Genetic Structure of Populations of Specially Protected Mollusk *Cepaea vindobonensis* (Mollusca, Gastropoda, Pulmonata) in the Northeastern Part of the Modern-Day Range

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Abstract—A research study on the population structure of relict and specially protected species *Cepaea vindobonensis* Fer. (Mollusca, Gastropoda, Pulmonata) in the conditions of the northeastern part of the modern-day distribution range is conducted with the use of allozymes and DNA markers. Groups with both high and low levels of genetic diversity are revealed. A high degree of differentiation in the studied populations ($F_{st} = 0.231$, $G_{st} = 0.265$, $\Phi_{st} = 0.261–0.263$, $N_m = 0.630–0.832$) is noted, which indicates a disruption of migratory channels and prolonged isolation from each other. Calculation of the effective population size and its comparison to analogous indices for background and vulnerable species of terrestrial mollusks demonstrate a high level of viability of the *C. vindobonensis* population within the studied area.

INTRODUCTION

The creation and management of the Red Lists of plants and animals has become an important aspect of work on preserving the living environment. Moreover, in the past few decades, this work has increasingly focused on monitoring regional systems, since the features of urbanization and the resulting ecological problems are specific to different landscapes. It is well known that the disappearance of a species begins with the disappearance of its population, as a result the distribution range is considerably reduced, genetic diversity decreases, and therefore loss of stability and extinction occur. In this case as a rule, the groups located at the borders of species ranges or some isolated territories suffer first, since during the distribution they inherited a small part of the species gene pool due to the gene flow. Moreover, loss of a portion of alleles is occasionally not replenished by the appearance of new genetic factors in the course of the mutational process. Caught in conditions of anthropogenic pressure, such populations are more vulnerable than other species of the community due to not having a genetic reserve of strength. It frequently occurs that a species included in the protection lists of separate regions is widespread in general and reaches large numbers within individual parts of the range; therefore, it does not need protection. However, this apparently flourishing state can change into a depressive state in a number of cases. In the normally varying environment, this extinction of the local peripheral groups is easily compensated by the influx of individuals from the central regions. At present, however, this maintenance of species stability is proving to be less and less feasible due to the destruction of biological corridors. By itself, the rare occurrence of a species in a region can only be an indirect argument, since it can be caused by the random drift of individuals from the adjacent satisfactory parts of the distribution range and can be considered an attempt at inhabiting new territories. Another version appears when the matter concerns native relict populations. Besides traditional demographic characteristics, profound knowledge of the state of the population gene pools is necessary to evaluate their viability, including the estimation of allelic and genotypic diversity, the degree of mutagenic pressure, and adaptive rearrangements at the genetic level. This information should become a serious argument in decision-making.

One such vulnerable species is the relict terrestrial gastropods, *Cepaea vindobonensis* Fer.[1] (Austrian snail), known from the Pleistocene deposits of Europe. Its contemporary distribution range covers Southeastern Europe, Crimea, and North Caucasus [1]. Our studies were conducted mainly on the southern macroslope of

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[1] In a number of studies on molecular taxonomy, this species is included in the genus *Caucasotachea* [16, 17].

Table 1. Collection points of *C. vindobonensis*

No.	Point	Point description	Coordinates
1	Golovchino	Vorskla River valley near Golovchino village, highland oak forest	50°33′57.29″ N 35°48′12.29″ E
2	Khotmyzhsk	Vorskla River valley, floodland meadow near Khotmyzhsk village	50°35′05.99″ N 35°52′24.83″ E
3	Bekaryukovskii Bor	Bekaryukovskii Bor Nature Monument, floodland of Nezhegol' River (Shebekinskii region)	50°25′43.87″ N 37°04′12.07″ E
4	Valyiki	Valyi River valley, bottom of chalk cliff, neighborhood of the city of Valu- iki, near the road	50°13′24.38″ N 38°00′34.61″ E
5	Rzhevka	Chalk cliff of west exposure in the floodland of Korocha River near Rzhevka village	50°26′32.63″ N 36°58′22.89″ E
6	Kupyansk	Floodland meadow of Oskol River valley, near the city of Kupyansk (Ukraine)	49°42′37.60″ N 37°37′26.18″ E
7	Zhitomir	Floodland of Teterev River, city of Zhitomir (Ukraine). Willow and maple thicket	50°14′31.06″ N 28°40′23.52″ E
8	Khortitsa	Khortitsa Island on Dnepr River. Vicinity of city of Zaporozhie (Ukraine)	47°50′31.45″ N 35°04′10.03″ E
9	Dubki	Dubki Park in vicinity of city of Nikolaev (Ukraine). Young oaks. Patches of bush thicket are present. Is subjected to relatively intense anthropogenic influence. Forest fires are frequent	46°58′42.47″ N 32°03′51.29″ E
10	STP	City of Nikolaev. Park near South Turbine Plant. Mainly composed of oaklets. Bush thickets are present in some places. It is subjected to relatively intense anthropogenic influence	46°56′26.17″ N 32°03′03.45″ E
11	Penza	City of Penza (Arbekovo district). Abandoned swamped area. Bush, remains of an orchard, birch and ash tree plantings	44°56′32.53″ N 53°13′30.05″ E

the Mid-Russian Upland (Belgorod and Kharkov regions), where the northeastern boundary of the natural distribution range of this species lies. In this area, the snails inhabit the relict communities of the preglacial and postglacial periods, such as the chalky pine forests and highland oak forests; they are also encountered on the dry, thoroughly warm slopes, chalky outcrops, and dry meadows [2]. The species is in the Red List of Belgorod Region [3].

There are a number of studies on the population structure of this species, which include the shell features and which cover the central and western parts of the distribution range of the species [4–13]. The aforementioned publications list the data on the correlation between the metric and coloration features of the shell of this mollusk and the microclimatic conditions of the natural and urbanized environment. Furthermore, one continuous population of *C. vindobonensis*, inhabiting the environment of the city of Nikolaev in southern Ukraine, was studied using molecular DNA markers (*RAPD*) [14]. In our previous studies, we obtained the preliminary data on the population structure of *C. vindobonensis* in the Southern Mid-Russian Upland, obtained using the conchological features and allozymes [15].

