 13 | 24 | 25 | 12 |
| EST5 | 13 | 23 | 34 | 35 | 33 |

Since it does not contain mucus (which impedes analysis), water-soluble proteins were extracted from mollusks’ leg retractor by freezing at −80 °C, followed by thawing and mechanical grinding with a Teflon homogenizer in a 0.05 M TrisH buffer (pH 6.7). Electrophoresis of isoenzymes was performed in a 10% polyacrylamide gel in a VE-3 chamber (Helicon). For this process, a TrisHCl gel buffer (concentration gel pH 6.7, separating gel pH 8.9), and a tris-glycine electrode buffer (pH 8.3) was used. The blocks were stained in a substrate mixture of TrisHCl (pH 7.4), α-naphtyl acetate, and fast red TR.

Four loci of non-specific monomeric esterases were used for analysis, namely, EST2 (with three alleles), EST3 (with three alleles), EST4 (with four alleles), and EST5 (with five alleles) (Fig. 2) [5, 6].

The data obtained were processed using the GenAlEx v.6.5 software package [7].
RESULTS

The allele frequencies of esterase-active enzymes in populations are presented in Table 2, whereas Table 3 presents averaged indicators of genetic heterogeneity.

Since we assessed the condition of population gene pools among Fr. fruticum, a limited number of samples were used containing only a small part of the population allele pool. In each group, the total number of multilocus genotypes ($N_{MLG}$) and the number of unique multilocus genotypes ($N_{MLG-1}$) were estimated, i.e., combinations were noted in one single population. In the future, based on the frequency distribution of multilocus genotypes, potential genetic diversity can be calculated for each population via an increase in sample size to infinity ($N_{max}$). The analysis was performed using two nonparametric methods, the Chao1-bc (bias-corrected form for the Chao1) method [8] and the first-order jackknife method [9]. All calculations were performed using the SPAdes software [10]. Table 4 presents the subsequent results.

The degree of differentiation in populations under study within the territory examined was estimated using genetic diversity characteristics proposed by Wright [11] and by analyzing molecular variance (AMOVA) [12]. The results are presented in Tables 5 and 6, respectively.

To clarify the degree of difference between individual populations, we calculated the values of pairwise indicators

### Table 2

| Locus | EST2 | EST3 | EST4 | EST5 |
|-------|------|------|------|------|
|       | 1    | 2    | 3    | 4    | 5    |
|       | 1    | 2    | 3    | 4    | 5    |
|       | 1    | 2    | 3    | 4    | 5    |
|       | 1    | 2    | 3    | 4    | 5    |
|       | 1    | 2    | 3    | 4    | 5    |
|       | 1    | 2    | 3    | 4    | 5    |
|       | 1    | 2    | 3    | 4    | 5    |
|       | 1    | 2    | 3    | 4    | 5    |
|       | 1    | 2    | 3    | 4    | 5    |
|       | 1    | 2    | 3    | 4    | 5    |
|       | 1    | 2    | 3    | 4    | 5    |
|       | 1    | 2    | 3    | 4    | 5    |
Genetic diversity indicators in *Fr. fruticum* populations, obtained from the analysis of esterase loci

