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Running head: Volga fishes DNA barcoding

Abstract

This study represents the first thorough genetic assessment of freshwater fishes from the Volga River basin – the longest (3,690 km) river in Europe. DNA barcode sequences (*COI*) were studied in 84 species (approx. 90% of all diversity) belonging to 57 genera and 25 families. Totally 1,327 DNA barcodes (1,151 sequences from 202 localities generated by this study) were used to (i) build a DNA barcode reference library and check for the presence of a barcoding gap; ii) assess the genetic and taxonomic diversity of Volga fishes, and iii) clarify the biogeography of Volga fishes. DNA barcodes are reliable means for the identification of over 95% of analyzed species showing barcode gaps. An overlap between intra- and interspecific genetic distances was present in four species of genera *Leuciscus* and *Rutilus*. DNA barcoding identified seven new species for the Volga. Among these are two aggressive alien species (*Hemiculter leucisculus* and *Pseudorasbora parva*), while five are aboriginal species in genera *Salmo*, *Leuciscus*, *Gobio*, and *Cobitis* from neighboring basins, which naturally or human-aided colonized the Volga basin. Moreover, three new phylogenetic lineages (candidate species) were discovered – specifically, in the genera *Leucaspius*, *Phoxinus*, and *Proterorhinus*. While the fish fauna of the Volga is highly mixed, the more ancient Kama displays regional endemism for rheophilic species (e.g., *Barbatula, Cottus*). Likely, the Kama served as refuge during Pleistocene climate oscillations. In sum, generated *COI* dataset fills the existing gap in *COI* database of fishes inhabiting freshwaters of Eastern Europe and will be highly useful in future studies.

Keywords: cryptic diversity, cytochrome *c* oxidase subunit I, Eastern Europe, mitochondrial DNA, molecular identification, phylogeography

Introduction

Biodiversity is a cornerstone for the functioning, sustainability, and efficiency of ecosystems (Bengtsson et al. 2000, Hooper et al. 2005; Tsoupas et al. 2022). Fishes are the most diverse group of vertebrates with a species number exceeding 37,000 (Fricke et al. 2025), although their diversity is still significantly underestimated (e.g. Zemlak et al. 2009; Wang et al. 2020; Tsoupas et al. 2022). Despite freshwater ecosystems cover only 0.8% of the Earth's surface, they disproportionately contribute to approximately half of the global known fish diversity (Barbarossa et al. 2021). In addition, fishes are an important food source that, in turn, need proper management for sustained resource use. Freshwater habitats are more threatened by human activities and environmental change, being more vulnerable compared to those in marine or terrestrial ecosystems (Sayer et al. 2025). Freshwater ecosystems in densely populated areas are degraded faster than in less-altered environments. The human pressure on aquatic ecosystems can result in the extinction of a significant part of fish diversity before it is scientifically documented (Dodds et al., 2013).

DNA barcoding is a well-established tool for species identification, biodiversity assessment, and monitoring of ecosystems, significantly contributing to the understanding of fish species diversity (Hebert and Gregory, 2005; Ward et al. 2009; Knebelsberger et al. 2015; Behrens-Chapuis et al. 2021; Sheraliev and Peng 2021; Tsoupas et al. 2022; Zangl et al. 2022; Appleyard et al. 2025). It is beneficial compared to morphology-based identification especially when cryptic/pseudocryptic species, larval or juvenile stage fish or parts of organisms are analyzed (Steinke et al. 2009). In addition, data on genetic polymorphism of wide-spread species based on DNA barcodes may provide information on population structure, genetic diversity, evolutionary history and might be helpful in the delineation of conservation units. Novel DNA barcoding approach that is based on the environmental source of DNA samples (environmental DNA, or eDNA) is rapidly developing and very promising in non-invasive assessment of biodiversity (Ushio et al. 2018; Snyder et al. 2020; Zhang et al. 2024). However, the accuracy of this approach may be greatly restricted by some factors, among which an absence or insufficiency of a comprehensive reference database is a major constraint (Marques et al. 2021; Xiong et al. 2022).

Volga is the longest European river (3,690 km) with a catchment area of about 1,360,000 sq. km. Volga goes across heterogeneous ecoregions from North to South – forests, forest steppe, steppe, and semi-desert, and borders to the Azov and Black Sea basin, Baltic Sea basin, White Sea basin, Ob' River system (Kara Sea), and the Ural River system in the Caspian Sea drainage (Mineeva et al. 2022). Water ecosystems in the Volga drainage are severely threatened by anthropogenic pressure because of their location in an economically developed and densely populated area - ca. 70 million people dwell in the Volga basin (Mineeva et al. 2022). In addition, the construction of numerous dams on the Volga River in the 20th century drastically altered its hydrological regime and interrupted or shortened the migration routes of anadromous fish, including valuable species of sturgeons (Ruban et al. 2019). All of this makes the indigenous fish fauna of the Volga River highly vulnerable to various direct and indirect human impacts, including the influence of alien species (Panov et al. 2007; Schletterer et al. 2018; Mineeva et al 2022).