The goal of this study was to estimate the state of the *C. vindobonensis* population's gene pools in the northeastern part of the distribution range for conservational purposes, based on the selectively significant loci of isozymes and neutral DNA markers (*RAPD, ISSR*).

MATERIALS AND METHODS

The tissue samples of *C. vindobonensis* specimens stored in a cryobank created in the Population genetics and Genotoxicology Scientific and Research Laboratory of Belgorod National Research University served as the material for this study. The sets of specimens from *C. vindobonensis* populations were collected during an expedition from 2006 to 2010 from six points in the Southern Mid-Russian Upland. We used the datasets from the western part of the species distribution range (points 7 to 10) and an adventive group from the city of Penza (point 11) for the comparison (Table 1, Fig. 1). In total, 999 specimens were studied for allozymes and 488 specimens were studied using DNA markers.

The allozymes were extracted from the feet of mollusks by freezing at −80°C with the subsequent thawing and mechanical fragmentation in a 0.05 M Tris-
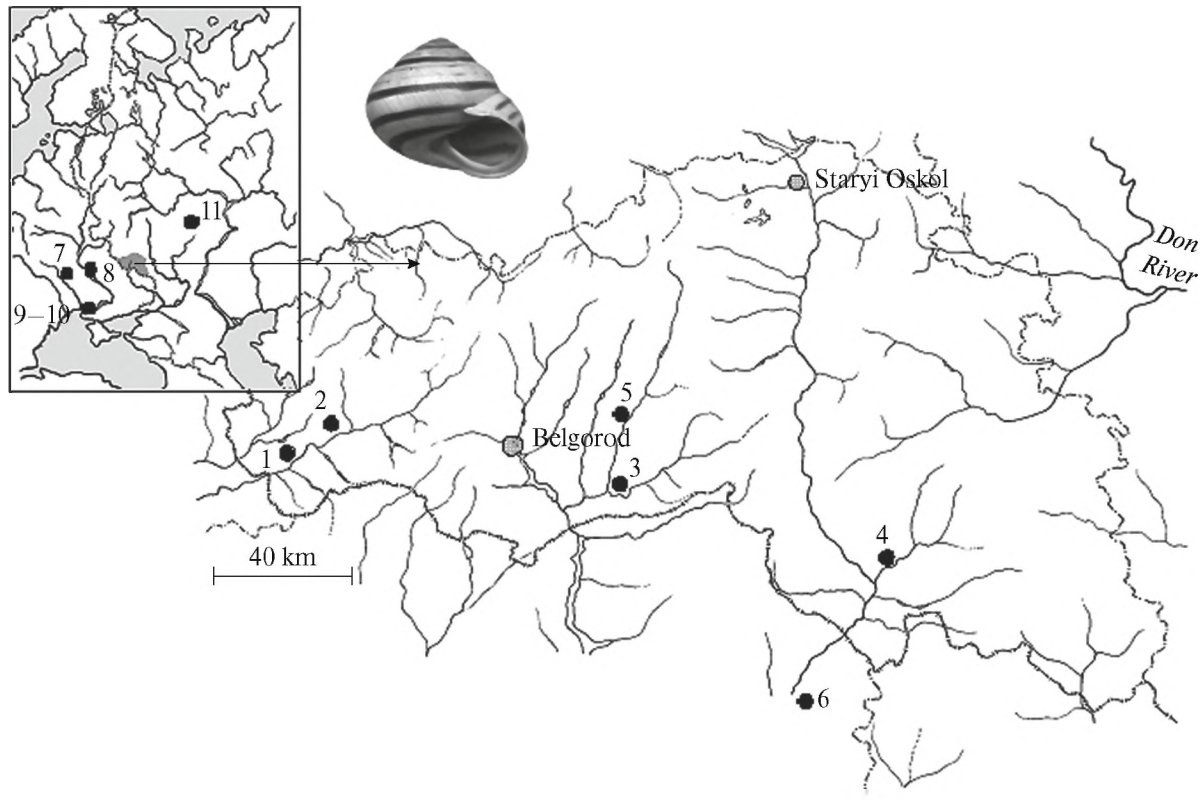
Fig. 1. Collection points of *C. vindobonensis*.

HCL buffer (pH 6.7). The electrophoresis of isozymes was conducted in a 10% polyacrylamide gel in a VE-3 chamber (Helicon, Russia); a Tris-HCl gel buffer (concentrating gel at pH 6.7 and separating gel at pH 8.9); an electrode Tris-glycine buffer (pH 8.3). To detect nonspecific esterases, the blocks were stained in a substrate mixture of Tris-HCl (pH 7.4), α-naphtyl acetate, and fast red TR. Three loci of nonspecific esterases were used as the genetic markers: *EST1*, a dimer with two alleles; *EST2*, a dimer with two alleles; and *EST8*, a monomer with three alleles [15].

We analyzed the variability of the DNA markers using Random amplified polymorphic DNA (*RAPD*) [18] and Inter simple sequence repeats (*ISSR*) [19] PCR methods. Two primers were used for the analysis: *OPA10* (5'-GTGATCGCAG-3'; *RAPD* method) and *SAS3* (5'-GAGGAGGAGGAGGC-3', *ISSR* method). Amplification was conducted in a MyCycler thermocycler (Bio-Rad, United States). The reaction was carried out in 20 μL of the mixture, which contained 20 ng of genomic DNA, a PCR buffer (10 mM of Tris HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$), 0.25 mM dNTP, 0.5 μM of the primer, and 1 unit of Taq DNA polymerase (inhibited for a hot start). The reaction was conducted under the following conditions: hot start, 2 min at 94°C, 35 cycles (denaturion, 45 s at 94°C; primer annealing, 15 s at T °C; synthesis, 1 min at 72°C), additional synthesis, 10 min at 72°C. The

annealing temperature T for the *OPA10* primer was 36°C, for *SAS3*, T was 55°C. The PCR products were separated by electrophoresis in 2% agarose gel using a TAE buffer (cooled to +4°C), 10 V/cm, 45 min. The blocks were stained with ethidium bromide.