| Population No. | N   | P, % | A   | $A_e$ | $I$   | $H_o$ | $H_e$ | $F$   |
|----------------|-----|------|-----|-------|-------|-------|-------|-------|
| 1              | 66  | 100  | 3.00| 1.137 | 0.252 | 0.064 | 0.116 | 0.362*|
| 2              | 127 | 100  | 2.75| 1.447 | 0.474 | 0.244 | 0.274 | 0.081 |
| 3              | 158 | 100  | 2.50| 1.284 | 0.359 | 0.174 | 0.206 | 0.132*|
| 4              | 31  | 100  | 3.00| 1.374 | 0.473 | 0.185 | 0.250 | 0.183 |
| 5              | 56  | 100  | 3.00| 1.476 | 0.472 | 0.259 | 0.251 | –0.028|
| 6              | 80  | 100  | 3.50| 1.664 | 0.627 | 0.441 | 0.365 | –0.151*|
| 7              | 60  | 100  | 2.75| 1.384 | 0.400 | 0.238 | 0.242 | 0.026 |
| 8              | 58  | 75   | 2.25| 1.537 | 0.462 | 0.233 | 0.274 | 0.114 |
| 9              | 94  | 100  | 2.50| 1.498 | 0.443 | 0.269 | 0.266 | 0.041 |
| 10             | 37  | 75   | 1.75| 1.446 | 0.411 | 0.203 | 0.273 | 0.222*|
| 11             | 62  | 75   | 2.50| 1.138 | 0.244 | 0.121 | 0.116 | –0.032|
| 12             | 20  | 75   | 2.50| 1.729 | 0.602 | 0.288 | 0.348 | 0.196 |
| 13             | 38  | 75   | 2.50| 1.436 | 0.414 | 0.145 | 0.231 | 0.301*|
| 14             | 39  | 50   | 2.00| 1.313 | 0.333 | 0.090 | 0.191 | 0.533*|
| 15             | 63  | 75   | 2.00| 1.612 | 0.463 | 0.163 | 0.282 | 0.425*|
| 16             | 57  | 75   | 2.50| 1.708 | 0.542 | 0.281 | 0.312 | 0.054 |
| 17             | 42  | 100  | 3.25| 1.748 | 0.711 | 0.262 | 0.416 | 0.401*|
| 18             | 36  | 75   | 2.00| 1.625 | 0.499 | 0.278 | 0.336 | 0.132 |
| 19             | 116 | 100  | 3.00| 1.321 | 0.388 | 0.205 | 0.219 | 0.036 |
| 20             | 37  | 100  | 3.50| 1.798 | 0.702 | 0.250 | 0.372 | 0.277*|
| 21             | 40  | 75   | 2.00| 1.392 | 0.355 | 0.331 | 0.225 | –0.322*|
| 22             | 20  | 75   | 1.75| 1.456 | 0.375 | 0.313 | 0.253 | –0.145|
| 23             | 20  | 75   | 2.50| 1.608 | 0.604 | 0.450 | 0.368 | –0.213|
| 24             | 37  | 75   | 2.50| 1.542 | 0.504 | 0.284 | 0.310 | 0.082 |
| 25             | 88  | 100  | 2.25| 1.699 | 0.552 | 0.278 | 0.336 | 0.126 |
| 26             | 100 | 100  | 3.25| 1.503 | 0.498 | 0.175 | 0.287 | 0.266*|
| 27             | 53  | 100  | 2.50| 1.288 | 0.364 | 0.146 | 0.208 | 0.249*|
| 28             | 33  | 100  | 2.75| 1.918 | 0.718 | 0.394 | 0.454 | 0.108 |

$M \pm m$ $88.4 \pm 2.7$ $2.58 \pm 0.38$ $1.503 \pm 0.220$ $0.473 \pm 0.018$ $0.242 \pm 0.094$ $0.278 \pm 0.097$ $0.123 \pm 0.037$

*Note.* $N$ is the number of individuals in the sample; $P$ is the percentage of polymorphic loci; $A$ is the average number of alleles; $A_e$ is effective number of alleles; $I$ is Shannon index; $H_o$ is observed heterozygosity; $H_e$ is expected heterozygosity; and $F$ is fixation index (inbreeding coefficient). *Cases of significant differences between the expected and observed heterozygosity ($p \leq 0.05$) are explained in the text.*

for genetic distance (according to Ney) between the studied bush snail groups (see Table 7). The genetic relationships of populations were further investigated using principal component analysis (PCA) (Fig. 3). Accordingly, the first principal component (PC1) reflected 34.06% of the registered variability of populations and the second (PC2) reflected 29.1% of the total variance.

In the final stage, we evaluated the effective population size of *Fr. fruticum* using a model based on a linear function coefficient between pairwise estimates of gene flow ($N_{m}$) and geographical distance between populations ($D_g$): $\log N_m = a + b \cdot \log D_g$. The effective population size (for all studied populations as a whole) was calculated as $N_e = 10^a$, where $a$ is the coefficient obtained in the equation [13]. The equation used is presented in Fig. 4, and the results are shown in Table 8. For comparison, similar data are given here for other terrestrial mollusk species, studied by us at an earlier stage.
**Table 4**

