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Discovery of *Unio gontierii* (Bivalvia: Unionidae), a freshwater mussel belonging to the *crassus*-group, on the Black Sea coast of Russia

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ABSTRACT. In the Kherota River (Russian Black Sea coast), freshwater mussels not previously known in the area were discovered and morphologically identified as *Unio gontierii* Bourguignat, 1856 (Bivalvia: Unionidae). The mussel species identity was verified by analysis of the mitochondrial gene sequence encoding cytochrome c oxidase subunit I (*COI*). The diversity of the eight studied microsatellite loci in the studied sample was rather high (the average number of alleles per locus was 4.75, the observed heterozygosity was 0.50±0.08). The aforementioned facts, in conjunction with the substantial distribution of the bitterling *Rhodeus colchicus* Bogutskaya et Komlev, 2001 along the Russian Black Sea coast, whose larvae develop in the bivalves, indicate that *Unio gontierii* is an indigenous inhabitant of the Russian part of the Caucasian coast and not a recently human-introduced species.

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Находка двустворчатого моллюска, относящегося к группе *Unio crassus*, *Unio gontierii* (Bivalvia: Unionidae), на черноморском побережье России

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РЕЗЮМЕ. В реке Херота (российское побережье Черного моря) были обнаружены неизвестные здесь ранее двустворчатые моллюски, по морфологическим признакам определенные как *Unio gontierii* (Bivalvia: Unionidae). Видовая принадлежность моллюсков подтверждена анализом последовательности митохондриального гена, кодирующего субъединицу I цитохромоксидазы (*COI*). Разнообразие восьми изученных микросателлитных локусов в изученной выборке оказалось довольно высоким (среднее число аллелей на локус составило 4,75, наблюдаемая гетерозиготность – 0,50±0,08). Все это, наряду с довольно широким распространением вдоль российского побережья Черного моря горчака *Rhodeus colchicus*, личинки которого развиваются в двустворчатых моллюсках, свидетельствует о том, что моллюск *Unio gontierii* – исконный обитатель российской части побережья Кавказа, а не вселился сюда в недавнее время по вине человека.

Introduction

In recent years, molecular genetic data, in combination with modern methods of morphological analysis, have significantly changed ideas about the systematics and evolution of the large bivalve molluscs – freshwater mussels or naiads (order Unionida) throughout Eurasia and beyond [Lopes-Lima *et al.*, 2021, 2024]. In particular, it appeared that the thick-shelled river mussel, *Unio crassus* Philipsson, 1788, is represented by a number of well-defined groups which deserve the status of separate species. These include *Unio gontierii* Bourguignat, 1856, which is currently identified as a valid species [Lopes-Lima *et al.*, 2024].

The discovery of new populations belonging to this molluscan group has been reported repeatedly, in which leads to a significant expansion of the established boundaries of the range occupied by a number of species, however, such findings are usually made in hard-to-access regions of Asia [Bolotov *et al.*, 2020; Bulakhova *et al.*, 2023]. Nevertheless, *Anodonta cygnea* (Linnaeus, 1758) was discovered recently in the small River Kherota, as well as in a water reservoir situated in the vicinity of the Sergey-Pole settlement located on the Russian Black Sea coast [Tuniev, 2002]. Despite the repeated studies of the benthic macroinvertebrates in the relevant watercourses, no other freshwater mussels have been found along the Russian Black Sea coast [Reshetnikov, Pashkov, 2009; Palatov *et al*., 2016; Baryshev *et al*., 2017].

In the present study, we describe the discovery of a freshwater mussel species from the *Unio crassus*-group inhabiting the Kherota River, and also perform its identification using molecular genetic methods. In addition, an assessment of the genetic diversity of molluscs from this sample is conducted, which provides an approximation of the effective abundance of a given population (precisely, the subpopulation inhabiting a particular section of the river).

Material and methods

The Kherota River (alternative spellings: Khorota and Huarota) is located on the Black Sea coast of the Northwest Caucasus (Fig. 1). It originates from a spring in a forest area east of the Sakharnaya Golovka Mt. and drains into the Black Sea in the Adler District of Sochi at the coordinates 43.4397°N, 39.9054°E. According to the Russia State Water Register, the length of the river is 14 kilometers, and the catchment area is 24.7 km^2.