Unexpectedly, fish diversity estimates for the Volga basin largely vary in the modern literature – from 79 (Schletterer et al. 2018) and 96 (Dyldin et al. 2020) to 114 (Slynko and Tereshchenko 2014) and even 117 (Froese and Pauli 2025). In our view, the two latter counts are greatly overestimated since they include all cases of historical introductions, naturalization of which has never been reported (e.g., *Oncorhynchus* spp., *C. baunti*, *Parabramis pekinensis* etc.). They also list the species from the surrounding waters in the Caspian Sea but never recorded in the Volga (e.g., *Acipenser persicus*, *Gasterosteus aculeatus* - Froese and Pauli 2025) or mistakenly listed species (e.g., *Proterorhinus marmoratus* that listed along with *P. nasalis*; *Ameiurus nebulosus* - Froese and Pauli 2025), or listed species of uncertain, probably, hybrid origin - e.g. *Alosa suworowi* (see Dyldin et al. 2020). According to our preliminary assessment, the list of aboriginal and naturalized fishes and lampreys inhabiting the Volga basin includes ca. 90 species, of which 78 are indigenous and 12 are considered alien.

In spite of traditionally high interest in the Volga fish diversity, a few genetic studies were devoted to the aboriginal Volga fishes (Mendel et al. 2008; Levin et al. 2016, 2017, 2018; Borovikova 2017; Artaev et al. 2021, 2024). Only 179 sequences of DNA barcodes of *COI* were deposited in the GenBank so far (30 Sept. 2025), 108 of which are focused on invasive fish fauna (Karabanov et al. 2022). Insufficiency of DNA barcodes of Volga's fishes highly impedes implementation of various genetic approaches to identify species for various purposes – monitoring of biodiversity, surveys of endangered or alien species (including eDNA applications), food control, and forensic research.

Our goal was threefold: i) to cover the majority of Volga fish species by *COI* DNA barcodes to provide a wide range of reference sequences, ii) to describe genetic diversity and to update the taxonomy of Volga species, and iii) to clarify the biogeography of Volga fishes based on new knowledge obtained.

Material and methods

Sampling

Fishes were sampled during field studies in 2011-2024. In particular, 1151 specimens from 80 species were collected from 202 sampling sites in all Volga sub-basins according to Mineeva et al. (2022): i) Upper Volga, ii) Middle Volga, iii) Kama, and iv) Lower Volga (Fig. 1; Supplementary File S1). Fin clips or scales were taken and stored in 96% ethanol. For most fin-clips, voucher specimens were stored at the Fish Collection of the Papanin Institute for Biology of Inland Waters of the Russian Academy of Sciences (**IBIW_FS**), Borok, Russia. The DNA samples of rare and endangered species (except for some *Acipenser ruthenus*) were obtained from the collection of the Russian Federal Research Institute of Fisheries and Oceanography, Moscow, Russia.

Specimens were identified in the field or in the lab using the most recent identification keys or species descriptions (Kottelat and Freyhof 2007; Mendel et al. 2008; Neilson, Stepien 2009; Mousavi-Sabet et al. 2011; Bogutskaya et al. 2013; Levin et al. 2016, 2017, 2018; Pereira et al. 2021; Segherloo et al. 2021; Artaev et al. 2024; Turbanov et al. 2025; Vasil'eva et al. 2025). Set of species includes nine Volga species protected by the IUCN Red List of Threatened Species (http://iucnredlist.org) and the Red Book of Russian Federation (2021): *Acipenser ruthenus* (EN), *A. gueldenstaedtii* (CR), *A. nudiventris* (CR), *A. stellatus* (CR), *Huso huso* (CR), *Coregonus albula* (EN - population of Lake Pleshchejevo in the Volga basin is protected by the national Red Book), *Stenodus leucichthys* (NT), *Hucho taimen* (VU), *Salmo caspius* (CR - resident populations in the Middle Volga and Kama are protected by the national Red Book).

DNA extraction, PCR and sequencing

DNA was extracted from 1151 samples belonging to 56 genera and 80 species, using the protocol of Aljanabi and Martinez (1997). The numbering of the samples, the species in which they belong, and their sampling sites are given in Supplementary Table S1. The barcoding fragment of mitochondrial *COI* gene was amplified with Polymerase Chain Reactions (PCR), using an M13-tailed primer cocktail with the primers VF2_t1, FishF2_t1, FishR2_t1, and FR1d_t1 (Ivanova et al. 2007) or LCO 1490 and HCO 1298 primers (Folmer, 1994) for some fish taxa; lampreys were amplified and sequenced using the primers COI-F1 (Ivanova et al., 2007) and COI-2R (Ward et al., 2005). A standard 25 µl PCR mix consisted of 2.5 µl PCR buffer (10x); 2.5 µl dNTP (2 mM); 1.5 µl 'Fish Cocktail' (2 µM); 0.2 Taq DNA Polymerase (5 units per µl); 16.75 µl mQ-H2O and 1.0 µl of the extracted DNA sample. The temperature profile for various amplification protocols is given in Table S2. PCR products were visually checked on 1.5% agarose gels, and purified using 96% ethanol and sodium acetate (3M). Sanger sequencing using M13 primers, LCO1490 and HCO1298 primers as well as COI-F1 and COI-2R primers was performed in IBIW RAS using an ABI's 3500 DNA Analyzer. All sequences were deposited to GenBank under Accession numbers PV840619-PV841769 (Table S1).