Number of marked multilocus genotypes and estimates of potential genetic diversity obtained by different methods for the studied *Fr. fruticum* populations

| Population No. | N<sub>MLG</sub> | N<sub>MLG-1</sub> | Chao1-bc | First-order jackknife |
|---|---|---|---|---|
| | | | N<sub>max</sub> ± SE | 95 % CI | N<sub>max</sub> ± SE | 95 % CI |
| 1 | 12 | 1 | 15.0 ± 3.4 | 12.5–29.7 | 17.9 ± 3.4 | 14.1–29.0 |
| 2 | 14 | 3 | 16.0 ± 2.9 | 14.2–29.9 | 18.0 ± 2.8 | 15.1–27.9 |
| 3 | 10 | 1 | 11.0 ± 2.3 | 11.1–23.9 | 12.0 ± 2.0 | 10.4–20.2 |
| 4 | 11 | 2 | 24.5 ± 12.8 | 13.8–76.0 | 18.7 ± 3.9 | 14.0–30.7 |
| 5 | 12 | 3 | 15.3 ± 4.1 | 12.5–33.7 | 16.9 ± 3.1 | 13.6–27.4 |
| 6 | 25 | 3 | 37.8 ± 9.2 | 28.7–70.1 | 38.8 ± 5.2 | 31.7–53.4 |
| 7 | 11 | 3 | 15.9 ± 6.0 | 11.8–42.6 | 15.9 ± 3.1 | 12.6–26.4 |
| 8 | 7 | 0 | 7.0 ± 0.5 | 7.0–7.0 | 8.0 ± 1.4 | 7.1–14.7 |
| 9 | 17 | 3 | 44.7 ± 21.1 | 24.4–121.4 | 24.9 ± 4.0 | 20.1–30.0 |
| 10 | 6 | 1 | 6.0 ± 0.2 | 6.0–6.0 | 7.0 ± 1.4 | 6.1–13.7 |
| 11 | 13 | 0 | 22.2 ± 8.7 | 14.9–57.3 | 20.9 ± 4.0 | 16.1–32.9 |
| 12 | 14 | 7 | 35.9 ± 16.6 | 20.3–97.6 | 26.4 ± 4.7 | 20.2–39.8 |
| 13 | 12 | 0 | 14.4 ± 3.1 | 12.4–28.6 | 16.9 ± 3.1 | 13.6–27.3 |
| 14 | 10 | 0 | 10.5 ± 2.5 | 9.1–23.7 | 11.9 ± 2.4 | 9.7–20.9 |
| 15 | 23 | 2 | 30.7 ± 6.2 | 24.9–54.0 | 33.8 ± 4.6 | 27.8–47.2 |
| 16 | 22 | 2 | 23.1 ± 2.5 | 21.3–34.2 | 26.9 ± 3.4 | 23.1–37.9 |
| 17 | 26 | 13 | 67.7 ± 27.5 | 38.9–161.3 | 44.5 ± 6.1 | 35.9–60.6 |
| 18 | 18 | 2 | 23.0 ± 4.5 | 19.1–40.9 | 26.8 ± 4.2 | 21.6–39.2 |
| 19 | 21 | 1 | 26.9 ± 5.3 | 22.3–47.9 | 29.9 ± 4.2 | 24.7–42.5 |
| 20 | 20 | 5 | 32.8 ± 10.0 | 23.3–69.3 | 31.7 ± 4.8 | 25.4–45.3 |
| 21 | 7 | 1 | 12.9 ± 6.9 | 7.9–43.5 | 10.9 ± 2.8 | 8.1–20.7 |
| 22 | 5 | 0 | 5.0 ± 0.2 | 5.0–5.0 | 6.0 ± 1.4 | 5.1–12.5 |
| 23 | 13 | 4 | 24.4 ± 10.3 | 15.5–64.7 | 21.6 ± 4.1 | 16.5–33.8 |
| 24 | 17 | 5 | 21.1 ± 4.2 | 17.8–38.8 | 23.8 ± 3.7 | 19.5–35.3 |
| 25 | 32 | 14 | 51.2 ± 12.5 | 38.0–93.6 | 48.8 ± 5.8 | 40.7–64.4 |
| 26 | 31 | 14 | 68.9 ± 25.4 | 42.5–156.0 | 48.8 ± 6.0 | 40.4–64.7 |
| 27 | 14 | 1 | 27.7 ± 13.0 | 16.9–79.9 | 21.8 ± 3.9 | 17.1–33.9 |
| 28 | 23 | 10 | 49.4 ± 17.7 | 31.0–109.9 | 39.5 ± 5.7 | 31.5–54.8 |

Note: N<sub>max</sub> is sample size to infinity; N<sub>MLG</sub>, total number of multilocus genotypes; and N<sub>MLG-1</sub>, the number of unique multilocus genotypes.

