

Genetic and Morphological Heterogeneity of Lake Baikal Endemic Gastropod *Benedictia fragilis* (Dybowski, 1875)

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Abstract—Baikal endemic *Benedictia fragilis* gastropods distributed in a wide range of depths (from sublittoral to abyssal) of three lake basins are studied. The analysis of the nucleotide sequence of the *COI* mitochondrial gene fragment and internal transcribed nuclear DNA spacer (ITS1) demonstrates that the studied gastropods are represented in Lake Baikal by three genetic groups. The results of the studies on genetic diversity, phenotypic traits, and distribution allow us to assume that the detected groups are incipient allopatric (geographical) species. On the basis of the data obtained and geological and climatic history of Baikal, possible pathways of the *B. fragilis* resettlement in the lake and the emergence of three genetic groups are hypothesized.

Keywords: *Benedictia fragilis*, *COI*, ITS1, genetic polymorphism, shell morphology, phenotypic variability

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INTRODUCTION

Like all ancient lakes in the world, Baikal has a unique endemic fauna represented by series of “flocks of closely related species” [1, 2]; their generation is usually explained by adaptations to different ecological conditions inside the lake ecosystem [3–8]. The geographical diversification in ancient lakes is usually associated with the history of the lake formation (the existence of fragmented reservoirs and their subsequent unification into a single lake) and the presence of natural barriers in the shallow zone generated by the coastline irregularity [1]. Lake Baikal is a unique lake among ancient lakes in the world in which the fauna (including gastropods) inhabiting all depth zones up to the maximum (>1600 m) exists. To date, there is no molecular genetic evidence about possible ways of animal diversification by geographical gradient in the deep lake zone.

Baikal has a long history of the development covering the period of 25–30 million years from the emergence and unification at first of the southern and middle basins, formation of the lake with depths of a few hundred meters, then attachment of the northern valley (0.8–0.5 million years ago), and until final formation (500000–150000 years ago) of a single ultradeep reservoir with depths more than 1 km [9–11]. Global climate changes from subtropical to sharply continental [9] and alternation of cold and warm periods, including during the last 1.5–1.8 million years, promoted the speciation in Baikal [12, 13].

The fauna of gastropods in Lake Baikal includes approximately 150 species, 78% of which are endemics; among them, allopatric species and subspecies with geographical confinement are mainly known in the shallow zone [14]. In addition, it was detected that some gastropod species that spread at the depths from 10 to 40 m along the entire coastline are represented by populations that occupy different geographical areas [15].

The Baikal eurybathic gastropods (approximately 10% of endemic species) are able to inhabit a wide range of depths (from 20–30 to 800–1300 m) at almost constantly low water temperature of approximately 4°C [14]. The rises of the bottom between Baikal basins (Buguldeika–Selenga Isthmus and Academic Ridge) have depths more than 100 m and are not natural barriers for fauna resettlement.

Among eurybathic species, three species, *Benedictia fragilis* Dybowski, 1875, *B. distinguenda* Lindholm, 1909, and *B. pulchella* Sitnikova, 1983, whose taxonomic independence was questioned and united in the *B. fragilis* group owing to morphological similarity, occupy a special place [14]. The snails from this group are characterized (as compared with remaining Baikal gastropods) by a “giant” shell (reaching a height of 50 mm) and a small cap (4–5 times smaller than the aperture size). A light shell (less than 0.2 mm in thickness and constituting approximately 22% of total mollusk weight) and a large foot promote the movement of these snails along the oozy grounds without sinking in viscous precipitate. Dwelling in “herds,” the snails attach the egg capsules to the shells of neighboring

individuals and thus compensate a need for a solid substrate. Being omnivorous, “giant” benedictiids mainly feed on the plant and animal detritus and inhabit the bottom regions with a high trophicity [16]. The habitat of “giant” benedictiids covers all of the Baikal water area with depths from 30 m to 1300 m. No strict confinement of the snails of these taxa to the depth zone was detected; no biotopic and trophic preferences for each of them were found either during separate and joint habitation. Thus, the Baikal *B. fragilis* gastropods are a good object for the study of bathymetric and geographical diversification in the deep zone within a single freshwater continental reservoir.