In the middle reaches, the water is characterized as calcium hydrocarbonate with mineralization from 0.15 to 0.30 g/dm^3, in the lower reaches – mixed chloride-carbonate-calcium-sodium composition, with mineralization increasing to the level of 0.6–0.7 g/dm^3 [Gudkova, Reneva, 2019]. The river is supplied by a mixed, predominantly precipitation-driven, sources, and it exhibits a flooding regime (Kherota (№ 0161089) / Registry of Names of Geographical Objects in Krasnodar Krai as of 17.12.2019 // The State Catalogue of Geographical Names https://dimastbkbot.toolforge.org/gkgn/?name=%D0%A5%D0%B5%D1%80%D0%BE%D1%82%D0%B0). The river is exposed to extensive landslide events [Lukashov, Ivanchenkova, 2020].

The substrate of the Kherota River is distinct from that of the majority of rivers in the region under study, which is due to the prevalence of clay banks and pools across many sites. The watercourse is under the influence of contaminated effluents from a variety of industrial and agricultural enterprises, transport infrastructure and the residential area, which lacks a centralized sewerage system and the river is also affected by pollutants from the municipal solid waste landfill site. The waterway is partially taken into the underground channel [Gudkova, Gorbunova, 2017].

The presence of large bivalves was first observed by us in the Kherota River in 2017, at geographical coordinates 43.4643° N and 39.9328° E (Fig. 2). In 2021 and 2022, soft tissue samples from 32 molluscs were collected and fixed in 96% ethanol at the same location using a non-lethal method [Berg *et al.*, 1995; Karlsson *et al.*, 2013]. Shells of seven dead individuals were also collected in the river, and their species identity was determined by morphological characteristics recommended by Lopes-Lima *et al.* [2024].

Total genomic DNA was isolated from mollusc tissues using a commercial QIAGEN DNA Investigator Kit (QIAGEN, Germany) according to the instructions recommended by the manufacturers. The sequences of the cytochrome c oxidase subunit I encoding gene fragment (*COI*) were amplified using primer pair LCO1490 and HCO2198 [Folmer *et al.*, 1994]. The amplification mixture contained 0.5 µl of cellular DNA (with an initial concentration of 200 ng/µl), 1 µl of each oligonucleotide primer (10 pmol/µl), 2.5 µl of a mixture of four deoxyribonucleotides (2 mmol each), 1 µl of $MgCl_2$ solution (50 mmol), 2.5 µl of 10-fold PCR buffer, 0.15 µl of Taq-polymerase (SibEnzyme Ltd., Russia, with an initial concentration of 5 units/µl). The volume of the mixture was adjusted to 25 µl with double distilled water. The amplification regime included an initial denaturation at +95°C during 4 min, followed by 28 cycles, each of which in turn included denaturation at +95°C during 50 sec, primer annealing at +54°C during 50 sec, and DNA chain elongation at +72°C during 50 sec, followed by a final elongation process: +72°C, 5 min.

Sequencing was performed with both forward and reverse primers on an automated sequencer (ABI PRISM3730, Applied Biosystems) using the ABI PRISM BigDye Terminator v.3.1 reagent kit at the