Based on the pictures of the amplified fragments obtained by electrophoresis, we comprised the binary matrices, where the presence of a band was designated as 1 (allele p) and the absence was designated as 0 (allele q). Since nonspecific amplification products can occur in the *RAPD* method, we used clearly visible and reproducible amplicons for the analysis. The criterion of reproducibility was the repeated presence of amplicons after PCR for the same studied specimens. We isolated 17 loci using the *OPA10* primer and 18 loci using the *SAS3* primer. The resulting DNA patterns and their interpretation are given in Fig. 2.

The obtained data were processed using the GenAlExv.6.5 [20], POPGENE32 [21], and MEGA6 [22] software.

RESULTS

The first stage of our studies was to estimate the degree of genetic heterogeneity of the studied *C. vindobonensis* populations. The allele frequencies and indices of genetic variability based on the utilized allo-
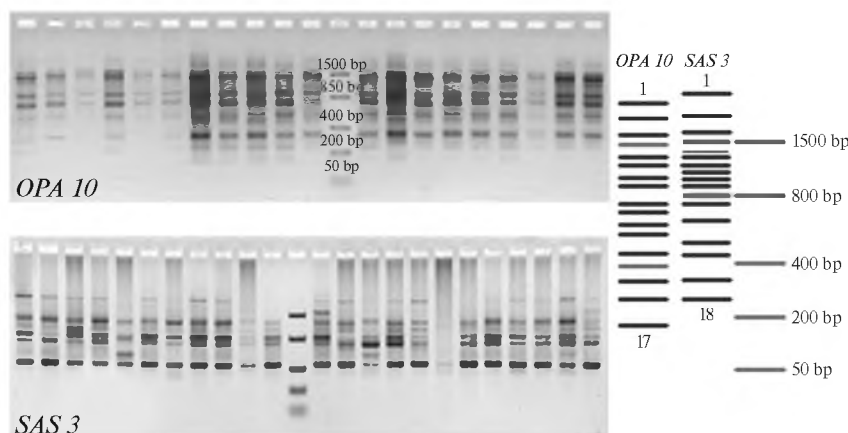
Fig. 2. RAPD and ISSR DNA patterns of *C. vindobonensis*.

zyme loci are listed in Tables 2 and 3, and on the DNA markers in Table 4.

Since we used limited datasets that included only a small portion of the population's allele pool to estimate the state of the population's gene pools of *C. vindobonensis*, the total number of multilocus genotypes (N_{MLG}) and the number of unique multilocus genotypes (N_{MLG-1}), i.e., the combinations that were only registered in a single dataset, were estimated in each group. The potential genetic diversity expected if a dataset size was increased ad infinitum (N_{max}) was subsequently calculated for each population based on the frequency distribution of the multilocus genotypes.

The analysis was performed using two nonparametric methods: Chao1-bc (bias-corrected form for Chao1) [23] and the 1st order jackknife method [24]. All the calculations were conducted in SPADE [25]. The results of the analysis of multilocus genotypes are listed in Table 5.

The next stage of our study was to assess the degree of subdivision of the *C. vindobonensis* populations in the studied territories. To describe the genetic differentiation of populations based on allozymes at different levels of the hierarchical structure, we used S. Wright's model [26] (Table 6). Furthermore, the level of dissociation of populations was calculated based on M. Nei's

Table 2. Frequencies of alleles and indicators of genetic variability of allozyme loci in populations of *Cepaea vindobonensis*

Locus	Allele	Populations										
		1	2	3	4	5	6	7	8	9	10	11
EST2	1	0.141	0.028	0.068	0.260	0.038	0.132	0.310	0.071	0.064	0.000	0.200
	2	0.859	0.972	0.932	0.740	0.962	0.868	0.690	0.929	0.936	1.000	0.800
	H_o	0.196	0.037	0.113	0.247	0.077	0.242	0.494	0.143	0.128	0.000	0.350
	H_e	0.243	0.054	0.127	0.385	0.074	0.229	0.428	0.133	0.120	0.000	0.320
	F	0.194	0.314	0.106	−0.358	0.040	−0.059	−0.154	−0.077	−0.068	−	−0.094
EST3	1	0.891	0.949	0.968	0.990	0.256	0.913	0.684	0.929	0.974	0.840	0.875
	2	0.109	0.051	0.032	0.010	0.744	0.087	0.316	0.071	0.026	0.160	0.125
	H_o	0.217	0.102	0.064	0.019	0.359	0.163	0.354	0.143	0.051	0.320	0.250
	H_e	0.194	0.097	0.062	0.019	0.381	0.159	0.433	0.133	0.050	0.269	0.219
	F	−0.122	−0.054	−0.033	−0.010	0.059	−0.029	0.181	−0.077	−0.026	−0.190	−0.143
EST8	1	0.141	0.005	0.085	0.003	0.000	0.089	0.013	0.000	0.103	0.100	0.763
	2	0.152	0.102	0.058	0.208	0.000	0.200	0.108	0.000	0.154	0.120	0.238
	3	0.707	0.894	0.857	0.789	1.000	0.711	0.880	1.000	0.744	0.780	0.000
	H_o	0.370	0.176	0.185	0.026	0.000	0.379	0.177	0.000	0.282	0.400	0.225
	H_e	0.458	0.191	0.256	0.334	0.000	0.447	0.214	0.000	0.413	0.367	0.362
	F	0.193	0.080	0.277	0.922	−	0.153	0.173	−	0.317	−0.089	0.379

Table 3. Parameters of genetic diversity in populations of *C. vindobonensis* derived from allozyme analysis