The use of various genetic markers is an effective tool to detect genetic heterogeneity within different animal groups, including gastropods. In multicellular organisms (including mollusks), the interspecies divergence by the *COI* is usually at least 2%, while intraspecies divergence is no more than 1% of nucleotide substitutions [17–19]. The genetic markers are also used for identification of biogeographical associations and pathways of allopatric speciation, for example, in marine gastropods [20]. In addition, a large amount of attention has been paid in recent years to incongruence between morphological and genetic traits, as well as between different genetic markers (in particular, in the freshwater Pleuroceridae gastropods [21] and Baikal endemic mollusks of the Baicaliidae family [15, 22]).

The present study is devoted to the problem of allopatric speciation by example of Baikal gastropods. The following tasks were set: (1) to detect whether genetic diversification by depths and geographical regions exists in the *B. fragilis* gastropods within middle and northern basins of Lake Baikal; (2) to see does whether congruence exists between morphological and genetic variability, as well as mitochondrial and nuclear markers; (3) on the basis of available evidence on geological and climatic changes in the history of Baikal, to suggest a hypothesis about possible pathways of the resettlement of the studied gastropods.

MATERIALS AND METHODS

The benedictiids individuals (92 specimens) were collected by a trawl in different regions of Lake Baikal. Information about places, depths, collection dates, and amount of analyzed material is presented in Fig. 1a.

Genetic polymorphism was studied using sequences of the mitochondrial DNA cytochrome *c* oxidase subunit I (*COI*) gene and internal transcribed nuclear DNA spacer (ITS1). Total DNA was isolated from the mollusk foot tissues by phenol and chloroform extraction [23]. The *COI* gene fragment was amplified with universal primers: L1490 (5'-ggtcaacaatcataaagatattgg-3') and H2198 (5'-taaacttcagggtgac-caaaaataca-3') [24]. The ITS1 fragment amplification was conducted using KP-2 (5'-aaaaagcttcctaggt-

gaacctgcg-3') [25] and 5.8S (5'-agcttggtgcgtttctcatcga-3') [26] primers. Sequencing was carried out using the automatic sequencer 3500xL (Applied Biosystems, United States). The preliminary analysis of nucleotide sequences and calculation of haplotype diversity (*Hd*) indices and levels of nucleotide variability (π) were performed using the DnaSP v. 4.10.3 [27]. Indices of neutrality of nucleotide substitutions (Tajima's *D* [28] and Fu's *Fs* [29]) were calculated in the ARLEQUIN v. 3 [30]. The construction of median haplotype network was made with the NETWORK v. 4.6.1.3 (<http://fluxus-engineering.com/>) using the median-joining algorithm [31]. Genetic distances for the *COI* were estimated using the MEGA v. 6.06 [32] with the Tamura 3-parameter model and gamma-distribution (T92 + G). The evolutionary model of nucleotide substitutions was selected in the jModelTest v. 2.1.6 [33, 34]. The calculation of the divergence time was conducted on the basis of genetic distances taking into account intrapopulation genetic polymorphism (net average distances, MEGA4) and using the mutation rate of the *COI* suggested for gastropods and being $1.83\% \pm 0.21$ substitutions per sequence per million years [35].

Species identification of snails was carried out by a comparative method [36], since isolation of *B. pulchella* and *B. distinguenda* was made on the basis of this method. It should be noted that *B. pulchella* was isolated earlier from the typical *B. fragilis* series, while *B. distinguenda* was initially considered as its subspecies [14]. The essence of the method is as follows: the outline of the spire of holotype or lectotype was drawn by means of a drawing apparatus like a camera Lucida, which is adapted to the stereomicroscope MBS-9 and DRS-Opton so that the shell axis lies exactly in the image plane. Subsequently, this contour (or template) was used for the comparison with other shells (independently of their size). The compared shell was established under the same magnification under which the template was drawn, and turning it along the axis, one of its turnovers was combined with one of the template turnovers. If all other shell spire turnovers coincided, it was concluded that the shells belong to one species. If there was no coincidence, the template of another species type was used. The level of differences in the species ratio among genetic groups was determined by means of Pearson's χ^2 test (STATISTICA-10).