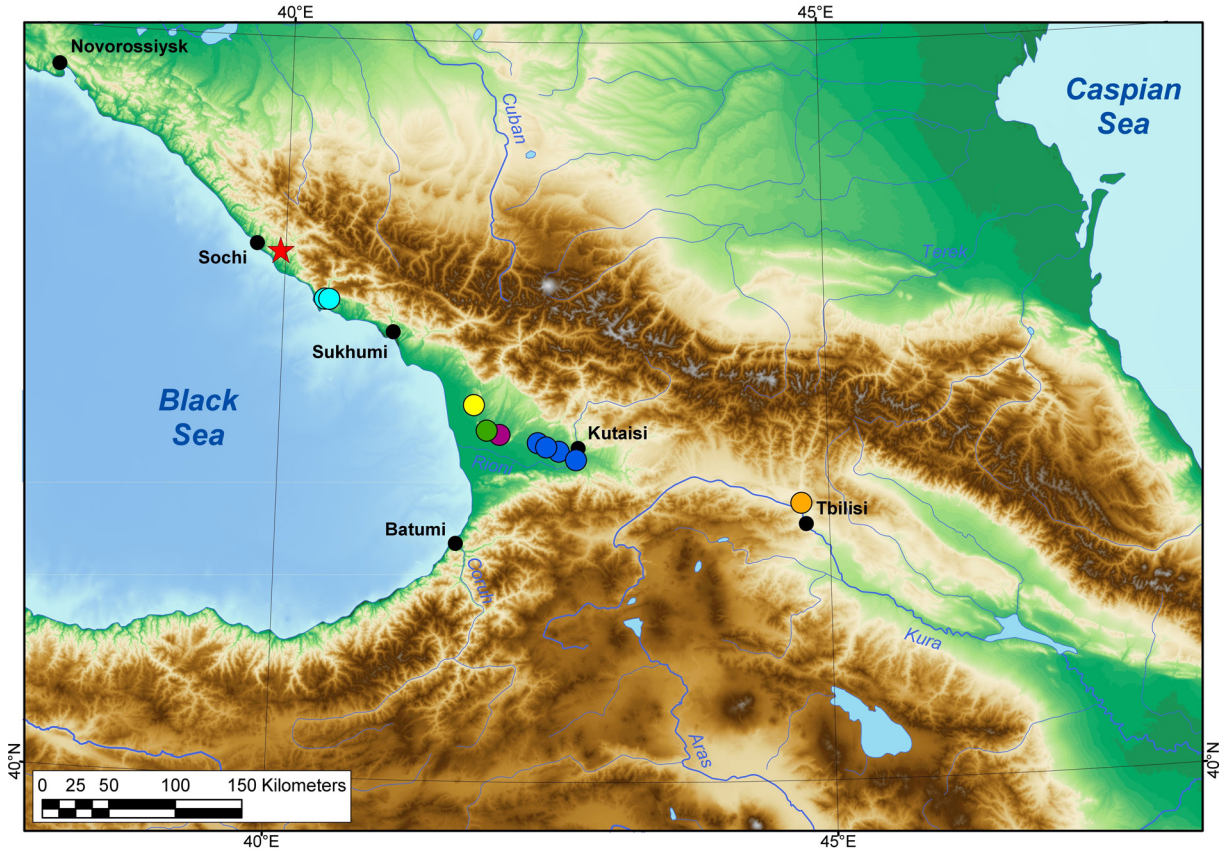

FIG. 1. Records of *Unio gontierii* Bourguignat, 1856 based on the Table S1. The red star indicates the new record from Kherota River; colored circles indicate other records of this species. Circles colors represent different haplotypes according to the Fig. 4. Map: Mikhail Y. Gofarov.

РИС. 1. Места находок *Unio gontierii* Bourguignat, 1856 по данным из Таблицы S1. Красная звездочка показывает место новой находки в реке Херота; цветные кружки показывают другие находки этого вида. Цвета, в которые окрашены кружки, соответствуют разным гаплотипам, согласно Рис. 4. Карта составлена М.Ю. Гофаровым.

Genome Laboratory of the V.A. Engelhardt Institute of Molecular Biology, RAS. We used the BioEdit 7.2.5 software [Hall, 1999] for sequences analyses. In addition, 30 *COI* sequences of *U. gontierii* were obtained from NCBI GenBank and BOLD Systems V4 (Table S1). Sequences were aligned using the MUSCLE algorithm implemented in MEGAX [Kumar *et al.*, 2018]. For the subsequent analyses, each COI sequence of the aligned datasets was trimmed, leaving a 657-bp fragment. The phylogeographic analyses of *U. gontierii* were performed based on a median-joining network approach using Network ver. 4.6.1.3 software with default settings [Bandelt *et al.*, 1999].

Microsatellite locus analyses were performed at the Northern (Arctic) Federal University named after M.V. Lomonosov (Arkhangelsk, Russia). Eight microsatellite loci (Uc5, Uc7, Uc15, Uc19, Uc25, Uc39, Uc77 and Uc57) previously developed for *U. crassus* were analyzed for each of the 32 specimens [Sell *et al.*, 2013]. The forward primers had fluorescent markers at the 5' end. In particular, Table 1 describes the markers used for each locus, the annealing temperature for each pair of microsatellite primers, and the number of amplification cycles for each locus.