Point	N	N_e	$P\%$	A	A_e	I_{sh}	H_o	H_e	F	N_e/N
1	46	42.3	100.0	2.33 ± 0.33	1.47 ± 0.19	0.520 ± 0.145	0.261 ± 0.055	0.298 ± 0.081	0.088 ± 0.105	0.919
2	108	96.9	100.0	2.33 ± 0.33	1.13 ± 0.05	0.229 ± 0.068	0.105 ± 0.04	0.114 ± 0.041	0.114 ± 0.108	0.898
3	265	237.5	100.0	2.33 ± 0.33	1.18 ± 0.08	0.299 ± 0.109	0.121 ± 0.035	0.148 ± 0.057	0.116 ± 0.09	0.896
4	154	108.1	100.0	2.33 ± 0.33	1.38 ± 0.18	0.387 ± 0.166	0.097 ± 0.075	0.246 ± 0.114	0.424 ± 0.271	0.702
5	39	29.1	66.7	1.67 ± 0.33	1.23 ± 0.19	0.244 ± 0.169	0.145 ± 0.109	0.152 ± 0.117	0.340 ± 0.331	0.746
6	190	186.1	100.0	2.33 ± 0.33	1.43 ± 0.19	0.488 ± 0.149	0.261 ± 0.063	0.278 ± 0.087	0.021 ± 0.066	0.979
7	79	74.0	100.0	2.33 ± 0.33	1.59 ± 0.16	0.550 ± 0.071	0.342 ± 0.092	0.358 ± 0.072	0.067 ± 0.11	0.937
8	14	10.9	66.7	1.67 ± 0.33	1.10 ± 0.05	0.172 ± 0.086	0.095 ± 0.048	0.088 ± 0.044	0.282 ± 0.359	0.780
9	39	36.3	100.0	2.33 ± 0.33	1.30 ± 0.20	0.366 ± 0.191	0.154 ± 0.068	0.194 ± 0.111	0.074 ± 0.122	0.931
10	25	20.2	66.7	2.00 ± 0.58	1.32 ± 0.17	0.373 ± 0.199	0.24 ± 0.122	0.212 ± 0.11	0.24 ± 0.381	0.806
11	40	38.2	100.0	2.00 ± 0.00	1.44 ± 0.08	0.475 ± 0.051	0.275 ± 0.038	0.300 ± 0.043	0.047 ± 0.166	0.955
Mean			90.9 ± 4.7	2.15 ± 0.32	1.33 ± 0.14	0.373 ± 0.128	0.191 ± 0.068	0.217 ± 0.080	0.165 ± 0.192	0.868 ± 0.028

N, number of analyzed specimens; N_e, effective population size; $P\%$, percentage of polymorphic loci; A, average number of alleles per locus; A_e, effective number of alleles; I_{sh}, Shannon index; H_e, expected heterozygosity; H_o, observed heterozygosity.

Table 4. Parameters of genetic heterogeneity in populations of *C. vindobonensis*, averaged over set of DNA loci

Point	N	Allele frequencies		$P\%$	A	A_e	H_e	I_{sh}
		p	q					
1	30	0.351	0.649	85.7	1.86 ± 0.06	1.35 ± 0.05	0.219 ± 0.029	0.345 ± 0.04
2	19	0.278	0.722	68.6	1.69 ± 0.08	1.22 ± 0.05	0.146 ± 0.026	0.242 ± 0.038
3	120	0.340	0.660	74.3	1.74 ± 0.07	1.24 ± 0.05	0.160 ± 0.028	0.257 ± 0.041
4	64	0.330	0.670	80.0	1.80 ± 0.07	1.31 ± 0.06	0.193 ± 0.03	0.303 ± 0.043
5	39	0.336	0.664	40.0	1.58 ± 0.01	1.08 ± 0.01	0.178 ± 0.005	0.29 ± 0.007
6	40	0.319	0.681	31.4	1.47 ± 0.002	1.01 ± 0.002	0.165 ± 0.001	0.267 ± 0.001
7	53	0.337	0.663	71.4	1.71 ± 0.08	1.22 ± 0.05	0.142 ± 0.028	0.232 ± 0.04
8	24	0.398	0.602	54.3	1.54 ± 0.08	1.23 ± 0.05	0.143 ± 0.03	0.223 ± 0.044
9	34	0.363	0.637	57.1	1.57 ± 0.08	1.26 ± 0.06	0.154 ± 0.032	0.237 ± 0.046
10	25	0.178	0.822	68.6	1.69 ± 0.08	1.32 ± 0.06	0.191 ± 0.033	0.292 ± 0.047
11	40	0.387	0.613	51.4	1.51 ± 0.09	1.18 ± 0.05	0.112 ± 0.028	0.178 ± 0.041
Mean		0.329 ± 0.018	0.671 ± 0.018	62.1 ± 1.5	1.65 ± 0.06	1.22 ± 0.04	0.164 ± 0.024	0.260 ± 0.035

model [27] (Table 7), as well as using the analysis of molecular dispersion (AMOVA) [28] (Tables 8, 9). The results of the cluster analysis based on the genetic distances unweighted pair group method (UPGMA) are presented in Fig. 3, and a plot of the correlation between the level of gene flow and geographical distances between groups is shown in Fig. 4.

At the final stage, we estimated the effective number of *C. vindobonensis* populations using different models. The first method of calculation was performed based on the inbreeding coefficient: $N_e = N/(1 + F)$ [29]. In order to obtain comparable data, we calculated the ratio of the effective dataset size to its total volume (Table 3).

The second calculation method was based on the linear function coefficient between pairwise estimations of the gene flow (N_m) and geographic distance between populations (Dg): $\log N_m = a + b\log Dg$. The

effective population size (for all the studied populations in general) was calculated as $N_e = 10^a$, where a is the coefficient obtained in the equation [30]. The utilized equations are shown in Fig. 4. The obtained results are listed in Table 10.