Morphological analysis of mollusks was performed on the basis of evaluation of the shell background coloration and analysis of its morphometric traits. The evaluation of the shell coloration was conducted in all individuals mainly using fresh material. The morphometric analysis was performed only on mature specimens. For this, mollusks were photographed and then measurements of 14 linear parameters of the shell were conducted using photos by means of the Image-Pro Plus program (Fig. 3). To determine the level of morphological differences, the nonparametric Kruskal–Wallis criterion was used for small samples with non

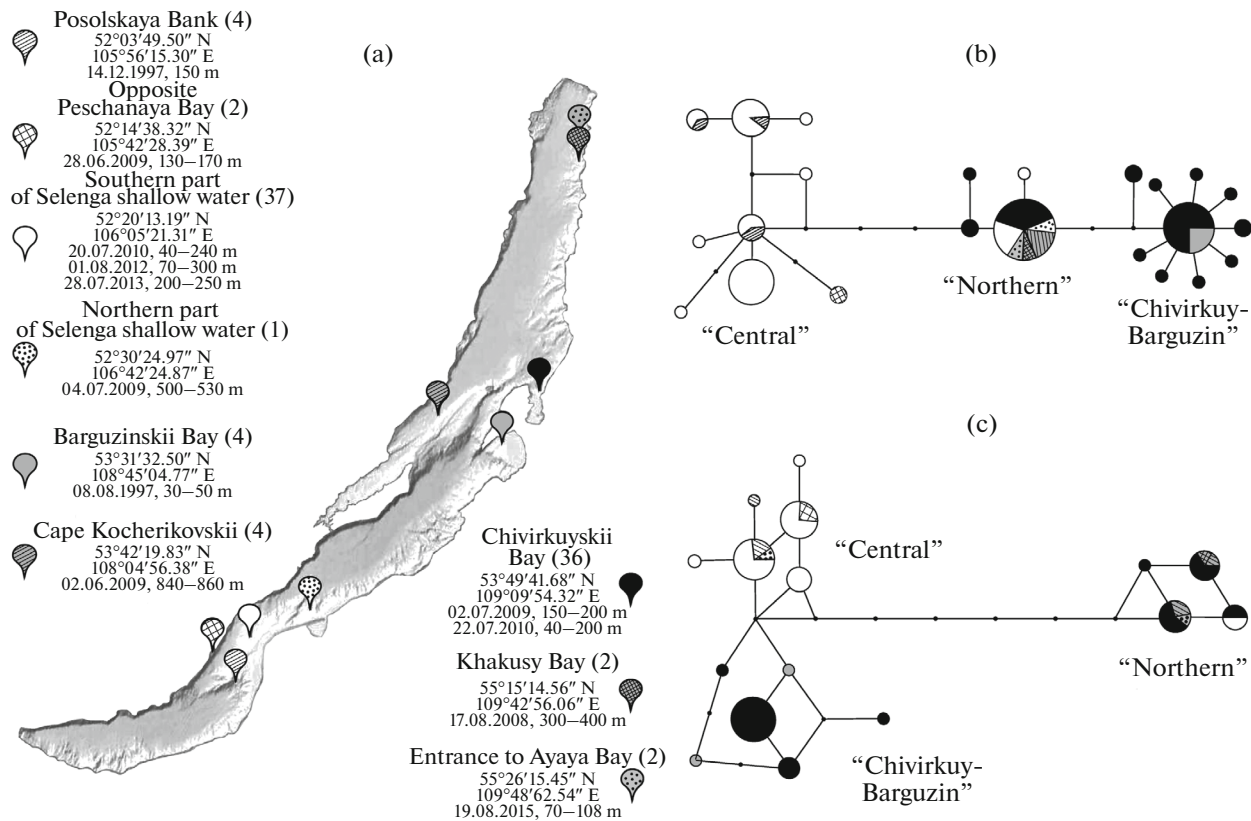


Fig. 1. (a) Location of material collection regions. The number of analyzed individuals is given in brackets; median network of haplotypes constructed on the basis of nucleotide sequences: (b) *COI*; (c) *ITS1*. See text.

normal distribution according to the Shapiro–Wilk test (STATISTICA-10). Medians were used as an estimate of the values of parameters.

RESULTS

Population Polymorphism by Mitochondrial Marker

A 622-bp fragment of the *COI* gene was sequenced from 92 individuals. Sequences of unique haplotypes were placed in GenBank under the accession numbers KX241824–KX241846. The analysis of obtained sequences detected 26 variable positions, out of which 14 were phylogenetically informative. Only one substitution in a single individual was nonsynonymous. The median network represented by 23 haplotypes (Fig. 1b) demonstrated the presence of three main haplogroups. The first group ("Central") was generated by nine haplotypes and 37 individuals, collected near the Selenga Isthmus, Peschanaya Bay, and Posolskaya Bank. The second group ("Northern") consisted of four haplotypes and united four individuals from the south of the Selenga Isthmus and one from northern part of this isthmus, as well as 22 individuals from northern regions of the lake (Chivirkuyskii Bay, Cape Kocherikovskii, Khakusy Bay, Ayaya Bay). The third group ("Chivirkuy-Barguzin") of ten haplotypes was

