In loving memory of Distinguished Professor of Moscow State University P.V. Matekin

Assessment of the Genetic Distances between Some Species of the Family Bradybaenidae (Mollusca, Pulmonata)

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Abstract—On the basis of inter-simple sequence repeat (ISSR) loci and the nucleotide sequences of nuclear (18S and ITS-1) and mitochondrial genes (COI and 16S), a phylogenetic analysis of the three species of terrestrial mollusks of the family Bradybaenidae (Mollusca, Pulmonata), *Bradybaena fruticum* Müll., *Bradybaena schrencki* Midd., and *Bradybaena transbaicalia* Shileyko, was conducted to clarify their taxonomic status. The analysis showed that *Br. fruticum* was far apart from the other two species (*Br. schrencki* and *Br. transbaicalia*). The genetic distance between the latter puts in doubt their status as distinct species. It is suggested that the species *Br. transbaicalia* can be treated as a form of *Br. schrencki* var. *transbaicalia*.

Keywords: ISSR, COI, ITS-1, 16S, 18S genes, terrestrial mollusks, phylogenetic analysis

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INTRODUCTION

The issues related to reliable species identification for more than 200 years (since Linnaeus) have provoked disputes in the scientific community. During this time, many approaches providing classification of the organisms considerably close to natural classification were discovered. Nevertheless, the search for such criteria continues, as in many taxonomic groups, the key morphological and other visual indicators are either absent or very unreliable. With the advent of the polymerase chain reaction and sequencing technique, it became possible to use the fragments of a DNA molecule as genetic markers. At present, the taxonomy actively implements the method of species identification by DNA barcoding, where the identifiers are the unique species-specific mutations in nuclear and mitochondrial DNA.

In respect of terrestrial mollusks, the important taxonomic criteria are the shell type, body structure (for slugs), and the structure of the reproductive system. However, in some cases, because of the so-called overlapping series of variation, it is not always possible to accurately determine the species assignment.

A similar problem arose with regard to three species of the family Bradybaenidae, *Bradybaena fruticum* Müll., *Bradybaena schrencki* Midd., and *Bradybaena transbaicalia* Shileyko[1] (Fig. 1).

Although the most well-known identification guide of terrestrial mollusks [1] presents the characters that make it possible with varying degree of probability to judge the species assignment of studied individuals, the author himself admits that these species are very similar in the elements of shell morphology and anatomy of the reproductive system (especially, *Br. fruticum* and *Br. schrencki*).

The situation is aggravated by the fact that the three species in Eurasia have overlapping ranges and inhabit similar biotopes. For example, the range of *Br. fruticum* was previously determined only within the boundaries of the European continent (from Western Europe to the Urals). However, at present, there is quite strong evidence on the presence of this species in the south of Western Siberia (Tomsk oblast, Tyumen oblast, and Krasnoyarsk krai) [2]. The range of *Br. transbaicalia* was previously restricted to the territory of the original description, the Khamar-Daban Mountain Range in Transbaikalia. Now this species is known from a variety of sites in Cisbaikalia, Kras-

[1] In some studies, these species are assigned to the genus *Fruticicola* [4–6].

Fig. 1. Shells of three mollusk species of the genus *Bradybaena*. (*1*) *Br. fruticum* from Belgorod sampling site; (*2*) *Br. schrencki* from Gorno-Altaisk sampling site; (*3*) *Br. transbaicalia* from Ural sampling site.

noyarsk (Akademgorodok), and the Middle Urals [3]. Moreover, earlier, the individuals of this species were probably assigned to *Br. schrencki* form *major* [7]. The range of truly *Br. schrencki* encompasses Northeastern Europe, Siberia, Altai, and Kamchatka.

These circumstances allowed P.V. Matekin to put in doubt the taxonomic independence of these species. The author especially insisted on the recognition of *Br. schrencki* and *Br. fruticum* as a single species [8].