For amplification of the tested loci, the standard for all primers reaction mixture containing 0.5 µl of DNA (with a concentration of 200 ng/µl), 2.5 µl of 10x PCR buffer supplied with Taq polymerase, 1 µl of $MgCl_2$ (50 mmol), 2.5 µl of dNTP's (with a concentration of 2 mmol each), 0.3 µl of fluorescently marked forward primer (10 pmol/µl), 1 µl of reverse

FIG. 2. Sampling site in the Kherota River.

РИС. 2. Место сбора материала в реке Херота.

Table 1. Characteristics of microsatellite loci used in the study.

Таблица 1. Характеристика микросателлитных локусов, использованных в работе.

Locus	Sequence of primers (5′– 3′)	Repeat Motif	Marker	T (°C)	Number of cycles	Allele lengths (bp) by Sell *et al.*, 2013
Uc 5	F: GCCAGAATTGTGCTGATGTG R: TTTTGGGGTTCAAGGTCAAG	$(CATA)_2(CATG)_2$ $(CATA)_{11}$	R6G	62	38	116–200
Uc7	F: TCATTTTCCAGGCTGTCACTT R: GGTGATGGTGATGCATGAAA	$(TCCA)_5$	6-FAM	63	38	112–126
Uc15	F: GTGTAAATGGAAACACTACATTTGTT R: ACAGAGTTGGTTGAGCCCTG	$(GTAT)_{13}$	ROX	65	38	149-197
Uc19	F: GCTTGTTACATGAGATGCTGTGAT R: GCCACCATATAATGTGCAGCTA	$(ATGT)_{15}$	TAMRA	58	36	153–205
Uc25	F: GGAACTTGTTCCCTGTTGCT R: CAAGATTATCGAAAATTGGGGA	$(GTAT)_{17}$	ROX	55	37	180–224
Uc39	F: ATCCATCTATCGATCTATCCATCT R: CGGGTGGATGAATCTCGTTA	$(ATCT)_5$	TAMRA	65	40	140–160
Uc77	F: CGTACAAACTGCCTGGGTTG R: ATGATCATTTCAGACAGCCATT	$(AC)_{17}$	R6G	59	36	226–256
Uc57	F: CACAAACTTCATGGGAAGCA R: CGTGATCAAGACGACCTTTTC	$(CAA)_7$	6-FAM	65	37	156–176

Note: T (°C) is the annealing temperature.

primer (10 pmol/µl), 0.2 µl of Taq polymerase (5 units/µl), and the total volume was adjusted to 25 µl of double distilled water.

PCR was performed at the following common for all loci temperature and time profile: initial denaturation at +94°C – 3 min; followed by 36-40 cycles (Table 1), including denaturation at +94°C - 30 sec, primer annealing at T°C (Table 1) – 30 sec, DNA strand elongation at +72°C – 30 sec; and eventually a final elongation step at +72°C – 3 min. To ensure that amplification was successful, amplification products were visualised in a 2% agarose gel.

The fragment lengths of amplified microsatellite loci were determined using NANOFOR 05 genetic analyser (Syntol, Russia) with PDMA4 polymer and SD450 length standard (Syntol, Russia). Interpretation of the obtained results was conducted using GeneMarker v.4.0 software (https://softgenetics.com/GeneMarker.php).

Each microsatellite locus was examined for the presence of null alleles using the four methods implemented in MICRO-CHECKER 2.2. [Van Oosterhout et al., 2004].

Population indices (allele number (N_A), effective allele number (Ne), expected (H_E) and observed (H_O) heterozygosity and inbreeding coefficient (FIS)) were analyzed using the GenAlEx v.6 programme [Peakall, Smouse, 2006]. Correspondence to Hardy-Weinberg (P_{H-W}) equilibrium for all loci was assessed using GlobalTest in GENEPOP v.4.7.3 programme [Raymond, Rousset, 1995].