generated by 28 individuals from Chivirkuyskii and Barguzinskii bays. Two fixed substitutions were detected between the groups "Chivirkuy-Barguzin"/"Northern"; four substitutions, between "Central"/"Northern" and "Chivirkuy-Barguzin"/"Central." The estimated time that elapsed from the beginning of the divergence of the "Central"/"Northern" groups was 392000–494000 years; "Northern"/"Chivirkuy-Barguzin," 245000–309000 years; and "Central"/"Chivirkuy-Barguzin," 490000–617000 years.

The comparative analysis of detected *B. fragilis* genetic groups demonstrated that the genetic diversity of the group "Central" was the highest ($Hd = 0.788$ and $\pi = 0.00335$). The haplotype diversity in the group "Chivirkuy-Barguzin" was somewhat lower ($Hd = 0.635$), while the nucleotide diversity was more than 2 times lower ($\pi = 0.00147$). A high haplotype diversity in combination with low nucleotide diversity serves as indicator of rapid growth of the "Chivirkuy-Barguzin" group. The "Northern" group, which covers a large water area of Baikal, was the least diverse genetically. The indices of haplotype diversity ($Hd = 0.297$) and levels of nucleotide variability ($\pi = 0.00061$) in the "Northern" group were several times lower than in "Central" and "Chivirkuy-Barguzin" groups.

Genetic differences within the “Central” group were 0.3%; within “Northern” and “Chivirkuy-Barguzin” groups, 0.1%. The following values of genetic distances were detected between haplogroups: “Northern”/“Chivirkuy-Barguzin,” 0.6%; “Central”/“Northern,” 1%; and “Central”/“Chivirkuy-Barguzin,” 1.3%. The intraspecific divergence by the *COI* within all group of “giant” benedictiids was 0.7%.

The neutrality indices of nucleotide substitutions (Tajima’s *D* and Fu’s *F_s*) for all haplogroups had negative values, but they were statistically significant ($p < 0.05$) only for the “Chivirkuy-Barguzin” group.