Barcoding analyses

Newly obtained barcode sequences along with the data available in GenBank were combined, totaling of 1327 individuals, and a multiple sequence alignment (MSA) was created with MUSCLE 3.8.31 (Edgar 2004). All sequences were then translated to protein and checked for the presence of unexpected stop codons. To examine the clustering of DNA barcode sequences in a gene tree, we used the maximum likelihood (ML) approach in IQ-TREE 2.4.0 (Minh et al. 2020). In total, 312 unique haplotypes were used. The best-fit models and partitioning scheme by codon position were selected with ModelFinder (Chernomor et al 2016; Kalyaanamoorthy et al. 2017) using Bayesian information criterion (BIC) as follows: TIM3+F+I+G4 for codon position 1, F81+F+R2 for codon position 2, and TN+F+R4 for codon position 3. Tree support was accessed with 1 000 ultrafast bootstrap replicates (Hoang et al. 2018) and 1 000 replicates of the Shimodaira–Hasegawa-like approximate likelihood ratio test (SH-aLRT; Guindon et al. 2010).

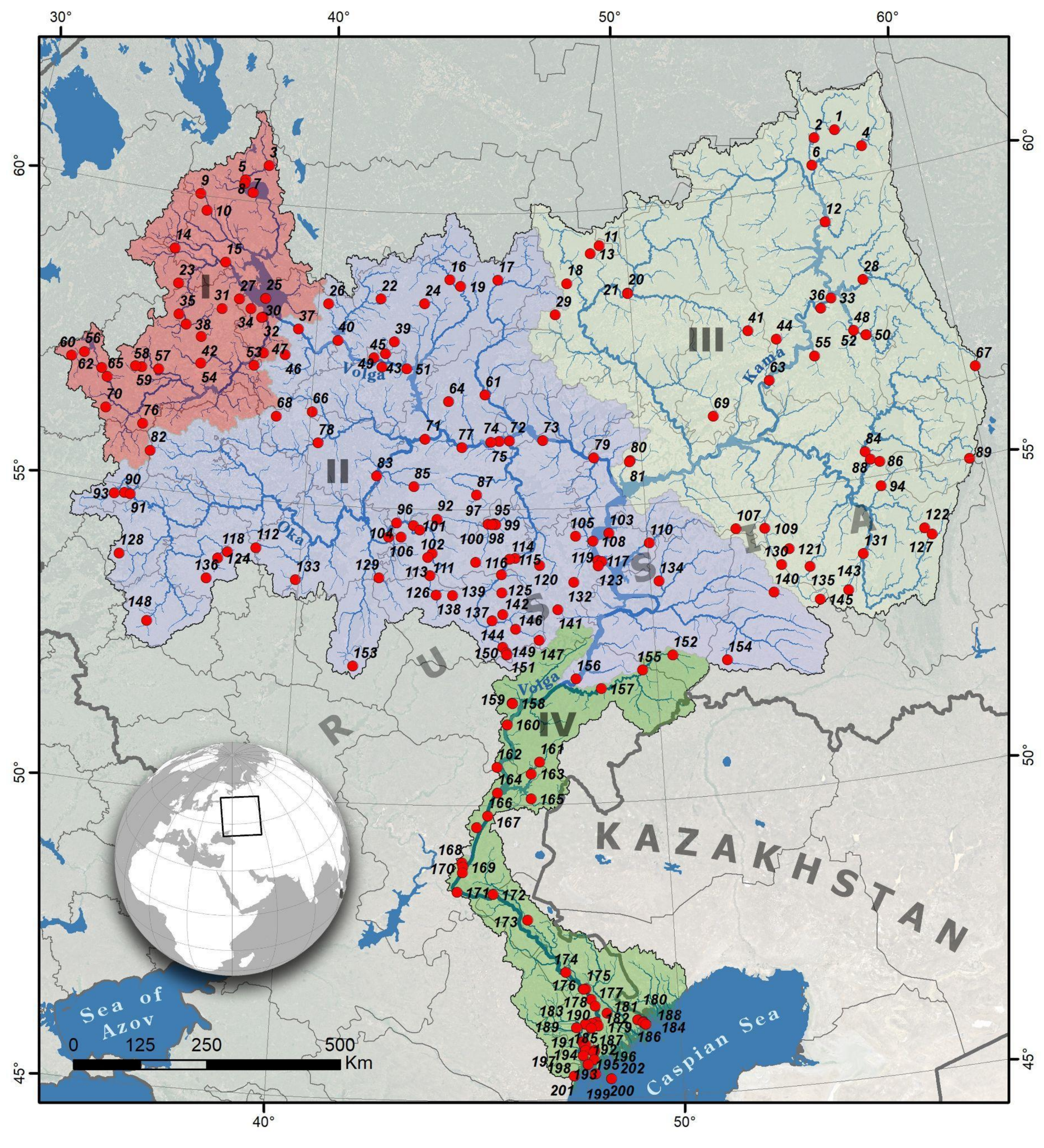

Figure 1. Map of fish sampling localities. Roman numbers designate four Volga's sub-basins: I – Upper Volga, II – Middle Volga, III – Kama, and IV – Lower Volga. Arabic numbers display sampling localities; geographic coordinates and other details of sampling localities are given in Table S1.