The effective population size Ne was calculated using the linkage disequilibrium (LD) method [Waples, Do, 2010] employing the programme NeEstimator v. 2.1 [Do *et al.*, 2014]. Recent demographic history, namely the probability of population declines in the recent past, was assessed using the BOTTLENECK v.2.1 programme. In the absence of acute recent changes in population size, the graph of the proportion of alleles plotted by the 10 allele frequency classes has an L-shape. In case of population passing through periods of its abundance decline, the graph ceases to be L-shaped, as the proportion of alleles with high frequencies increases [Luikart *et al.*, 1998].

The distribution of *U. gontierii* was mapped based on published georeferenced records and original data (Table S1) using ESRI ArcGIS 10 software (www.esri.com/arcgis). The base of the map was created from free online resources.

Results

All studied mussel shells from the Kherota River corresponded to the species *Unio gontierii* Bourguignat, 1856 according to their morphological characteristics (Fig. 3). The shape and proportions of the examined shells of dead individuals corresponded to the external features of the discovered living mussels, whose soft tissues were used in molecular genetic studies.

Sequence analysis of the *COI* gene also demonstrated that all individuals studied belonged to the species *U. gontierii*. All studied specimens from the

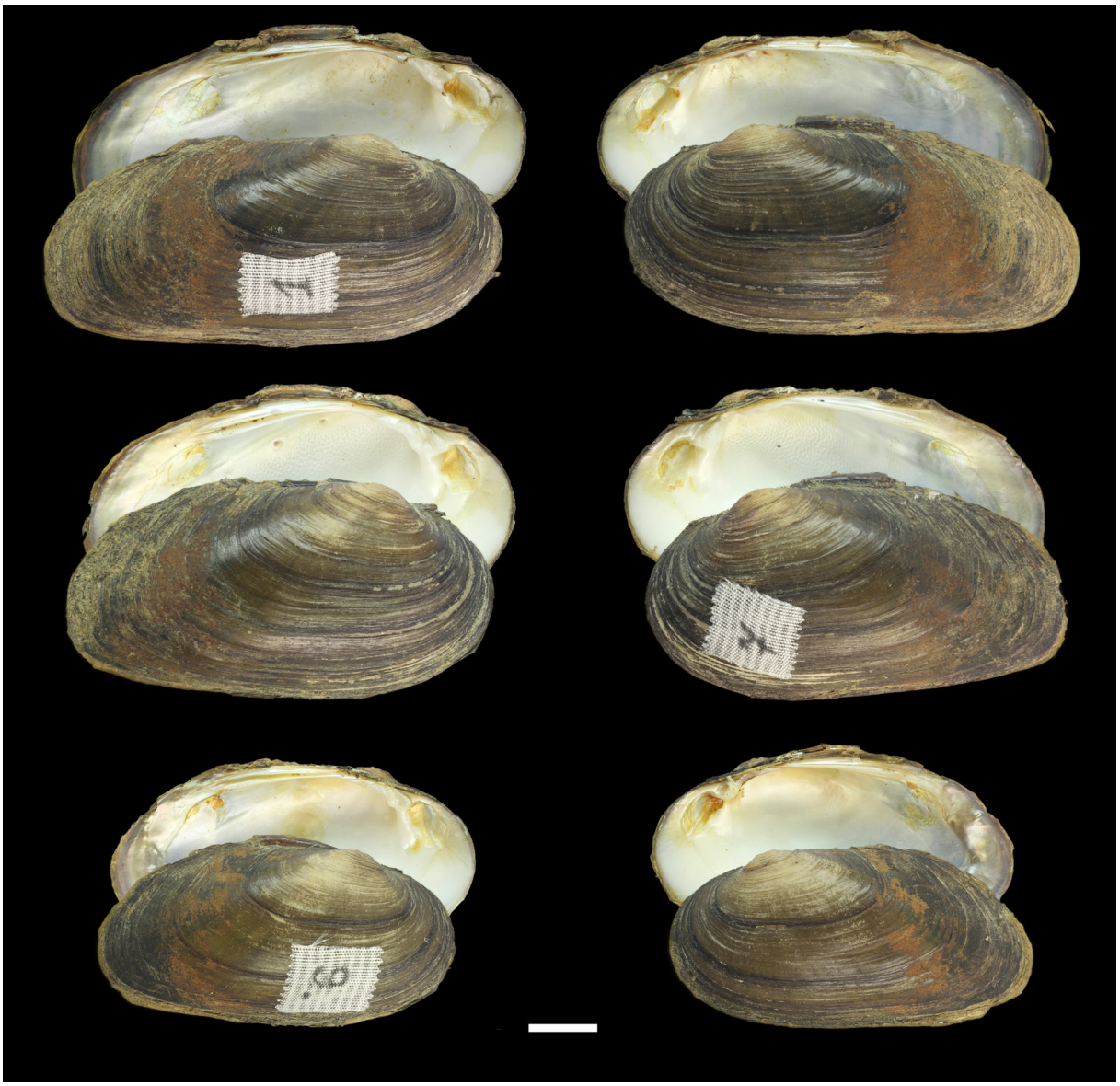

FIG. 3. Shells of *Unio gontierii* from the Kherota River. Scale bar = 10 mm.

РИС. 3. Раковины *Unio gontierii* из реки Херота. Масштабная линейка – 10 мм.