Another method used by us to calculate the effective size of the populations is based on the model in which the values of the subdivision index of the population are considered [31]:

$$F_{st} = \frac{1 - t_k}{1 + t_k}, \quad \text{where} \quad t_k = \exp - \left\{ \left(\frac{1}{N_e} [\ln(K - 0.5) \right. \right.$$
$$+ 0.5772] + \left(\frac{1}{2N_e^2} \right) \left[1.6449 \frac{2}{2K - 1} \right]$$
$$+ \left(\frac{1}{3N_e^3} \right) \left[1.202 \frac{2}{(2K - 1)^2} \right] \right\},$$

Table 5. Allozyme genotypes and estimations of potential genetic diversity obtained by different methods for investigated populations of *C. vindobonensis*

Population	N_{MLG}	N_{MLG-1}	Method			
			Chao1-bc		1st order jackknife	
			$N_{max} \pm SE$	95% CI	$N_{max} \pm SE$	95% CI
1	16	0	22.8 ± 6.5	17.4–49.1	23.8 ± 3.9	19.1–35.8
2	9	0	10.5 ± 2.6	9.1–24.0	12.0 ± 2.4	9.7–21.1
3	17	0	20.3 ± 4.1	17.5–39.0	22 ± 3.2	18.6–32.5
4	8	1	8.5 ± 1.3	8.0–16.2	10.0 ± 2.0	8.4–18.2
5	5	0	5.2 ± 0.7	5.0–9.6	6.0 ± 1.4	5.1–12.7
6	20	1	23.3 ± 4.1	20.5–42.0	25.0 ± 3.1	21.6–35.5
7	20	5	28.9 ± 7.5	22.1–57.6	29.9 ± 4.4	24.3–42.9
8	2	0	2.0 ± 0.0	2.0–2.0	2.0 ± 0.0	2.0–2.0
9	9	0	10.5 ± 2.5	9.1–23.7	11.9 ± 2.4	9.7–20.9
10	6	0	7.0 ± 2.1	6.1–19.2	7.9 ± 1.9	6.4–15.9
11	11	4	12.0 ± 1.8	1.1–21.5	13.9 ± 2.4	11.7–23.0

Table 6. Measures of genetic differentiation and heterogeneity of population of *C. vindobonensis* averaged on set of allozyme loci

Parameters	EST2	EST3	EST8	Mean
H_e	0.192	0.183	0.277	0.217
H_o	0.184	0.186	0.202	0.191
F_{is}	0.040	−0.014	0.271	0.099
F_{it}	0.123	0.299	0.487	0.303
F_{st}	0.087	0.309	0.297	0.231
N_m	2.628	0.558	0.591	0.832
df	9	9	22	40
χ^2	38.4	6.0	218.9	263.3
P	0.000	0.700	0.000	0.000

F_{it}, inbreeding coefficient of individual relative to large population; F_{is}, inbreeding coefficient of individual relative to subpopulation; F_{st}, inbreeding coefficient of subpopulation relative to large population; P, probability of significance of χ^2 criterion; df, degrees of freedom.

where K is the number of populations used. Due to the fact that, in order to determine the degree of population subdivision in the present study, we used two integral and interchangable indices G_{st} and Φ_{st}, together with the F_{st} index, we found it possible to modify the indicated formula, introducing the values of these indices into it alternately. The results are given in Table 11.

DISCUSSION

According to the data obtained on allozymes (Table 2), a significant deficiency of the heterozygotes is observed in 30.3% of the cases[2], in the remaining

[2] Significance of heterozygote deficiency was estimated according to the formula $\chi^2 = F^2 N(k − 1)$, $df = k − 1$, where F is the inbreeding coefficient, N is the dataset size, and k is the number of alleles for the given locus [29].

versions, significant differences between the observed and theoretical heterozygosity were not found. The data given in Fig. 5, where an insignificant trend towards the scarcity of heterozygotes is present, indicate the same; at the same time, the regression coefficient was 0.958 ± 0.195 ($p = 0.05$).

According to the data listed in Table 3, the smallest level of variability and an increased value of the inbreeding coefficient based on the isozyme loci among the six studied populations of the Southern Mid-Russian Upland (points 1–6) are noted in the groups from the points Valuiki (no. 4), Rzhevka (no. 5), and Khotmyzhsk (no. 2). Beyond the borders of the Mid-Russian Upland, the group from Khortitsa Island (no. 8) proved to be the most monomorphic. In the latter case, the low indices of genetic variability are possible due to the isolation and small population of the island group. Natural populations from the points

Table 7. Parameters of genetic differentiation of populations of *C. vindobonensis* based on DNA loci

Locus	No	H_t	H_s	G_{st}
OPA10	1	0.163	0.135	0.172
	2	0.167	0.147	0.118
	3	0.414	0.247	0.405
	4	0.123	0.091	0.258
	5	0.497	0.348	0.300
	6	0.157	0.141	0.106
	7	0.221	0.175	0.207
	8	0.100	0.061	0.395
	9	0.081	0.064	0.210
	10	0.197	0.166	0.158
	11	0.387	0.149	0.616
	12	0.232	0.152	0.345
	13	0.037	0.036	0.028
	14	0.043	0.037	0.149
	15	0.274	0.140	0.489
	16	0.424	0.210	0.505
	17	0.021	0.021	0.035
SAS3	1	0.500	0.376	0.248
	2	0.045	0.042	0.053
	3	0.212	0.193	0.087
	4	0.216	0.203	0.061
	5	0.054	0.052	0.043
	6	0.362	0.292	0.193
	7	0.024	0.023	0.034
	8	0.494	0.363	0.266
	9	0.015	0.014	0.016
	10	0.334	0.269	0.196
	11	0.031	0.030	0.031
	12	0.405	0.279	0.310
	13	0.054	0.048	0.100
	14	0.120	0.115	0.043
	15	0.371	0.334	0.100
	16	0.202	0.165	0.181
	17	0.028	0.028	0.022
	18	0.006	0.006	0.031
Mean		0.200 ± 0.028	0.147 ± 0.020	0.265

G_{st}, portion of interpopulation genetic diversity in total diversity; H_t, expected portion of heterozygotic genotypes in whole population; H_s, average value of intrapopulation diversity for all subpopulations.