This supposition led us to test the taxonomic status of these species with the use of modern molecular diagnostic methods. This task was defined by one more fact. The reason is that *Br. fruticum* is currently used as a model for population genetic studies serving as an indicator of anthropogenic impacts on various ecosystems in different landscapes [6, 9–15]. Solution of the outlined above taxonomic problems will make it possible to avoid generation of false results.

MATERIALS AND METHODS

DNA was extracted from mantle specimens thoroughly removed from mucus. DNA was isolated using silica-based sorbent according to the instructions to the Silica uni reagent kit (Biokom, Russia).

The DNA markers for the analyses were represented by ISSR amplicons and nucleotide sequences of nuclear (18S and ITS-1) and mitochondrial (COI and 16S) genes that provided good taxonomic signals and that were earlier used for phylogenetic reconstructions of gastropods. PCR was performed in a Verity thermal cycler (Applied Biosystems, United States) in a total volume of 20 μL of reaction mixture containing 20 ng genomic DNA, PCR buffer (10 mmol Tris-HCl (pH 8.3), 50 mmol KCl, 2 mmol $MgCl_2$), 0.25 mmol dNTP, 0.5 μmol primer, and 1 unit of *Taq* DNA polymerase (inhibited for hot start). The primer sequences and amplification conditions are presented in Table 1.

The fragment analysis and sequencing of PCR products was carried out using an ABI PRISM 3500

Table 1. Primers and amplification conditions used for the gene sequencing

DNA markers	Primers	Temperature regime
ISSR (UBC827)	FAM 5'-ACACACACACACACACG-3'	1st cycle 2 min/94°C; 40 cycles (30 s/94°C, 30 s/55°C, 2 min/72°C); 1 cycle 10 min/72°C
ISSR (SAS1)	FAM 5'-GTGGTGGTGGTGGC-3'	
COI [16, 17]	5'-GGTCAACAATCATAAAGATATTGG-3' 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'	1st cycle 2.5 min/94°C; 40 cycles (30 s/90°C, 1 min/48°C, 1 min/72°C); 1 cycle 10 min/72°C
16S rRNA [17, 18]	5'-CGGCCGCCTGTTTATCAAAAACAT-3' 5'-GGAGCTCCGGTTTGAACTCAGATC-3'	1st cycle 2.5 min/90°C; 10 cycles (50 s/92°C, 30 s/44°C, 40 s/72°C); 36 cycles (30 s/92°C, 40 s/48°C, 40 c/72°C); 1 cycle 3 min/72°C
ITS-1 [17, 19, 20]	5'-TAACAAGGTTTCCGTAGGTGAA-3' 5'-GCTGCGTTCTTCATCGATGC-3'	1st cycle 3 min/94°C; 40 cycles (30 s/92°C,30 s/52°C, 1 min/72°C); 1 cycle 5 min/72°C
18S rRNA [17, 21]	5'-CTGGTTGAT(CT)CTGCCAGT-3' 5'-CTGAGATCCAACTAGGAGCTT-3'	1st cycle 2 min/94°C; 40 cycles (30 s/92°C, 30 s/52°C, 40 s/72°C); 1 cycle 3 min/72°C

Table 2. Sampling sites of the mollusk specimens for fragment analysis and sequencing