Population Polymorphism by Nuclear Marker

Nuclear DNA ITS1 fragment (450–470 bp in length) were sequenced for 65 individuals. The ITS1 fragments contain insertions and deletions; this significantly complicates the analysis of sequences. Therefore, all insertions were reduced to one nucleotide for the median tree construction; this made it possible to take into account the insertion/deletion as a single mutation event. The median ITS1 network (Fig. 1c), consisting of 16 haplotypes, was represented by the same three groups (“Central,” “Northern,” and “Chivirkuy-Barguzin”) as the network of *COI* haplotypes. However, as opposed to the mtDNA network, five individuals from the “Northern” group in ITS1 network were located in the “Central” group. Seven fixed mutations (three substitutions and four insertions/deletions) were detected between the groups “Chivirkuy-Barguzin”/“Northern” and “Central”/“Northern.” There were no fixed mutations between the groups “Chivirkuy-Barguzin”/“Central.”

Nuclear DNA of the studied mollusks carried the traces of genetic information exchange between the groups “Chivirkuy-Barguzin” and “Central.” In the second group, 24.2% heterozygous individuals were detected in one site. For this site, one part (44.8%) of homozygous individuals had the nucleotide typical only of the group “Central,” while another part (31%) had the nucleotide typical of the “Chivirkuy-Barguzin” group. The presence of heterozygotes indicates the gene flow from this group to the “Central” group. No signs of the gene flow were found in the “Northern” group.

The genetic distance between the groups “Central” and “Chivirkuy-Barguzin” by ITS1 was lower than by the *COI* and was 0.3%. The genetic distances between pairs “Northern”/“Chivirkuy-Barguzin” and “Central”/“Northern” were close (0.8 and 0.6%, respectively).

Genetic Homogeneity by Habitat Depths

No genetic differentiation by bathymetric zones was detected in the studied gastropods collected in the range of depths from sublittoral (40 m) to abyssal (860 m) of the lake (Fig. 1a). According to both genetic mark-

ers, individuals inhabiting different depth zones were presented in each of three groups. Thus, the group “Central” was represented by individuals from depths from 40 to 530 m; “Northern,” from 40 to 860 m; “Chivirkuy-Barguzin,” from 30 to 200 m. In addition, it was revealed that no bathymetric isolation between the groups “Northern” and “Chivirkuy-Barguzin” exists in Chivirkuyskii Bay: individuals of both groups were found at depths of 40–60 m.

Ratio of *B. fragilis*, *B. distinguenda*, and *B. pulchella* Taxa in Genetic Groups

Out of 90 individuals analyzed by a comparative method, only two immature specimens from the group “Central” were attributed to *B. fragilis* (Fig. 2). The *B. distinguenda* and *B. pulchella* members were present in all identified genetic groups, but their ratio within the groups were significantly different (Pearson’s χ^2 , $P < 0.05$). The amount of *B. distinguenda* was significantly larger (96%) in the group “Chivirkuy-Barguzin” ($P \leq 0.027$), while *B. pulchella* prevailed (76%) in the group “Central” ($P \leq 0.0002$). In the group “Northern,” the ratio between the *B. pulchella* and *B. distinguenda* was 1 : 2. Two mature individuals from the northern region of the Selenga water area combined signs of the spire of two taxa.

The results obtained indicate that the spire form (the main shell sign limiting the discussed taxa by a comparative method) varied significantly within each of allocated genetic groups and cannot be used for their taxonomic differentiation. These results confirm a previously expressed opinion about the reduction of taxonomic species in synonyms [14].