To examine the species identification success we applied Best match (BM), Best close match (BCM) and All Species Barcodes (ASB) approaches (Meier et al. 2006) as implemented in SpeciesIdentifier v 1.9 (http://taxondna.sourceforge.net). The Automatic Barcode Gap

Discovery (ABGD) method (Puillandre et al. 2012) for the detection of the “barcoding gap” in the distribution of Kimura 2-parameter (K2P) pairwise distances in the dataset was used. The ABGD was run with the default parameters except for K2P distance (option -d 0). In addition, we used the recently developed Assemble Species by Automatic Partitioning (ASAP) method (Puillandre et al. 2021), which is relying on a hierarchical clustering algorithm of pairwise genetic distances to build species partitions. The ASAP was run on the web server (https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html) with split groups below 0.05 probability and K2P distances. Additionally, we checked the dataset for the presence of the “barcoding gap” (Meyer and Paulay 2005) by calculating the furthest intraspecific distance and the closest non-conspecific distance (“nearest neighbour”) for each individual in the dataset using Spider v. 1.5.1 R-package (Brown et al. 2012).

To show different phylogeny patterns, a haplotype network was constructed using the median joining algorithm (Bandelt et al. 1999) in POPART 1.7 (Leigh and Bryant 2015) with the default value of epsilon (0).

Results

A total of 1151 specimens from the Volga system were successfully newly barcoded in this study. They belonged to 25 families, 56 genera, and 80 species. The comprehensive dataset consisted of our and previously published data totaling 1327 individuals of 57 genera and 84 species (Table 1; Fig. 2; File S3). After alignment and trimming, all barcode sequences were 643 bp long without any ambiguities or missing sites. The mean nucleotide frequencies of the entire dataset were A (24.1%), T (29.1%), G (19.1%), and C (27.8%). The genetic distance K2P within species ranged from 0.0 to 2.4 %.

For 14 species, *Atherina caspia*, *Babka gymnotrachelus*, *Benthophilus granulosus*, *Caspiosoma caspium*, *Gobio caucasicus*, *Hypophthalmichthys molitrix*, *Knipowitschia longecaudata*, *Leuciscus baicalensis*, *Neogobius pallasi*, *Phoxinus* sp., *Ponticola syrman, Pseudorasbora parva, Pungitius pungitius*, and *Vimba vimba*, only a single sequence was obtained. Of all other species, at least two, but mostly substantially more sequences (3–150, average 15.8) were obtained, providing the opportunity to assess intraspecific variation (Table 1, File S4).

Table 1. Summary of basic barcode statistics.

Statistics	Value
Total individuals	1327
This study	1151
Previously published	176
Number of species	84
Number of genera	57
Mean number of individuals per species (range)	15.8 (1-150)
Number of singletons	14
Mean intraspecific K2P distance (range)	0.21% (0.0-2.38%)

Mean interspecific, intrageneric K2P distance (range)	5.69% (0.0-15.77%)