Species	Analysis	Sampling locality	Coordinates
Bradybaena schrencki	ISSR $N = 19$ S	Gorno-Altaisk. The city of Gorno-Altaisk (Russia); a small forest area along ulitsa Lenina	51°57′19.40″ N, 85°58′09.31″ E
Bradybaena transbaicalia	ISSR $N = 19$ S	Ural. Sverdlovsk oblast, Sukholozhskii raion, the city of Sukhoi Log (Russia); foot of the limestone cliff on the right bank of Pyshma River, steep rocky slope	56°55′15.90″ N, 62°01′26.90″ E
Bradybaena fruticum	ISSR $N = 20$	Kislovodsk. The city of Kislovodsk; spa park; valley of Olkhovka River; overgrowth of nettles	43°53′21.40″ N, 42°43′52.40″ E
	ISSR $N = 19$	Ural. Sverdlovsk oblast, Nizhniserginsky raion (Russia); Olen'i Ruch'i Nature Park; pine–fir forest with birch and larch, opening, overgrowth of meadowsweet, raspberry	56°31′01.00″ N, 59°14′49.00″ E
	ISSR $N = 15$	Stenki Izgorya. Belgorod oblast, Novooskolskii raion (Russia); Stenki Izgorya Reserve area; wetland habitat, alder thickets, in the undergrowth burdock and nettles	50°41′23.25″ N, 37°49′12.22″ E
	ISSR $N = 14$	Kirov. The city of Kirov (Russia); city park area; Vyatka River floodplain; overgrowth of nettles and meadowsweet	58°34′57.11″ N, 49°41′50.75″ E
	ISSR $N = 10$	Divnogorie. Voronezh oblast (Russia); Divnogorie Nature Sanctuary; floodplain of Tikhaya Sosna River, overgrowth of burdock, nettles, hop	50°57′48.99″ N, 39°17′40.35″ E
	ISSR $N = 12$	Voronov Kamen. Lipetsk oblast (Russia). Vorgolskoe Reserve area; rocky outcrops of Devonian limestones in the floodplain of Vorgol River	52°34′25.32″ N, 38°21′05.34″ E
	S	Belgorod. Outskirts of the city of Belgorod (Russia); floodplain of Seversky Donets River, thickets of willow and maple	50°36′38.40″ N, 36°37′19.19″ E
Helicopsis striata	S	Belgorod. Outskirts of the city of Belgorod (Russia), southwest steep chalk slope of the right bank of Seversky Donets River	50°37′28.66″ N, 36°37′15.97″ E

N, number of individuals used for ISSR analysis; S, groups used for sequencing.

automated capillary DNA sequencer (Applied Biosystems, United States), using 50-cm capillaries and the POP-7™ polymer matrix.

ISSR amplicons were generated using primers labeled with the 6-FAM fluorescent tag (DNK-Sintez, Russia). After PCR, the obtained ISSR fragments were purified using the sorbent-based Diatom®DNA Clean-Up kit (Isogene, Russia). After amplification, 1 μL of PCR product was mixed with 9 μL of Hi-Di™ formamide and 0.3 μL of GS 600LIZv2.0 size standard solution. The prepared samples were analyzed on the DNA sequencer. Size analysis of the fragments was performed using the GeneMapper® Software v. 4.1 (Applied Biosystems). In total, in the range from 100 to 600 bp, we diagnosed 497 loci with primer SAS1 and 462 loci with primer UBC827. The data obtained were used for the construction of binary matrices, where the peak presence was designated as "1" (allele *p*) and the peak absence was designated as "0" (allele *q*). Analysis of molecular variance (AMOVA) [22] was carried out in the GenAlEx v. 6.5 software program [23]. The calculation of Nei and Li genetic distances [24], clustering of the samples using the neighbor-joining (NJ) method [25], and bootstrapping (1000 replicates) was carried out in the TREECON ver. 1.3b software program [26]. The binary vector of each population, consisting of 959 fragments, characterized the presence or absence of a fragment of defined size in at least one individual from the total population. In total, using ISSR analysis, 128 individuals from eight populations of the genus *Bradybaena* were analyzed (Table 2).

For DNA sequencing, a single individual from each examined species was used. After amplification, the DNA fragments were purified by electrophoresis in 2% agarose gel. The gel segments with target DNA were excised, and DNA was eluted by using the sorbent-based Diatom®DNA Elution kit (Isogene, Russia). Preparation of the specimens for sequencing was performed using the BigDye® Terminator Sequencing kit (Applied Biosystems) according to the protocol of the manufacturer. Then, forward and reverse sequencing of the specimens was conducted. The data were initially processed in the Sequencing Analysis® Software 6 program (Applied Biosystems). Alignment of forward and reverse sequences was performed in the BioEdit v. 7.0.0 software program [27]. To select the

Table 3. Accession numbers of the sequenced genes deposited in the GenBank database