Golovchino (no. 1) and Kupyansk (no. 6) are the most polymorphic in the eastern part of the distribution range, and in the western part, the population from the point Zhitomir (no. 7). Fairly high indices of genetic variability also characterize the adventive colony from the city of Penza (no. 11). The analysis of the multilocus variability of allozymes in many respects confirmed these conclusions (Table 5). The greatest observed and potential genetic diversity is noted at points 1, 3, 6, and 7. The greatest number of unique

Table 8. Result of AMOVA of populations of *C. vindobonensis*

Markers	Variability	df	SS	MS	V	%	Φ_{st}	P	N_m
Allozymes	Between populations	10	241.052	24.105	0.277	26	0.263	0.010	0.676
	Within populations	988	767.090	0.776	0.776	74			
	Total	998	1008.142	24.882	1.053	—			
DNA	Between populations	10	401.573	40.157	0.882	26	0.261	0.010	0.630
	Within populations	477	1187.796	2.490	2.490	74			
	Total	487	1589.369	42.647	3.372	—			

Table 9. Pairwise genetic differentiation (Φ_{st}) between populations of *C. vindobonensis* based on allozyme loci and DNA markers

Allozymes

Point	1	2	3	4	5	6	7	8	9	10	11
1		0.010	0.010	0.030	0.010	0.410	0.010	0.010	0.260	0.090	0.010
2	0.124		0.020	0.010	0.010	0.010	0.010	0.170	0.010	0.010	0.010
3	0.076	0.021		0.010	0.010	0.010	0.010	0.110	0.020	0.010	0.010
4	0.050	0.129	0.119		0.010	0.010	0.010	0.030	0.010	0.010	0.010
5	0.546	0.708	0.665	0.542		0.010	0.010	0.010	0.010	0.010	0.010
6	0.000	0.090	0.072	0.049	0.549		0.010	0.010	0.260	0.040	0.010
7	0.136	0.296	0.297	0.146	0.360	0.175		0.030	0.010	0.010	0.010
8	0.106	0.032	0.028	0.099	0.673	0.112	0.177		0.030	0.010	0.010
9	0.008	0.062	0.025	0.063	0.649	0.008	0.230	0.110		0.040	0.010
10	0.030	0.084	0.070	0.134	0.587	0.042	0.188	0.135	0.036		0.010
11	0.463	0.729	0.683	0.513	0.765	0.513	0.567	0.667	0.544	0.571	

DNA

Point	1	2	3	4	5	6	7	8	9	10	11
1		0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010
2	0.174		0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010
3	0.165	0.283		0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010
4	0.071	0.165	0.087		0.010	0.010	0.010	0.010	0.010	0.010	0.010
5	0.172	0.215	0.164	0.150		0.010	0.010	0.010	0.010	0.010	0.010
6	0.410	0.527	0.412	0.399	0.489		0.010	0.010	0.010	0.010	0.010
7	0.082	0.117	0.179	0.103	0.085	0.437		0.010	0.010	0.010	0.010
8	0.135	0.305	0.337	0.222	0.378	0.602	0.169		0.010	0.010	0.010
9	0.183	0.310	0.164	0.107	0.301	0.522	0.156	0.252		0.010	0.010
10	0.338	0.247	0.402	0.321	0.438	0.427	0.368	0.482	0.436		0.010
11	0.215	0.392	0.184	0.176	0.347	0.588	0.204	0.353	0.161	0.521	

Estimations of Φ_{st} are listed under diagonal; level of their significance (based on 99 permutations) are listed above diagonal.

genotypes (N_{MLG-1}) is also noted at points 7 and 11. The group from Khortitsa Island (no. 8) proved to be the most uniform.

Judging by the indices of genetic heterogeneity averaged based on the combination of DNA loci (Table 5), the populations Rzhevka (no. 5) and Kupyansk (no. 6) were the most monomorphic. This result is natural for the fifth group, since a reduction in the allelic diversity here was also observed based for the isozyme markers[3]. However, the sixth group dif-

[3] This phenomenon is possibly determined by the population waves due to the burning of dry vegetation during the spring period observed at this point; as a result, individuals in their active phase die.

(a) (b)
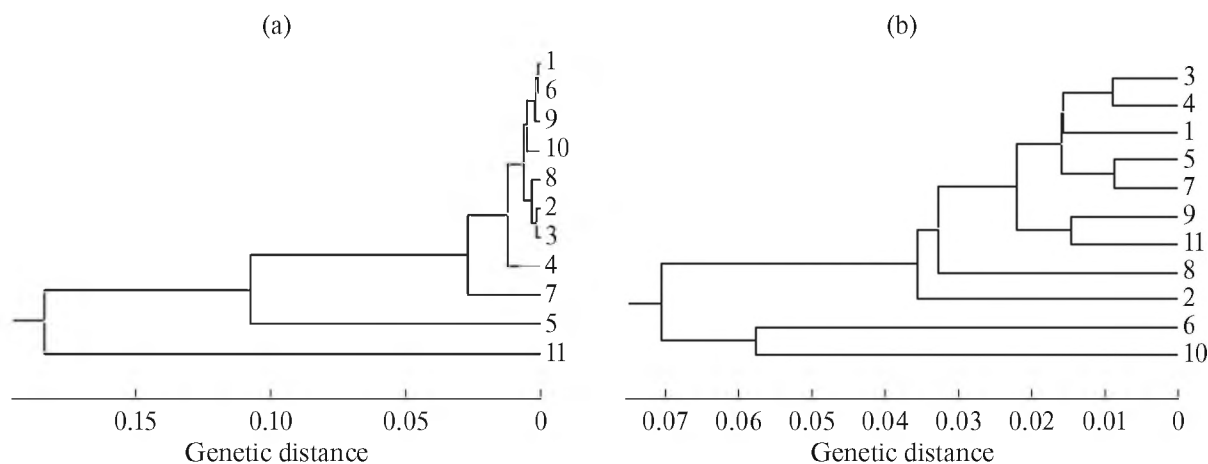
Fig. 3. Dendrogram of genetic distances by Nei [33] (UPGMA) between populations of *C. vindobonensis* based on allozyme loci (a) and DNA markers (b).
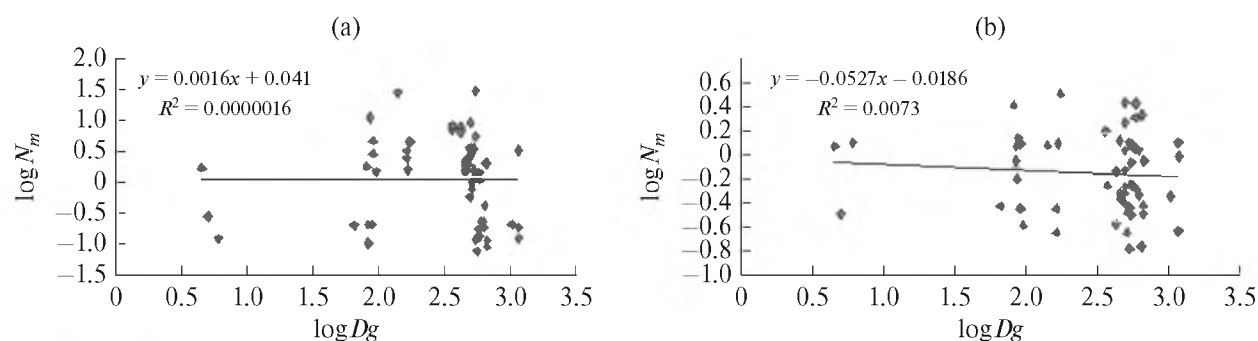
Fig. 4. Linear regression logarithm of gene flow N_m between population pairs of *C. vindobonensis* on logarithm geographic distance Dg between them ((a) for allozymes; (b) for DNA markers).