Morphological Analysis of Shell

Two variants of the background coloration of the mollusk shell were detected in allocated genetic groups: light with yellow-beige tone and darker green. Regardless of the belonging to the taxon and habitat, individuals from the groups “Northern” (including the snails inhabiting the Chivirkuyskii Bay) and “Central” were represented by mollusks only with light shell of yellow-beige tone, while members of the group “Chivirkuy-Barguzin” had a darker shell of greenish tone (Fig. 2). And it was noted that light shells of yellow-beige tone were more fragile and brittle as compared with dark shells of green color, whose structure was much more plastic and resistant to the mechanical effect. The analysis of 14 plastic shell traits in mollusks of the allocated genetic groups demonstrated (Fig. 3) that mature individuals of the group “Chivirkuy-Barguzin” were significantly larger (Kruskal–Wallis test, $P < 0.05$) with respect to three parameters (*W*, *aw*, *wl*) than the members of the groups “Central” and “Northern.” The shell of mollusks from the group “Northern” was significantly smaller than shells of the group “Chivirkuy–Barguzin” additionally with

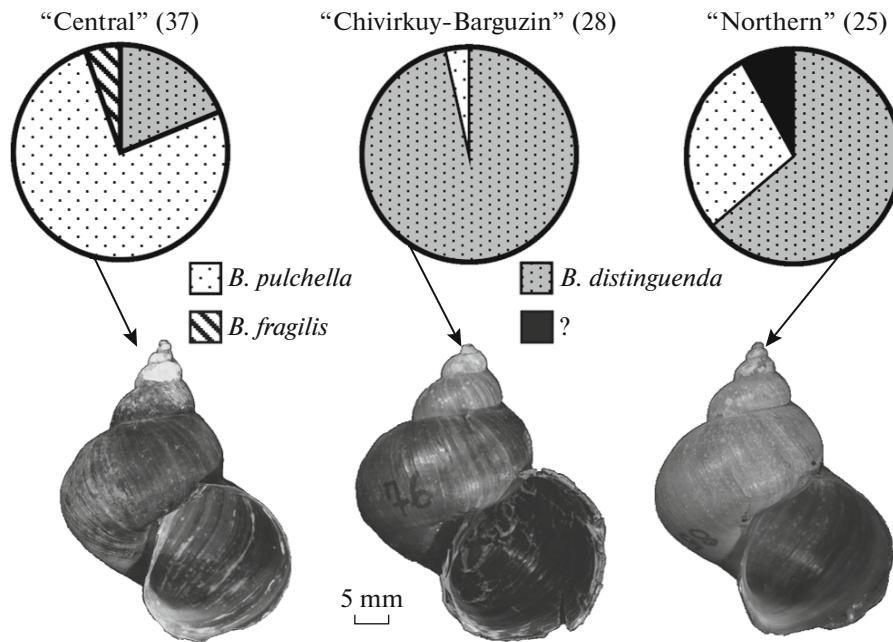


Fig. 2. Ratio of *B. fragilis*, *B. distinguenda*, and *B. pulchella* taxa in isolated genetic groups. Number of analyzed individuals is indicated in brackets.

respect to five parameters (*al*, *h2*, *h3*, *w2*, and *w3*). In addition, the shell of mollusks from the group “Chivirkuy-Barguzin” differed from shells of two other groups in the aperture form: the columellar lip in them was rounded and thickened as a fold, while shells of the members of the groups “Central” and “Northern” mainly had straightened or beveled and only slightly thickened columellar lip of the mouth. No differences between two latter groups of benedictiids were detected.

Thus, the shell of adult mollusks from the group “Chivirkuy-Barguzin” differed from the shells of two other groups (“Central” and “Northern”) in qualitative and some morphometric traits with significant overlapping of indices of most analyzed shell parameters among all genetic groups.

DISCUSSION

The topologies of median networks constructed by mitochondrial and nuclear markers were different. The groups “Central” and “Chivirkuy-Barguzin” were the most distant from each other in the first case (mtDNA *COI*) and, on the contrary, the closest in the second case (nDNA ITS1) (Figs. 1b and 1c). The topology differences may indicate heterogeneity in the evolution rates of mitochondrial and nuclear DNA in the studied gastropods.

The largest genetic heterogeneity of the group “Central” and the presence in its composition of individuals carrying simultaneously “Northern” mitochondrial DNA and “Central” nuclear DNA can

reflect mitochondrial DNA introgression in the past. The presence in the group “Central” of ITS1 alleles of the group “Chivirkuy-Barguzin” in the homozygous and heterozygous state indicates that the gene flow from the group “Chivirkuy-Barguzin” to the group “Central” occurred at present or the recent past.