Kherota River shared the same *COI* gene haplotype (Table S1). Previously, this haplotype was discovered in individuals of *U. gontierii* from the Bzyb River in Abkhazia (Fig. 4) [Lopes-Lima *et al.*, 2024]. The data of the analysed sequences were deposited in GenBank, and their accession numbers are listed in Table S1.

The presence of null alleles was not detected during testing using the MICRO-CHECKER 2.2.3 programme in the studied microsatellite loci. The analysis of the molecular diversity indices of the investigated loci for the *U. gontierii* population indicated that all the microsatellite loci used were polymorphic, with the number of alleles ranging from 2 to 10. A significant heterozygote deficiency was observed for the Uc15 and Uc39 loci (Table 2).

The effective population size (Ne) value for the studied locality of *U. gontierii*, calculated based on the LD method, equaled 52.2 specimens (with a minimum allele frequency of 0.05; with a CI 95% confidence interval (CI 95%, Parametric 21.1-∞)). The shift in allele frequency distribution of microsatellite loci was calculated in the BOTTLENECK programme. The allele proportion graph for the 10 allele frequency classes exhibited the typical L-shape that is customary for relatively stable populations (Fig. 5).

It should be noted, however, that we studied a small sample, relatively few loci, and two of them demonstrated deviation from Hardy-Weinberg equilibrium. In addition, hermaphrodites have previously been observed, although at a low frequency, in populations of species belonging to the *crassus* group [Shevchuk *et al.*, 2019], and self-fertilisation