**Table 5**

Values of locus values of inbreeding coefficients and the level of gene flow in *Fr. fruticum* populations under study

| Locus | F<sub>r</sub> | F<sub>st</sub> | F<sub>se</sub> | N<sub>m</sub> |
|---|---|---|---|---|
| EST2 | 0.281 | 0.409 | 0.177 | 1.159 |
| EST3 | 0.015 | 0.261 | 0.250 | 0.752 |
| EST4 | 0.176 | 0.383 | 0.251 | 0.747 |
| EST5 | −0.183 | 0.395 | 0.489 | 0.261 |

M ± m | 0.072 ± 0.101 | 0.362 ± 0.034 | 0.292 ± 0.068 | 0.730 ± 0.184 |

Note: F<sub>r</sub> is an inbreeding coefficient of an individual relative to the subpopulation; F<sub>st</sub>, an inbreeding coefficient of an individual relative to a large population; F<sub>se</sub>, an inbreeding coefficient of a subpopulation relative to a large population; and N<sub>m</sub>, an average indicator of the intensity of gene exchange between populations.
Values of the analysis of molecular variance (AMOVA) by esterases in *Fr. fruticum* populations

| Source of variability | df | SS     | MS    | V     | %  | Φst | P     | Nm   |
|-----------------------|----|--------|-------|-------|----|-----|-------|------|
| Interpopulation       | 27 | 668.73 | 24.768| 0.205 | 28 | 0.276| 0.001 | 0.654|
| Intrapopulation       | 3308|1773.98 | 1.074 | 0.537 | 72 |
| Total                 | 3335|2442.71 |       | 0.742 | 100|

**DISCUSSION**

The data obtained (see Table 3) indicates a significant deficiency of heterozygotes in 11 studied populations (39.3%), and their significant excess was noted in two cases (no. 6, 21) \((p \leq 0.05)\). There were no significant differences in the remaining variants between observed and expected heterozygosity. This was also evidenced by data presented in Fig. 4, which indicated a trend toward a deficit of heterozygotes, whereas the regression coefficient resulted as \(0.631 \pm 0.111\) \((p \leq 0.001)\).
### Table 7

| Population No. | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 |
|----------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| 1              | 0.000 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 2              | 0.028 | 0.000 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 3              | 0.026 | 0.004 | 0.200 | 0.000 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 4              | 0.025 | 0.028 | 0.098 | 0.000 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 5              | 0.017 | 0.014 | 0.088 | 0.000 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 6              | 0.026 | 0.028 | 0.098 | 0.000 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 7              | 0.026 | 0.014 | 0.088 | 0.000 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 8              | 0.025 | 0.028 | 0.098 | 0.000 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 9              | 0.026 | 0.004 | 0.200 | 0.000 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 10             | 0.017 | 0.014 | 0.088 | 0.000 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 11             | 0.025 | 0.028 | 0.098 | 0.000 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 12             | 0.026 | 0.028 | 0.098 | 0.000 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 13             | 0.025 | 0.014 | 0.088 | 0.000 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 14             | 0.026 | 0.028 | 0.098 | 0.000 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 15             | 0.017 | 0.014 | 0.088 | 0.000 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 16             | 0.025 | 0.028 | 0.098 | 0.000 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 17             | 0.026 | 0.028 | 0.098 | 0.000 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 18             | 0.025 | 0.014 | 0.088 | 0.000 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 19             | 0.026 | 0.028 | 0.098 | 0.000 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 20             | 0.017 | 0.014 | 0.088 | 0.000 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 21             | 0.025 | 0.028 | 0.098 | 0.000 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 22             | 0.026 | 0.028 | 0.098 | 0.000 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 23             | 0.025 | 0.014 | 0.088 | 0.000 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 24             | 0.026 | 0.028 | 0.098 | 0.000 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 25             | 0.025 | 0.028 | 0.098 | 0.000 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 26             | 0.026 | 0.014 | 0.088 | 0.000 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 27             | 0.025 | 0.028 | 0.098 | 0.000 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 28             | 0.026 | 0.028 | 0.098 | 0.000 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
Values of effective size calculated based on linear function coefficients between pairwise estimates of gene flow ($N_e$) and geographical distance between populations of various species of terrestrial mollusks