Species	GenBank accession numbers			
	COI	16S	18S	ITS-1
Br. schrencki	KX270740*	KX270729*	KX270737*	KX270733*
Br. transbaicalia	KX270741*	KX270730*	KX270739*	KX270734*
Br. fruticum (1)	KX270742*	KX270731*	KX270738*	KX270735*
Br. fruticum (2)	AY546264	AY546344	AY546384	AY546304
Br. similaris	AB902872	GQ851164	–	AB852967
Br. phaeogramma	–	AF098714	–	–
Br. phaeogramma phaeogramma	AB902879	–	–	AB902905
Br. ravida	KF765753	–	–	–
Br. brevispira	KF765755	–	–	–
Br. virgo virgo	KF765751	–	–	–
Br. blakeana	–	AB893777	–	AB893912
Br. circulus circulus	AB902860	–	–	AB902885
*H. striata**	AY546282	KX270732*	AY546402	KX270736*

Br. fruticum (1), individual from the city of Belgorod, Russia; *Br. fruticum* (2), individual from the city of Schlüchtern, Germany.
* Data obtained by the authors of the study.

best-fit model of nucleotide substitution and calculate the genetic distances, the jModelTest software program was used [28], while for all used genetic markers the generalized time-reversible model (GTR) of nucleotide substitution with gamma correction was selected. To reconstruct the phylogenetic trees and assess the reliability of their topology, the MrBayes software program was used [29, 30]. In the Bayesian approach, five runs of the program were used, and the trees were generated using 2000000 Markov chains. A consensus tree was built sampling every 1000th generated tree. Visualization of phylogenetic trees was carried out in the FigTree software program (http://tree.bio.ed.ac.uk/software/figtree/).

For comparison, the GenBank sequences of the analyzed genes from other species of the genus *Bradybaena* were used [17, 31–35]. The gene sequencing data for *Helicopsis striata* (family Hygromiidae) were used as a family-level control. Table 3 shows the accession numbers of nucleotide sequences deposited in the GenBank (NCBI) database.

RESULTS

The dendrogram constructed from the results of fragment analysis is given in Fig. 2. According to the data, the population sample of *Br. transbaicalia* was far apart from both the populations of *Br. fruticum* and *Br. schrencki*. Pairwise Φ_{st} values of genetic differentiation (Table 4) based on 959 inter-simple sequence repeat loci also showed that *Br. transbaicalia* was considerably distanced from both the populations of *Br. fruticum* ($\Phi_{st} > 0.3$), and *Br. schrencki* ($\Phi_{st} = 0.272$). At the same time, these genetic distances were comparable to the intergroup differences between some populations of *Br. fruticum*, for example, between *Br. fruticum* (Kislovodsk), on one hand, and *Br. fruticum* (Ural) and *Br. fruticum* (Stenki Izgoriya), on the other hand. The differences between the group of *Br. schrencki* and the populations of *Br. fruticum* also fit into interpopulation distances of *Br. fruticum*.

Phylogenetic trees constructed on the basis of the nucleotide sequences of the analyzed genes are shown in Fig. 3.

According to the data, the sequence of the mitochondrial gene of the cytochrome c oxidase subunit I (COI), containing 463 bp, showed a great similarity between *Br. schrencki* and *Br. transbaicalia* (D = 0.062). The genetic distance between these two species is comparable with the distance between two individuals of *Br. fruticum* from two far distant localities (the city of Schlüchtern, Germany, and the city of Belgorod, Russia; D = 0.016). Furthermore, the genetic differences between other relatively close species of the genus *Bradybaena* with respect to this marker are more considerable. For example, between *Br. circulus circulus* and *Br. phaeogramma phaeogramma*, the genetic distance is D = 0.395, and between *Br. ravida* and *Br. virgo virgo*, D = 0.368. It is noteworthy that the genetic distance between the clusters of *Br. fruticum* and *Br. schrencki* + *Br. transbaicalia* was found to be D = 0.502.