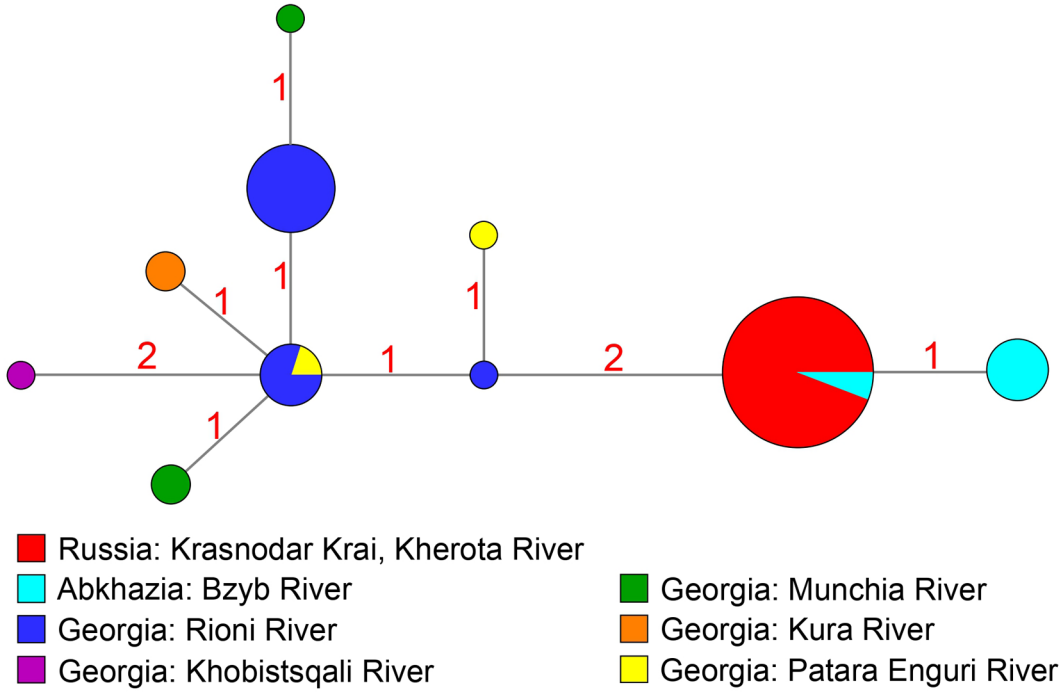

FIG. 4. Median joining network of the *COI* sequences of *Unio gontierii*. Circle symbols represent different haplotypes, with the size reflecting their frequency (smallest = 1). Numbers near branches are numbers of nucleotide substitutions. The dataset contains 62 *COI* sequences (length = 657 bp).

РИС. 4. Медианная сеть последовательностей *COI Unio gontierii*. Круги представляют собой разные гаплотипы, их размер отражает частоту гаплотипов (самый редкий гаплотип встречен один раз). Числа у ветвей – количество нуклеотидных замен. Набор данных включает 62 последовательности *COI*, длина последовательностей 657 п.н..

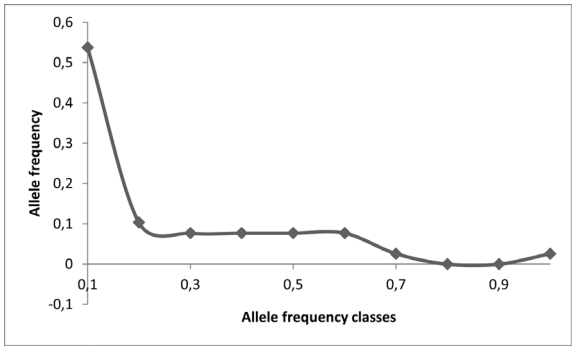

FIG. 5. Allele frequency distribution of microsatellite loci in the *Unio gontierii* population from the Kherota River.

РИС. 5. Распределение частот аллелей микросателлитных локусов в популяции *Unio gontierii* реки Херота.

reduces Ne values [Waples, 2025]. Therefore, the statistical estimates we obtained should be considered as indicative and related to the studied segment of the river only.

Discussion

The *COI* gene haplotype detected in all 32 studied specimens from the Kherota River is identical or similar to haplotypes of *Unio gontierii* from neighboring regions of Trans-Caucasus (Fig. 4); thus, the affiliation of the studied molluscs to this species is confirmed. This species has never before been documented in the region of the Black Sea coast of Russia, although many other rivers in the region have been surveyed by hydrobiologists [Tuniev, 2002; Reshetnikov, Pashkov, 2009; Palatov *et al.*, 2016; Baryshev *et al.*, 2017].

Meanwhile, it is highly probable that *Unio gontierii* also occurs in some other rivers along the Black Sea coast of the Caucasus. It is known that representatives of the bitterlings *Rhodeus* spp. (Cyprinidae: Acheilognathinae) of Eurasian water bodies spawn in the mantle cavity of freshwater mussels (Unionidae and Margaritiferidae) [Holčík, 1999; Smith *et al.*, 2004; Bogutskaya *et al.,* 2009; Klishko, 2012; Sayenko, Palatov 2023], this is also highly possible for species *Rhodeus colchicus* Bogutskaya et Komlev, 2001, as well inhabiting the reservoirs of the Caucasus Black Sea coast.

Meanwhile, apart from the Kherota River, the Georgian bitterling (*Rhodeus colchicus*) has also been recorded in other rivers of the Russian Black Sea coast – Loo, Kudepsta, Mzymta, lower reaches of the Psou River, as well as in some water bodies of the Imereti Lowlands [Tuniev, 2007; Tuniev, Tuniev, 2017].

The relatively widespread distribution of *Rh. colchicus* using *Unio gontierii* shells as spawning substrate in the Russian part of the Caucasian coast indicates that this freshwater mussel species is na-

Table 2. Indicators of genetic diversity of *Unio gontierii* from the Kherota River.