| Species                        | $N_e$ | 95%, CI            |
|-------------------------------|-------|--------------------|
| *Helix pomatia*               | 9.8   | 3.9–25.1           |
| *Helicopsis striata*          | 7.9   | 2.4–25.1           |
| *Chondrula tridens*          | 3.8   | 2.6–5.7            |
| *Cepaea vindobonensis*        | 1.1   | 0.14–8.5           |
| *Fruticicola fruticum*        | 4.1   | 2.2–7.6            |

Note: Data on *C. vindobonensis*, *Ch. tridens*, *H. striata*, and *H. pomatia* were taken from our previous publications [14–16].

The results pertaining to level of variability between populations are only partially consistent with data on multilocus genotypes (Table 4). Among populations of the Central Russian Upland, the largest number of multilocus genotypes was observed for groups 17 (Seversky Donets) and 6 (Dmitrievka). The largest number of unique combinations was also noted for 17, where calculations also revealed the highest values of potential genetic diversity. Outside the Central Russian Upland, the most genetically diverse populations were groups 25 (Olenyi ruchyi) and 26 (Avrig) living in mountainous areas. The smallest number of multilocus genotypes, both real and potential, was observed for groups 8, 10, and 22.

Assessment of the degree of differentiation among populations by allozyme loci was performed using F-statistics devised by Wright and, on average, showed a rather large disunity among the studied snail groups by esterase loci (Table 5). We observed a similar result when comparing data obtained via AMOVA (Table 6). In both cases, the average indicator of the gene exchange intensity between populations ($N_{eu}$) was less than unity, which according to the theory of evolution with shifting equilibrium indicates a violation of gene exchange between populations [18]. This was also indicated by the relatively high values of the $F_g$ inbreeding coefficient. In this case, the largest contribution to interpopulation diversity estimated by inbreeding coefficient $F_g$ (Table 5) was for the EST5 locus. The level of gene flow between groups ($N_{eu}$) resulted in higher than unity at the EST2 locus only, whereas the highest $F_g$ value was noted at this locus. It is worth noting that this locus has long been used as a marker for studying the population structure of bush snails in various Eastern European landscapes [1, 5, 19].

According to PCA, it was not possible to identify any isolated aggregate groups of bush snails (Fig. 3). The populations were distributed quite randomly, whereas geographically distant populations living in different natural zones were found to be genetically similar (for example, 9, 14, and 27 or 10 and 21). Conversely, populations living close to each other in similar biotopes were found to be genetically different (for example, 1–5). Populations 12, 15, 25, 26, and 28 distanced themselves significantly from the remaining groups.

The PCA data were confirmed by a graph illustrating dependence of gene flow ($N_{eu}$) level between populations, based on the geographical distances between them ($D_g$) (Fig. 4). The graph demonstrates the absence of a reliable relationship between these parameters (Mantel correlation coefficient $R_m = 0.007, p = 0.422, 9999$ permutations).

The above information indicates a violation of the migration channels between the studied groups of *Fr. fruticosa* and their prolonged isolation from each other. Moreover, their population gene pools may potentially be formed under the influence of Genetic drift processes. At the same time, an increase in the degree of subdivision of populations against a decrease in allelic diversity, as observed by us in many groups of bush snails, can be regarded as a shift in genetic equilibrium toward an increase in interpopulation diversity (based on Wright’s model). It is also known that if there is no effect of isolation by distance (as observed in our study), it is likely that the loci will be exposed to stabilizing selection [13]. In this regard, in our opinion, it is significant that the main feed plant for the species under study is stinging nettle (*Urtica dioica* L.), regardless of landscape, and that the frequency distribution aspects of esterase alleles in snail populations may therefore be associated with genetic (and as a result, biochemical) peculiarity among the populations of this plant. We believe that to clarify the situation in the future, it will be extremely useful to study the population structure of this feed object. Also, in some situations (for example, under conditions of high snail density or the absence of nettles), some individuals shift to other plants (burdock, hops, and meadowsweet), which can also affect the frequency ratio of esterase alleles in snail populations.
Russian Upland. According to average values, the effective size occupies an intermediate position between specially protected relict species (Helicopsis striata and Cepaea vindobonensis) on the one hand and background (Chondrula tridens) and adventive species (Helix pomatia) on the other.