The absence of the gene flow signs, as well as a large amount of fixed substitutions in analyzed ITS1 sequences, indicates the reproductive isolation of the members of the group “Northern” from representatives of the other two groups, including during their joint habitation in Chivirkuyskii Bay with the group “Chivirkuy-Barguzin.” Exactly here (in Chivirkuyskii Bay), individuals of both genetically isolated groups visually (without additional measurements) differ in coloration, shell fragility, and form of the columellar lip, but small genetic distances between them (<2%) by two genetic markers do not make it possible to give them a rank of species taxon.

The detected low level of genetic diversity in the groups “Northern” and “Chivirkuy-Barguzin” can indicate their smaller evolutionary age as compared with the group “Central”. Negative neutrality indices of the group “Chivirkuy-Barguzin” indicate its expansive growth or the effect of negative selection. The expansive genetic growth was also detected among shallow Baikal endemic *Maackia herderiana* gastropods (Lindholm, 1909) inhabiting the rocky littoral [37].

On the basis of the results obtained and available evidence about of the formation history of Lake Baikal, we assume that the group “Central” (the most

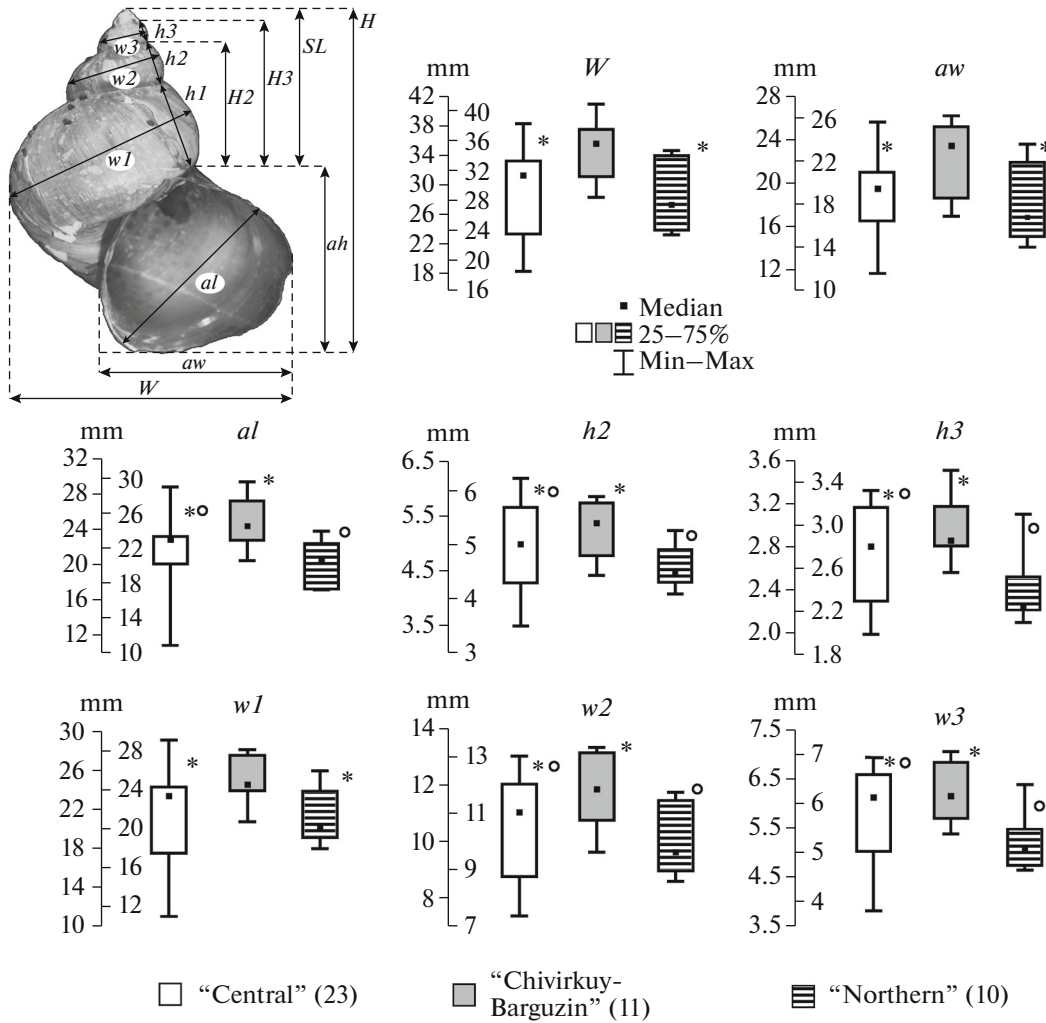


Fig. 3. Scheme of shell measurements and values of differing parameters between isolated genetic groups. The same symbols designate the absence of significant differences (Kruskal–Wallis test, $p < 0.05$). Number of analyzed individuals is indicated in brackets.

genetically diverse and initially occupying the water area of Selenginskii region (from Posolskaya Bank to Proval Bay)) was the initial group for three currently existing groups of “giant” benedictiids. The first stage of the resettlement of a part of individuals to the north (which we date on the basis of genetic differences between the groups “Central” and “Chivirkuy-Barguzin” approximately 500000–600000 years ago) started after the integration of southern-central basins with the northern basin. This period of time in the climatic history of Lake Baikal corresponds to the interglacial period (621000–560000 years ago), during which a significant increase in the number of plankton diatom algae and accumulation of biogenic silica in bottom sediments occurred (unlike the preceding cold period (663000–621000 years ago)) [13]. A constant supply of food (as a phytoplankton) to the bottom, regardless of the alternation of cold and warm periods in all lake basins, could have favored an increase in the number of gastropods and their resettlement. On the