fered in terms of the increased variability based on allozymes. From the populations outside the borders of the Mid-Russian Upland, the adventive population from the city of Penza (no. 11) proved to be the most monomorphic. The obtained result appears somewhat unexpected, since this group is separated from the rest by the larger heterogeneity based on the allozymes. Furthermore, according to the data obtained earlier, this colony differed by the high diversity of the color variants of the shell [15]. In this respect, we assume that this case once again demonstrates the difference in genetic processes which take place in the coding part of the genome subjected to natural selection and which comply with the genetically automatic laws in the remaining part of the "silent" DNA. Earlier we already attempted to explain the significant morphological variability of this colony by the special microclimatic conditions of the urban environment, as well as by the competitive vacuum and the absence of predators [15]. In the line of this reasoning, an increased monomorphism revealed based on the DNA markers is a possible result of the genetic drift caused by the "founder effect."

Regarding individual DNA loci, loci 3, 5, and 16 were the most polymorphous among the *RAPD* markers, and loci 1, 8, and 15 were more variable among the *ISSR* markers (Table 7). Loci 8, 9, 13, 14, and 17 were included in the group of more monomorphic loci based on the *OPA10* primer, and loci 2, 5, 7, 9, 11, 13, 17, and 18 were included based on the *SAS3* primer. It is interesting to note that on average the heterozygosity of the *RAPD* loci ($H_t = 0.208 \pm 0.035$, $H_s = 0.136 \pm 0.020$) does not significantly differ from the heterozygosity of the *ISSR* loci ($H_t = 0.192 \pm 0.041$, $H_s = 0.157 \pm 0.031$).

In general, it is interesting to note that the level of variability of the *C. vindobonensis* populations in the Southern Mid-Russian Upland is not inferior, and in certain cases exceeds the analogous level of variability in the western groups, which we assume is a positive signal as an estimation of the viability of the species' populations.

Estimation of the degree of differentiation of populations based on the allozyme loci with the use of *F*-statistics of S. Wright on the average demonstrated a sufficiently large dissociation of the studied groups of

Table 10. Values of effective size calculated based on linear function coefficients between pairwise estimates of gene flow (N_m) and geographic distances between populations of different species of terrestrial mollusks

Species	Isozymes		DNA	
	N_e	95%, Δ	N_e	95%, Δ
Helix pomatia	9.8	3.9–25.1	3.1	2.0–4.9
Bradybaena fruticum	4.1	2.2–7.6	1.7	1.2–2.5
Chondrula tridens	3.8	2.6–5.7	1.2	0.8–1.8
Helicopsis striata	5.1	2.6–10.0	1.9	1.3–2.7
Cepaea vindobonensis	1.1	0.14–8.5	0.96	0.36–2.6

Data on *Br. fruticum*, *Ch. tridens*, *H. striata*, and *H. pomatia* were obtained from our previous publications [38–44]. Same for Table 11.

Table 11. Values of effective size of populations of different species, calculated based on subdivision index (F_{st}, G_{st}, and Φ_{st})

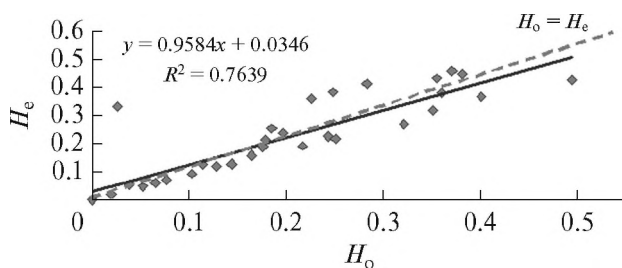| Index	*Br. fruticum* ($K = 35$)	*Ch. tridens* ($K = 25$)	*H. striata* ($K = 19$)	*H. pomatia* ($K = 12$)	*C. vindobonensis* ($K = 11$)
N_e (F_{st} based on allozymes)	9.2	10.5	4.9	5.3	6.0
N_e (Φ_{st} based on allozymes)	7.0	6.2	3.5	3.1	6.1
N_e (G_{st} based on DNA)	7.3	10.3	4.9	5.9	6.6
N_e (Φ_{st} based on DNA)	6.2	9.9	4.4	3.8	6.3
Mean	7.4 ± 0.6	9.2 ± 1.0	4.4 ± 0.3	4.5 ± 0.6	6.3 ± 0.1

snails (Table 6). We observe a similar picture while comparing the data obtained based on the analysis of the molecular variance (AMOVA, Table 8). In both cases, the index of the intensity of the exchange of genes between populations (N_m) proved to be less than one, which, according to the "shifting balance theory," disrupts panmixia between the populations [31]. The relatively high values of the inbreeding coefficient F_{it} also indicate the same. Note that the *EST3* locus makes the greatest contribution to the interpopulation diversity, evaluated based on the inbreeding coefficient F_{st} (Table 6). However, the heterogeneity test using the χ^2 criterion did not reveal a significant separation of the groups based on this locus ($p = 0.7$).