**CONCLUSION**

On the basis of the above results, we can state a continuing trend of reduction in allelic diversity and a change in the ratio of genotype frequencies in populations of bush snails in the study area, which partly reflects changes in the structure of the entire forest steppe and steppe landscapes of the Central Russian Upland as a result of anthropogenic pressure. Concurrently, the absence of clear dependence of the frequencies of alleles of esterase-active loci is less dependent on the geoclimatic and microbiotopic factors.

**REFERENCES**

1. Снегин Э.А. Эколого-генетические аспекты расселения Bradybaena fruticum (Mollusca, Gastropoda, Pulmonata) в элементах лесостепного ландшафта // Экология. – 2005; 36(1):33-40. (In Russ.)
2. Снегин Э.А. Оценка состояния популяционных генофондов наземных моллюсков в условиях влияния горно-обогатительных комбинатов на примере Bradybaena fruticum (Mollusca, Gastropoda, Pulmonata) // Экологическая генетика. – 2010; 8: 45-55. [Snegin EA. Assessment of the state of population gene pools of terrestrial mollusks in conditions of influence of ore dressing combines from the example of the land snail Bradybaena fruticum (Mollusca, Gastropoda, Pulmonata).] Ecological genetics. 2010;8:45-55. (In Russ.)
3. Снегин Э.А. Временная динамика генетической структуры эстераз и пространственная структура вида у кустарниковой улитки (Bradybaena fruticum Müll.) // Журнал общей биологии. – 1977; 38(4):1495–1510. [Makeeva VM, Belokon’ MM, Malyuchenko OP. Estimating the gene pool condition in natural populations of invertebrates in the fragmented landscape of Moscow and Moscow Region with special reference to bush snail Bradybaena fruticum Müll. Russian Journal of Genetics. 2005;41(11):1230-1244. (In Russ.)]
4. Снегин Э.А., Снегина Е.А. Оценка состояния популяционных генофондов малоподвижных видов животных на примере наземного моллюска Bradybaena fruticum Müll. (Gastropoda, Pulmonata) с использованием ДНК-маркеров // Экологическая генетика. – 2017; 15(3):4-19. [Snegin EA, Snegina EA. Assessment of the state of population gene pools of the slightly mobile animal species on the example of the land snail Bradybaena fruticum Müll. (Gastropoda, Pulmonata) based on the DNA markers. Ecological genetics. 2017;15(3):4-19. (In Russ.)]
5. Матекин П.В., Макеева В.М. Полиморфная система эстераз и пространственная структура вида у кустарниковой улитки (Bradybaena fruticum Müll.) // Журнал общей биологии. – 1977; 38(4):1495–1510. [Makeeva VM, Belokon’ MM, Malyuchenko OP. Estimating the gene pool condition in natural populations of invertebrates in the fragmented landscape of Moscow and Moscow Region with special reference to bush snail Bradybaena fruticum Müll. Russian Journal of Genetics. 2005;41(11):1230-1244. (In Russ.)]
6. Макеева В.М., Белоконь М.М., Малюченко О.П. Оценка состояния генофонда природных популяций беспозвоночных животных в условиях фрагментированного ландшафта Москвы и Подмосковья (на примере кустарниковой улитки Bradybaena fruticum (Müll.)) // Генетика. – 2005; 41: 11. – С. 4-19. [Makeeva VM, Belokon’ MM, Malyuchenko OP. Estimating the gene pool condition in natural populations of invertebrates in the fragmented landscape of Moscow and Moscow Region with special reference to bush snail Bradybaena fruticum Müll. Russian Journal of Genetics. 2005;41(11):1230-1244. (In Russ.)]
7. Peakall R, Smouse PE. GenALEx 6: genetic analysis in Excel. Population genetic software for teaching and research – an update. Mol Ecol Notes. 2006;6(1):288-295. https://doi.org/10.1111/j.1471-8286.2005.01155.x.