basis of the fact that the estimated time of divergence of the groups “Central” and “Northern” is 392000–494000 years, we assume that the second resettlement stage occurred after the generation of a single ultradeep reservoir (~500000 years ago [9–11]); a part of individuals from the Selenginskii region (initial for the group “Northern”) migrated into the northern end of the lake. The group “Northern” had the lowest indices of haplotype diversity and nucleotide variability of mtDNA *COI* (which is evidence of the passage through the “bottleneck” stage). The absence of signs of resettlement in mollusks from the group “Chivirkuy-Barguzin” from the water area that they utilized can be due to the presence of a sufficient amount of food, as a result of which they did not need migration for its search. The food resources in Chivirkuyskii and Barguzinskii bays are present as dead plankton settling to the bottom, plant detritus coming from the river flow (Bol’shoi and Malyi Chivyrkui, Bol’shaya

and Malaya Cheremshanaya, Barguzin), and animal detritus (fish dead bodies).

The genetic homogeneity of individuals from the group “Chivirkuy-Barguzin” can be explained by the proximity of bays (distance less than 100 km) and by the absence of large natural barriers between the gates of bays along the seaward side of the lake; this supports a high panmixia level inside the group and promotes an increase in the constancy of phenotypic traits typical of this group.

Thus, two out of three genetically isolated groups among Baikal *B. fragilis* gastropods (“Central” and “Northern”) are the closest by phenotypic traits, but occupy different parts of the species area. The area of the third group (“Chivirkuy-Barguzin”), which inhabits large bays, overlaps with the area of the group “Northern,” with which it differs in genetic and phenotypic traits. The emergence of genetic-geographical groups, which occurred as a result of repeated resettlement of the initial group of individuals in a relatively short period of time (~600000 years), is associated with the geological and climatic history of the lake and search for food resources. It is possible that the detected genetic groups are incipient allopatric (geographical) species, whose development occurs inside a large continental freshwater reservoir.

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