Таблица 2. Показатели генетического разнообразия *Unio gontierii* из реки Херота.

Locus	N	N_A	H_O	H_E	P_{H-W}
Uc5	32	5	0.625	0.623	0.576
Uc7	32	2	0.188	0.172	1.000
Uc15	32	7	0.250	0.532	**
Uc19	32	10	0.781	0.850	0.304
Uc25	32	6	0.750	0.735	0.561
Uc39	28	3	0.357	0.610	*
Uc77	32	3	0.438	0.552	0.144
Uc57	32	2	0.625	0.492	0.967
Average value	31.5	4.75	0.502±0.080	0.571±0.070	-

Note: N - sample size; N_A – number of alleles, H_E – expected and H_O – observed heterozygosity; P_{H-W} – deviation from Hardy-Weinberg equilibrium (*p≤0.05, **p≤0.01)

tive to the region and was not introduced artificially. This is evidenced by the rather high levels of genetic diversity for the sample we studied, as well as the presumed absence of significant fluctuations in the recent history of the population, based on the distribution of allele frequencies of microsatellite loci.

Molluscs previously classified as *Unio crassus* were found in the Upper Pliocene sediments on the Taman Peninsula [Burjak, 1969]. At periods of sea-level lowering, when various freshwater systems were connected into a united network, or during periods of desalination, which occurred repeatedly in the Pleistocene history of the Black Sea [Yanina, 2014], these molluscs had the opportunity to disperse into rivers in other regions of the Black Sea basin.

At the same time, during periods of sea salinization or some major habitat changes, evolutionary lineages seem to have been isolated, leading to the formation of new species, as the molluscs of the *crassus*-group do not tolerate high salinity [Ercan, Tarkan, 2014]. A comprehensive study demonstrated that *Unio gontierii* is distributed in the water ways of the north-eastern zone of the Black Sea coast, while the water bodies of the south-western part of the Black Sea coast are inhabited by another significantly diverged species of the *crassus*-group, *Unio bruguierianus* Bourguignat, 1853 [Lopes-Lima *et al.*, 2024].

Endemic phylogenetic lineages have been identified in populations of two other freshwater mussels, *Anodonta anatina* (Linnaeus, 1758) and *Pseudanodonta complanata* (Rossmässler, 1835) in the Azov Sea basin; in the Black Sea basin, there are other phylogenetic lineages of these species [Tomilova *et al.*, 2020; Vikhrev *et al.*, 2023].

Two or more phylogenetic lineages have also been identified in a range of fish species which are potential hosts for glochidia of the *crassus*-group mussels inhabiting the basins of both the Black and Azov seas. These are representatives of the genera *Salmo* [Turan *et al.*, 2009; Ninua *et al.*, 2018, 2023; Artamonova *et al.*, 2020], *Proterorhinus* [Sorokin *et al.*, 2011; Slynko *et al.*, 2013], *Barbus* [Levin *et al.*, 2019], *Rhodeus* [Bohlen *et al.*, 2006], *Phoxinus* [Bogutskaya *et al.*, 2023; Artaev *et al.*, 2024]. At the same time, some phylogenetic lineages of potential hosts represent independent species.

It can be assumed that there has been a significant reduction and fragmentation of the regional habitats of both *Unio gontierii* and *Rhodeus colchicus* in recent decades. This hypothesis is based on the data on the coastal reclamation and wetland draining carried out in the 20th and 21st centuries in the Greater Sochi area, including the Imereti Lowland, where there used to be a system of deep overgrown and silt and peat-filled lakes [Zenkovich, 1958]. Moreover, there is the decreased river water availability on the Black Sea coast.

The relict populations of *U. gontierii* inhabiting watercourses and reservoirs of the Russian Black Sea coast undoubtedly deserve protection and can be sensitive bioindicators of water pollution. The Georgian bitterling (*Rh.colchicus*) from these rivers and lakes, which is closely ecologically connected with this mollusc, is listed in the Red Data Book of Krasnodar Region [Tuniev, Tuniev, 2017] and the Red Data Book of the Russian Federation [Vasiljeva, 2021].

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