8. Chao A. Species richness estimation. In: Balakrishnan N, Read CB, Vidakovic B, ed. Encyclopaedia of statistical science. New York: Wiley; 2005. pp. 7907-7916.
9. Burnham KP, Overton WS. Estimation of the size of a closed population when capture probabilities vary among animals. Biometrika. 1978;65(3):625-633. https://doi.org/10.1093/biomet/65.3.625.
10. Chao A, Shen TJ. SPADE [cited 12.03.2015]. 2009. Available from: http://chao.stat.nthu.edu.tw/wordpress/.
11. Wright S. The genetical structure of populations. Ann Eugen. 1951;15(4):323-354.
12. Excoffier L, Smouse PE, Quattro JM. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochon-
drial DNA restriction data. Genetics. 1992;131(2):479-491.
13. Slatkin M. Isolation by distance in equilibrium and non-equilibrium populations. Evolution. 1993;47(1):264-279. https://doi.org/10.1111/j.1558-5646.1993.tb01215.x.
14. Снегин Э.А. Пространственные и временные аспекты эколого-генетической структуры популяций беспозвоночных животных (на примере наземных моллюсков и насекомых юга Среднерусской возвышенности): Дис. ... докт. биол. наук. – Белгород, 2012. – 473 с. [Snegin EA. Prostranstvennyye i vremennyye aspekty ekologo-geneticheskoi struktury popul'yatsiy bespozvonochnykh zhivotnykh (na primere nazemnykh mol'lyuskov i nasekomyykh yuga Srednerusskoy vozvyshennosti). [dissertation] Belgorod; 2012. 473 p. (In Russ.). Доступно по: https://www.dissercat.com/content/prostranstvennyye-i-vremennyye-aspekty-ekologo-geneticheskoi-struktury-populyatsiy-bespozvonotch Decision made on 16.07.2019.
15. Снегин Э.А., Артемчук О.Ю. Морфогенетический анализ популяций Helix pomatia L. (Pulmonata, Helicidae) юго-восточной и восточной части современного ареала // Экологическая генетика. – 2014. – Т. 12. – № 4. – С. 25–37. [Snegin EA, Artemchuk OYu. Morphogenetic analysis of Helix pomatia L. (Pulmonata, Helicidae) populations from south-eastern and eastern parts of the modern area. Ecological genetics. 2014;12(4):25-37. (In Russ.).] https://doi.org/10.17816/ecogen12425-37.
16. Снегин Э.А., Снегина Е.А. Генетическая структура популяций особо охраняемого моллюска Cepaea vindobonensis (Mollusca, Gastropoda, Pulmonata) в условиях северо-восточной части современного ареала // Экологическая генетика. – 2016. – Т. 14. – № 3. – С. 13–27. https://doi.org/10.17816/ecogen14313-27. [Snegin EA, Snegina EA. Genetic structure of populations of specially protected mollusk Cepaea vindobonensis (Mollusca, Gastropoda, Pulmonata) in the north-eastern part of the modern-day range. Ecological genetics. 2016;14(3):13-27. (In Russ.).] https://doi.org/10.17816/ecogen14313-27.
17. Ли Ч.Ч. Введение в популяционную генетику / пер. с англ. Е.А. Салменковой, Е.Я. Тетушкина, под ред. Ю.П. Алтухова, Л.А. Животовского. – М.: Мир, 1978. – 555 с. [Li CC. First course in population genetics. Transl. from English E.A. Salmenkova, E.Ya. Tetushkin, ed. by Yu.P. Altukhov, L.A. Zhivotovskiy. Moscow: Mir; 1978. 555 p. (In Russ.).]
18. Wright S. Random drift and shifting balance theory of evolution. Mathematical topics in population genetics. Berlin: Springer Verlag; 1970, pp. 1-31.
19. Макеева В.М. Роль естественного отбора в формировании генотипического своеобразия популяций моллюсков (на примере кустарниковой улитки Bradybaena fruticum (Mull.)) // Журнал общей биологии. – 1989. – Т. 50. – № 1. – С. 101–107. [Makeeva VM. The role of natural selection in the formation of the genotypic originality of the populations of mollusks (on the example of the shrub snail Bradybaena fruticum (Mull.)). Journal of general biology. 1989;50(1):101-107. (In Russ)].