Sequence analysis of the internal transcribed spacer separating the individual nuclear rRNA genes (ITS-1, 619–626 bp) showed even greater similarity between *Br. schrencki* and *Br. transbaicalia* (D = 0.004). The genetic distance between these two species at this marker was smaller than between the repre-

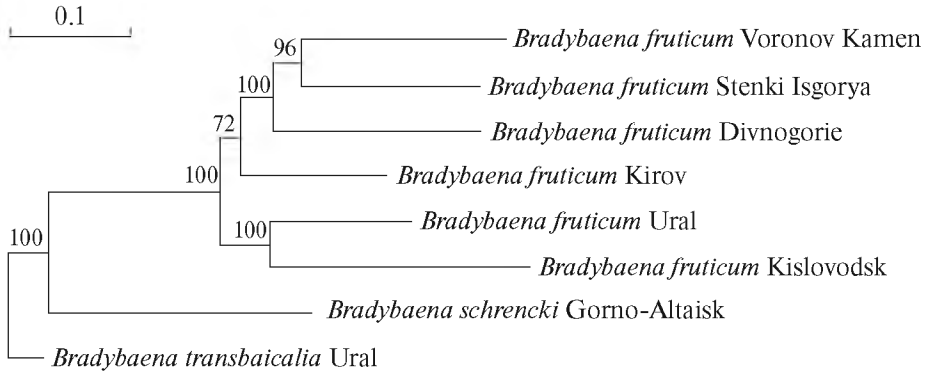

Fig. 2. Dendrogram of genetic distances constructed using the neighbor-joining (NJ) approach between the populations of *Bradybaena* sp. and derived from the analysis of ISSR loci. Numbers at the nodes indicate the bootstrap support values (%).

sentatives of different populations of *Br. fruticum* (D = 0.014). However, it is worth noting that, according to the phylogram (Fig. 2), the genetic distance between good species *Br. phaeogramma phaeogramma* and *Bradybaena similaris* at this marker was also small (D = 0.012). It should also be noted that *Br. transbaicalia* at positions 39–42 bp has the CAGC insertion, absent from other species of the family. Furthermore, *Br. schrencki* (at position 458–460 bp) and *Br. transbaicalia* (at position 462–464 bp) contain the original TGC insert.

Phylogenetic closeness of *Br. schrencki* and *Br. transbaicalia* was also demonstrated in sequence analysis (399 bp) of the mitochondrial DNA 16S ribosomal gene (D = 0.006). At the same time, the genetic distance between two individuals of *Br. fruticum* (D = 0.013) was twice as high, and the distance between *Br. phaeogramma* and *Bradybaena similaris*, located in the same cluster, was D = 0.331. Note that *Br. fruticum*, unlike other species of the family, has the GAACC insert at position 254–258 bp.

The 618-bp sequences of nuclear 18S rRNA in the compared three species of the family Bradybaenidae were found to be completely identical. Certain genetic distances could be determined only between the members of different families.

It should be noted that, with respect to the whole set of COI, 16S, and ITS-1 sequences, the genetic distances in the examined species constituted 0.019 between *Br. schrencki* and *Br. transbaicalia*, 0.012 between two individuals of *Br. fruticum*, and 0.169 between the cluster of *Br. fruticum* and *Br. schrencki* + *transbaicalia*. It is noteworthy that these results are comparable with similar data for other terrestrial mollusks. For instance, with respect to this set of genes, the genetic distances between the species of the same genus constituted, for example, 0.110 in the genus *Monacha*, 0.100 in *Helicella*, 0.100 in *Candidulla*, and 0.140 in *Caracolliana* [17]. Furthermore, with respect to a separate COI sequence, for example, in the genus *Satsuma*, the genetic distances between the species ranged from 0.103 to 0.285, and the genetic distance between different forms of *S. mellea* was 0.082 [34].