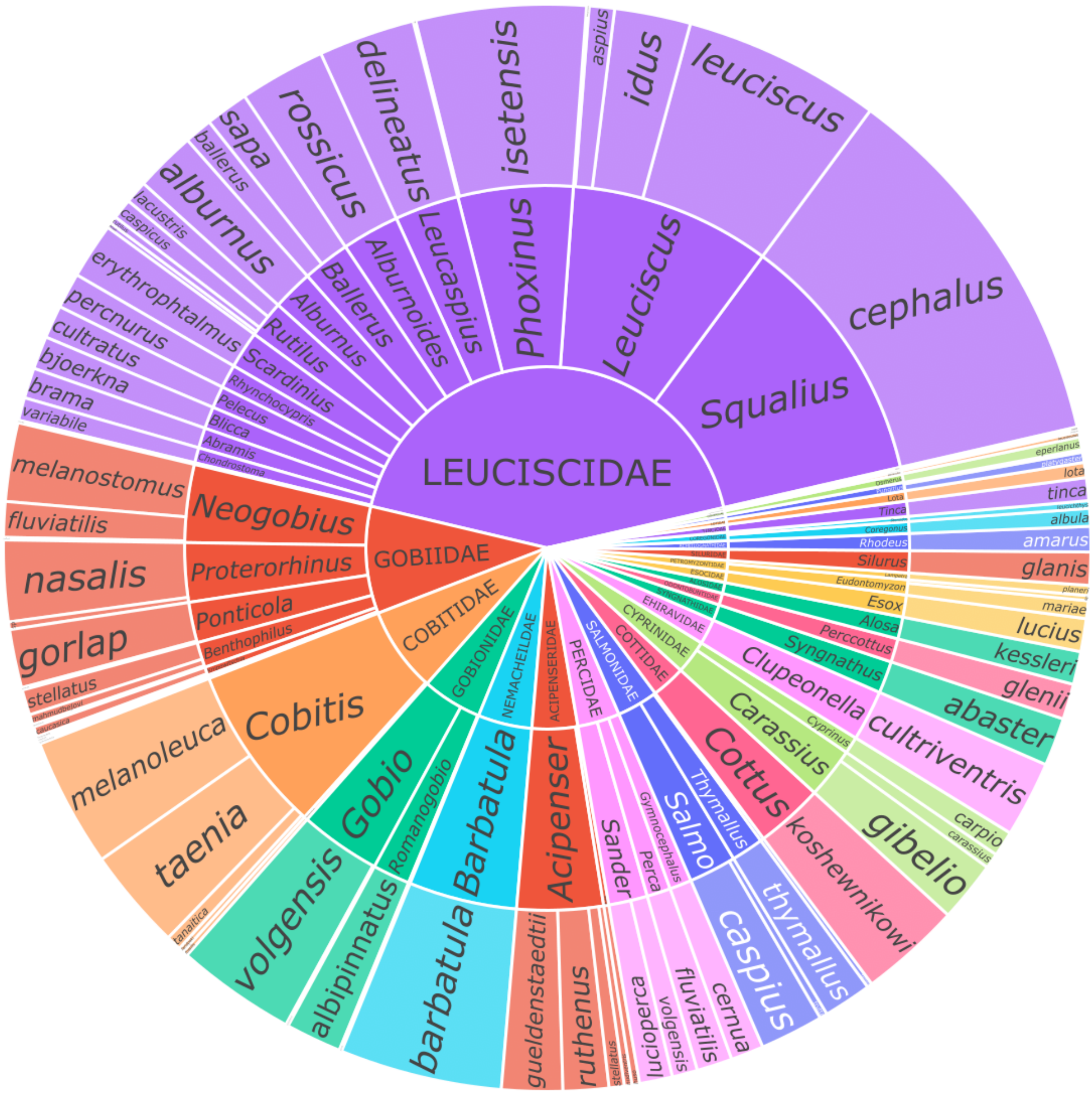

Figure 2. Pie chart showing the taxonomic composition of the examined *COI* dataset of fishes from the Volga River basin by family, genus, and species. Interactive version of the plot is available as a supplementary file S3.

Barcoding Analyses

Identification success rates using BM, BCM, and ABS approaches at 2% K2P threshold distance provided in Table 2. The ASB approach was less powerful with numerous ambiguous identifications (102 out of 1327, 7.68%). A small fraction (16 out of 1327, 1.2%) of ambiguous matches in BM and BCM were in *Rutilus caspius* / *R. lacustris* and *Leuciscus idus* / *L. leuciscus*. All incorrect identifications in BM and absence of any matches closer than the threshold in BCM and ABS were due to the singleton species in the dataset. In BCM and ABS analyses, a single incorrect identification was for *Knipowitschia longecaudata* (identified as *K. caucasica* with 1.09% distance).

ASAP yielded 84 subsets/species with the lowest (best) ASAP score of 7.0, at the threshold distance of 0.035 (see Table S5). The identical subsets were also produced in a range of threshold distances from 0.015 to 0.032. While this number (84) corresponds to the total number of predefined species in the specified dataset, it does not exhibit complete concordance due to the presence of both split and lumped taxa. *Benthophilus* species are lumped into one group, as well as in *Knipowitschia*, *Leuciscus idus / L. leuciscus*, and *Rutilus caspicus / R. lacustrus*. On the other hand, *Carassius gibelio*, *Leucaspius delineatus*, *Perccottus glenii* and *Squalius cephalus* species each split into two groups. The ABGD recursive partitions with prior maximal distance (P) of 0.013, 0.022 returned 80 groups, while a recursive partition with P=0.008 showed 85 groups, which is close to the number of predefined species in the dataset. However, the resulting partitions also split or combined some predefined species (see Table S6). For instance, with 80 groups the species in *Benthophilus*, *Knipowitschia, Leuciscus idus / L. leuciscus, Rutilus caspicus / R. lacustrus,* and *Salmo* are lumped, while *Leucaspius delineatus* split into two groups.