Based on the utilized DNA markers, the greatest fragmentation is noted for loci nos. 11, 15, and 16 (*OPA10*), and nos. 1, 8, 10, and 12 (*SAS3*) (Table 7). It

Fig. 5. Ratio of expected (H_e) and observed heterozygosity (H_o) in populations of *C. vindobonensis*.

is known that the average G_{st} values correspond to the level of genetic differentiation with the neutral selective process. In that case, loci with large G_{st} values can most probably experience the action of disruptive selection, and loci with low values of the subdivision index are subjected to the influence of stabilizing selection [32]. The differences based on the *RAPD* spectra ($G_{st} = 0.264$) proved to be higher than those for the *ISSR* spectra ($G_{st} = 0.112$).

The analysis of molecular dispersion both for allozymes and DNA markers demonstrated a similar picture. In either case, the intrapopulation variability contributed to 26%, and the Φ_{st} subdivision indices of the populations insignificantly differed from the F_{st} and G_{st} indices. Accordingly, the level of the gene flow (N_m) was 0.630–0.676 individuals per generation (Table 8).

The results of the cluster analysis demonstrated an explicit difference in the divergence of groups into clusters between the dendrograms reconstructed based on the allozyme and DNA markers (Fig. 3). At the same time, in both cases the geographical location of the populations does not have any effect on the similarity in the ratio of allele frequencies and their combinations. For example, the adjacent populations that inhabit the valley of Vorskla River (points 1, 2), despite close three-dimensional arrangement, proved to be, according to the diagrams, in different clusters. The differentiation level between them was $\Phi_{st} = 0.124$ based on allozymes, and $F_{st} = 0.174$ ($p = 0.01$) based on

DNA markers (Table 9). It is interesting to note that anthropogenic pressure could contribute to this originality. The Khotmyzhsk group (no. 2) inhabits the floodland sections plowed previously, which were neglected by man for more than 30 years. The development of this territory probably occurred from the preserved natural islets of the floodland vegetation, in which small groups of snails remained. The group experienced the so-called bottleneck effect and possibly the genetic revolution effect [34] and became more monomorphic than the Golovchino population (no. 1), which inhabited a natural forest biotope. This possibly indicates the tendency of the species towards the formation of a metapopulation structure under the conditions of the urbanized territory of the Southern Mid-Russian Upland [35].

The most original group regarding the genetic combinations of the isozyme markers is the colony from the city of Penza (no. 11). Specifically, a high frequency of the *EST8–1* allele (0.763) is found here. Its portion does not exceed 0.141 in all other populations. This phenomenon can be considered either a consequence of the genetic drift because of the founder principle or a result of natural selection in the urban environment.

The Kupyansk (no. 6) and STP groups from the city of Nikolayev (no. 10) are the most distanced based on the DNA markers. Moreover, the latter population differs significantly not only from the remote groups but also from the adjacent group, which inhabits the same city (no. 9). The level of differentiation between them reached $\Phi_{st} = 0.436$ ($p = 0.01$) (Table 9), which indicates a significant isolation of the snail colonies in an urbanized environment. It should be noted that the data obtained are partially inconsistent with the assumption of S.S. Kramarenko [14], according to which the studied group of *C. vindobonensis* in urban conditions exists as many discrete semi-isolated colonies that meet Wright's island model.

The cluster analysis data are confirmed by the graphs and equations of linear regression (Fig. 4), which demonstrate the absence of a correlation between the geographical distances and pairwise estimations of the gene flow both according to the allozymes (Mantel test $R_M = 0.001$, $p = 0.518$, 9999 permutations) and according to the DNA markers ($R_M = -0.085$, $p = 0.315$, 9999 permutations). All this speaks about the disturbance of the migratory channels between the studied groups of *C. vindobonensis* and their prolonged isolation from each other.

The calculation of the effective size of *C. vindobonensis* populations using the inbreeding coefficient demonstrated that the ratio of the effective size to the total size of the population (N_e/N) for the species in general is 0.868 ± 0.028 (Table 3), which fits within the overall range of the share of N_e proposed by Crow et al. [36, 37][4]. Furthermore, the average value of the

portion of N_e obtained for *C. vindobonensis* significantly exceeds the analogous values obtained by us earlier for the aboriginal background species of terrestrial mollusks that inhabit the studied region: *Br. fruticum*: 0.800 ± 0.021 and *Ch. tridens*: 0.661 ± 0.013 [38, 39], as well as for the relict mollusk *Helicopsis striata*: 0.720 ± 0.080 [39, 40]. The portion of the effective population size of *C. vindobonensis* proved comparable to the Roman snail *Helix pomatia* inhabiting this location that has a high life potential: 0.870 ± 0.043 [41]. These data indirectly indicate a high level of viability for the studied populations of *C. vindobonensis*.

Comparison of the obtained estimations of the effective size of the *C. vindobonensis* populations, calculated based on the equation of linear regression with the analogous data on other species of mollusks demonstrates an explicit decrease of this index for the groups of this land snail (Table 10), although the calculated confidence intervals show that significant differences are absent.

We obtained a somewhat different result during the calculation of the effective population size while taking into account the level of the subdivision of the populations (Table 11). According to the obtained data, *Ch. tridens* and *Br. fruticum* have the largest effective population size. In the *C. vindobonensis* populations, the effective size was lower than in these two background species, and higher than in the *H. striata* and *H. pomatia*, which are considered vulnerable mollusks.

CONCLUSIONS

Thus, based on the given data it can be established in general that from the point of view of genetic parameters in comparison with, for example, the vulnerable species *H. striata*, which often inhabits the same communities as *C. vindobonensis*, the studied populations of the latter have higher life opportunities. This is determined partially by the fact that *C. vindobonensis* is an euritopic species. Populations of these snails can inhabit open steppificated slopes, forest communities, and sections adjacent to floodlands due to their polymorphism. The cases of the successful development of gardens and orchards by the mollusks were noted. Nevertheless, reduction in the allelic diversity, an increase in the inbreeding coefficient in a number of groups, and the high level of isolation of *C. vindobonensis* populations in the northeastern part of the distribution range raises concerns and requires efforts to be made to preserve this species.

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[4] The authors determined that for the majority of the organisms the portion of N_e is 0.75 on average.

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