Table 4. Pairwise genetic differentiation estimates (Φ_{st}) between the examined populations of the genus *Bradybaena* over all 959 ISSR loci

Populations	1	2	3	4	5	6	7	8
1. *Br. schrencki* (Gorno-Altaisk)	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.001
2. *Br. transbaicalia* (Ural)	0.272	0.000	0.001	0.001	0.001	0.001	0.001	0.001
3. *Br. fruticum* (Kislovodsk)	0.326	0.438	0.000	0.001	0.001	0.001	0.001	0.001
4. *Br. fruticum* (Ural)	0.268	0.391	0.272	0.000	0.001	0.001	0.001	0.001
5. *Br. fruticum* (Stenki Izgorya)	0.264	0.368	0.282	0.205	0.000	0.001	0.001	0.001
6. *Br. fruticum* (Kirov)	0.208	0.311	0.214	0.109	0.130	0.000	0.001	0.001
7. *Br. fruticum* (Divnogorie)	0.208	0.337	0.252	0.174	0.080	0.073	0.000	0.001
8. *Br. fruticum* (Voronov Kamen)	0.253	0.363	0.265	0.219	0.106	0.104	0.069	0.000

Φ_{st} values below the diagonal; statistical significance levels above the diagonal.

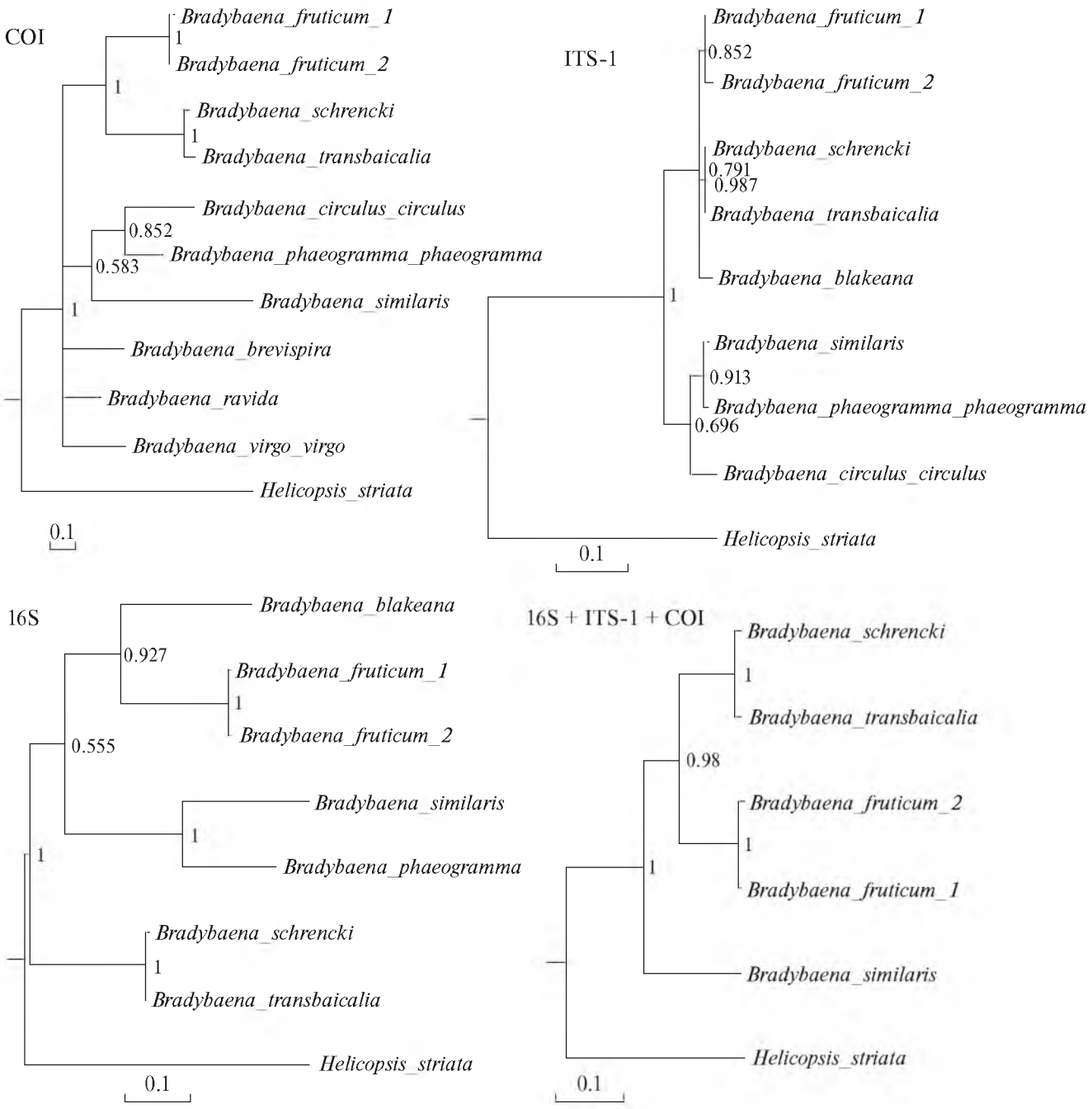

Fig. 3. Phylogenetic trees of *Bradybaena* sp. constructed using the Bayesian method on the basis of the ITS-1, 16S, and COI sequences. Numbers at the nodes indicate the posterior probability values >0.5.

DISCUSSION

In view of the fact that, at the ISSR loci, the genetic distances between the studied species fit into interpopulation distances, it seems likely that fragment analysis cannot give a definite answer on the taxonomic status of the studied groups of mollusks. The reason for this can be great variability of ISSR loci within populations and at the interpopulation level. Given that the studied species are phylogenetically very close, we can observe similar series of genetic variation that neutralize the interspecies differences. Probably, ISSR loci are more effective as genetic markers for studying the population structure of the species with well-established taxonomic reputation.

The genetic distances inferred from the nucleotide sequences of the examined genes showed that *Br. fruticum* was clearly distant from the two probably vicarious Asian species *Br. schrencki* and *Br. transbaicalia*.