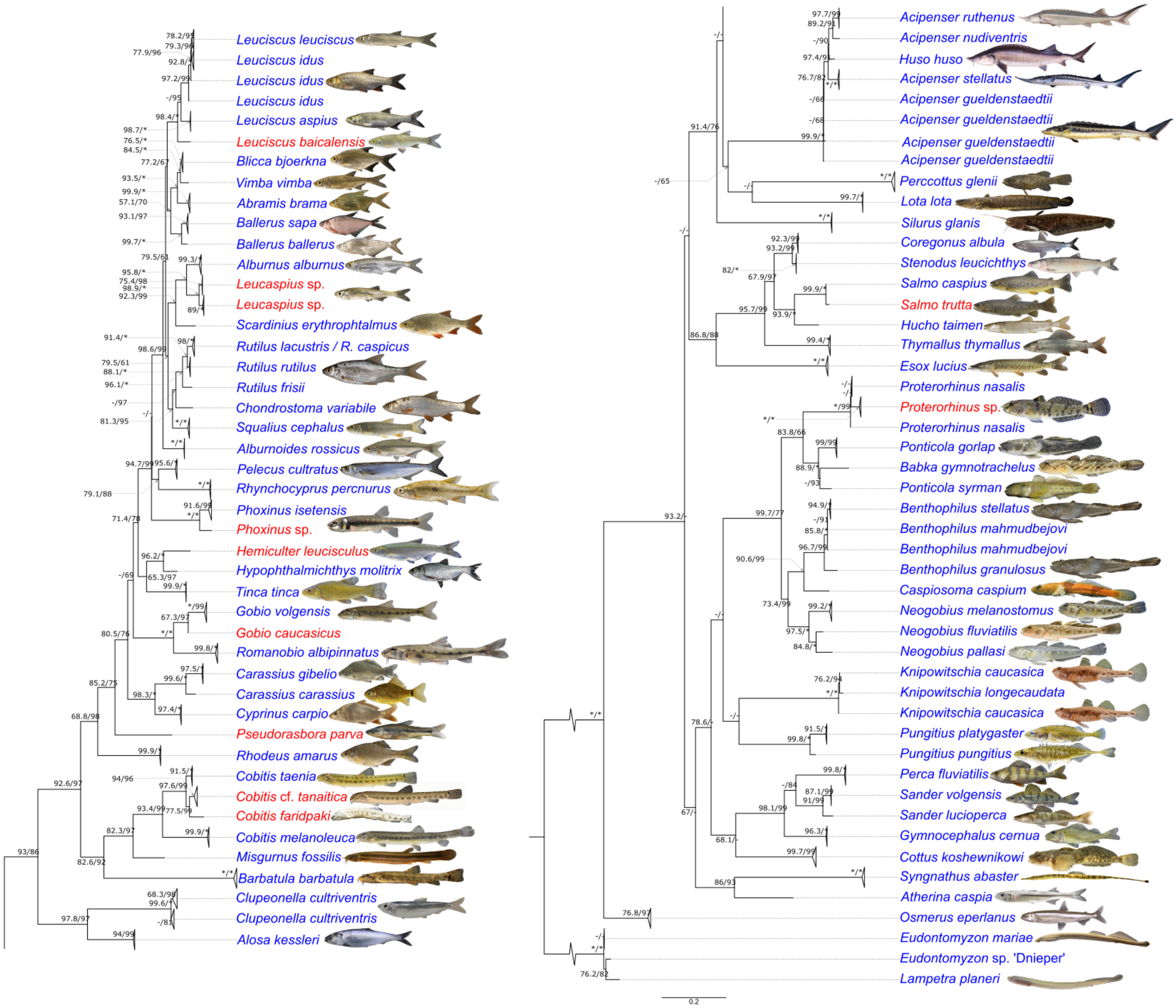

Figure 3. DNA barcode (*COI*) gene tree inferred using the maximum likelihood method. The tree comprises 312 unique haplotypes from 84 species of fish and lampreys from the Volga River basin. Numbers near the nodes are SH-aLRT support (%) / ultrafast bootstrap support (%) above 50%. The units of the scale bar and branch lengths are expected substitutions per site. Red color is for species (or new evolutionary lineages, possibly cryptic species) detected in the Volga basin for the first time.

Table 2. Results of identification success analysis using various criteria. BM – best match, BCM – best close match, ASB – all species barcodes; threshold is 2% K2P distance.

Identification result	BM, %	BCM, %	ASB, %
Correct	97.73	97.73	91.25
Ambiguous	1.2	1.2	7.68
Incorrect	1.05	0.07	0.07
Sequences without any match closer than threshold	NA	0.97	0.97