Given considerable genetic closeness of the latter, the species independence of these forms is in doubt. It should be noted in this respect that *Br. schrencki* was described much earlier (Middendorff, 1851) and was recorded in numerous localities of Eurasia, while *Br. transbaicalia* (Shileyko, 1978) was known from several localities in the Lake Baikal region and in one locality in the Urals and in the city of Krasnoyarsk. From here, it follows that the latter species can be either a recent isolate from *Br. schrencki* or, as mentioned earlier [4], the *maior* form of this species (according to a modern interpretation, *Br. schrencki* var. *transbaicalia*).

It is worth noting that taxonomic revision of the family Bradybaenidae on the basis of genetic markers already questioned the independence of some of its species, the isolation of which was based on morphological characters (the structure of shell and reproductive system). An example is the study of T. Hirano et al. [33], focused on the revision of some island species of the genera *Phaeohelix* and *Bradybaena*.

Interestingly, analysis of the COI and ITS-1 sequences showed that *Br. fruticum*, *Br. schrencki*, and *Br. transbaicalia* were members of one cluster and were phylogenetically distant from the other species of the family Bradybaenidae[2]. Given that the place of origin of this family is East Asia [4], it can be suggested that these three (or two) species are monophyletic. During the dispersal over the Eurasian continent in the Pliocene, *Br. fruticum* mostly moved westward, populating the European continent, and *Br. schrencki* adapted to more severe conditions of Siberia. In some isolated populations of this species, the elements of allopatry could have occurred, leading to the formation of *Br. transbaicalia* (or var. *transbaicalia*).

In conclusion, it should be noted that analysis of phylogenetic relationships of the three examined species of the family Bradybaenidae on the basis of a larger number of markers and different populations will make it possible to build a more realistic evolutionary history of this group of terrestrial mollusks.

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[2] The exception was *Bradybaena blakeana* (W. Newcomb, 1865) from the island of Hokkaido (Japan), which with respect to the ITS-1 marker was included in one clade together with the three species examined in our study. At the same time, with respect to 16S marker, this species was in the same group with *Br. fruticum*. We think that the genetic closeness of this island species to the continental mollusks will be discussed in the future.

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