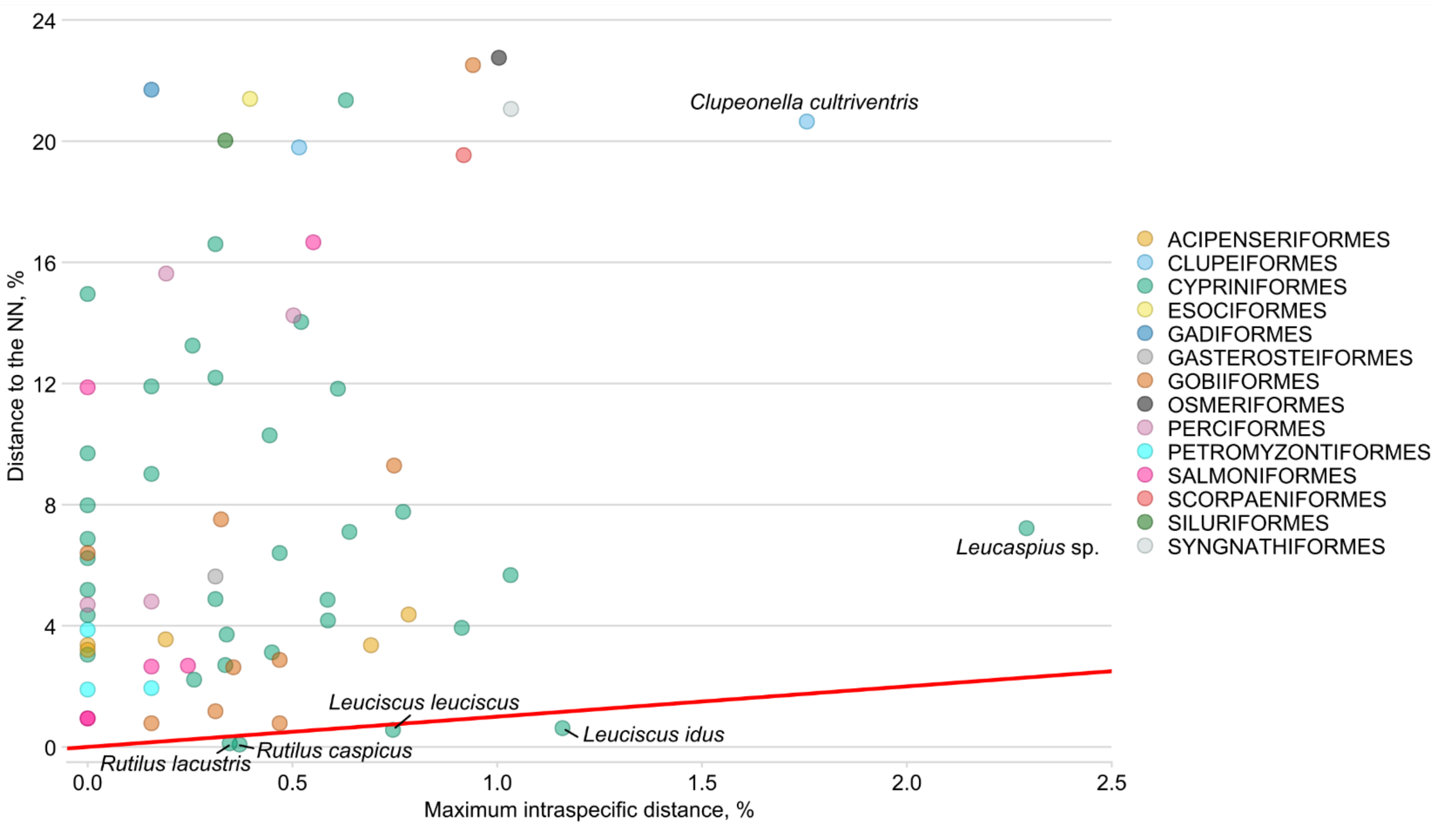

Figure 4. Barcode gap plot of the relationship between the distance to the closest non-conspecific (nearest neighbor) and maximum intraspecific K2P distance calculated as an average for each species with sample size above one. Dots above the 1:1 red line indicate the presence of a barcode gap.

Phylogeography patterns

Various phylogeography patterns of Volga's fishes were revealed. Below we consider four of the most representative examples of phylogeography patterns (Fig. 5). A single haplotype was for the limnophilous species - rudd *Scardinius erythrophthalmus* (n = 22) in spite of its broad geographic distribution. In particular, studied sites are ranged from the Upper Volga (58.4754 N, 35.4646 E) to the Lower Volga (46.2752 N, 47.8622 E) (Fig. 5A). Contrary, highly polymorphic, but geographically not structured haplotype net is for the rheophilic species, common dace *Leuciscus leuciscus* (n = 78; 21 haplotypes). Although the haplotype net of the *L. leuciscus* is complex, fish from the Upper and Middle Volga as well as from the Kama basins share the same haplotypes (Fig. 5B). Another phylogeography pattern – similar to that of *L. leuciscus* in terms of haplotype diversity, but showing some geographical subdivision within the Volga basin – is for more rheophilic species preferring the clear and cold water, the stone loach *Barbatula barbatula* (n = 64; 18 haplotypes – Fig. 5C). In particular, most of the Kama samples are sorted mainly in one of two haplogroups, while samples from the Upper and Middle Volga share both haplogroups (Lower Volga is represented by a few samples). Another pattern of phylogeography - lower haplotype diversity but strong sorting in geography – is displayed by even more rheophilic and more cold-water species, the Koshewnikow's sculpin *Cottus koshewnikowi* (n = 40; 6 haplotypes – Fig. 5D). Its Kama's populations are moderately diverse (three haplotypes) as populations from the Upper and Middle Volga taken together (*Cottus* is absent in the Lower Volga), but Kama and Volga samples do not share the haplotypes (Fig. 5D). Moreover, some populations of the Kama are rather divergent from Volga populations (six mutational steps) that can be considered as species level divergence for European sculpins of the genus *Cottus*, characterized by low interspecific divergence levels in *COI* (Knebelsberger et al. 2015; Sideleva and Zhidkov 2024).

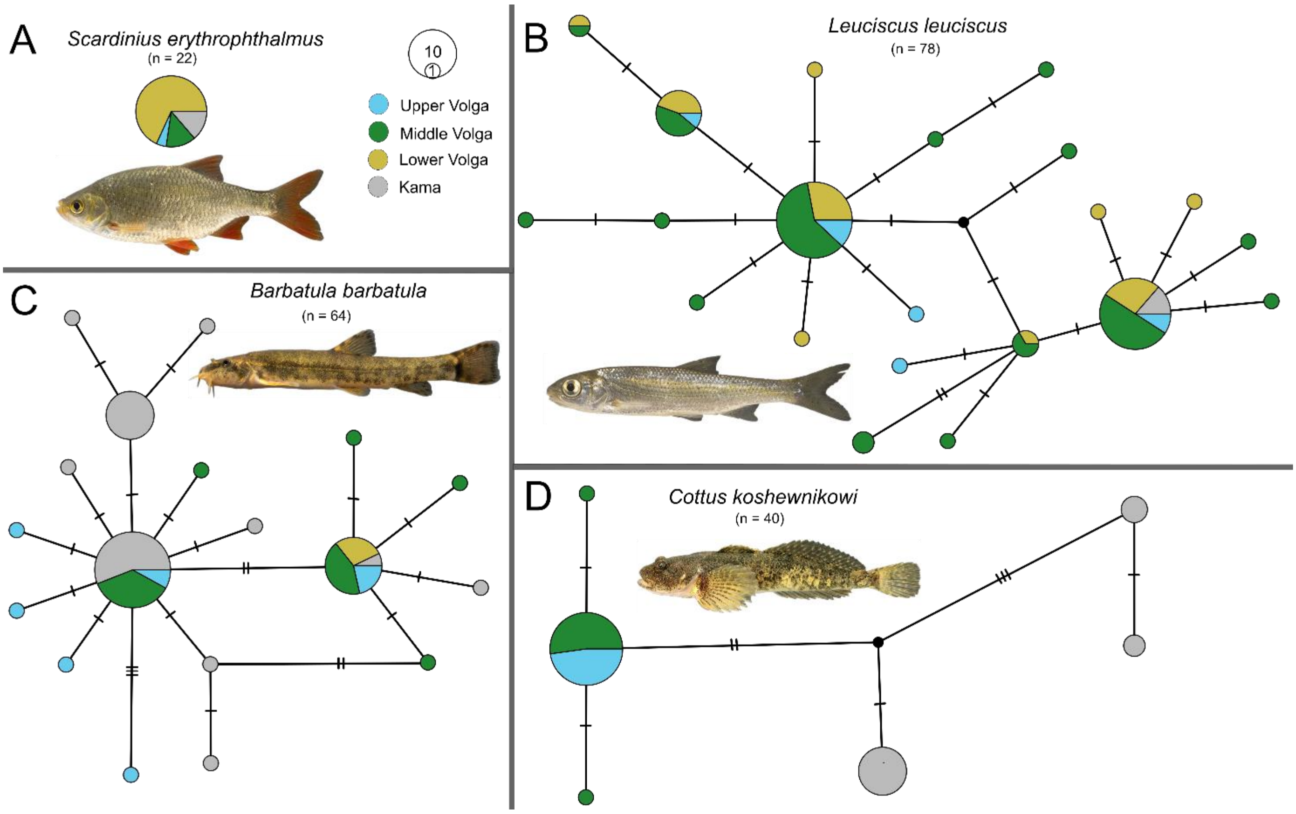

Figure 5. Haplotype networks of four selected species of the Volga basin represented different phylogeographical patterns. **A**. Monomorphic *Scardinius erythrophthalmus* (Leuciscidae); **B**.

Diversified but not geographically structured of *Leuciscus leuciscus* (Leuciscidae); **C**. Diversified and partially geographically structured of *Barbatula barbatula* (Nemacheilidae); **D**. Not diversified but strictly geographically subdivided of *Cottus koshewnikowi* (Cottidae). Black dots represent hypothetical intermediate haplotypes. Each short line signifies a single nucleotide substitution.

Discussion

Our study provided the first detailed and comprehensive genetic assessment of fishes and lampreys inhabiting the largest European riverine basin – the Volga River system. Generated *COI* barcodes showed high identification success (>95% of analyzed species). This is a very high ratio given that the success rate of DNA barcoding in most studies ranges from 76.4% to 100% (e.g., Hubert et al. 2008; Chen et al. 2015; Sheraliev and Peng 2021; Tsoupas et al. 2022; Muhala et al. 2024) and is comparable to that in the study of Zangl et al. (2022) for fish diversity in Austria, Central Europe. Therefore, obtained reference sequences might be used for various DNA-based techniques (including eDNA) for purposes of species identification, biodiversity assessment and monitoring, phylogeography, phylogeny, population genetics studies, as well as conservation genetics and food control. In addition to creating a reference database, our study largely contributed to knowledge on the diversity of fishes in various aspects and clarified the biogeography of Volga fishes. We consider these issues in more detail below.

Novel fish species for the Volga basin

Seven new species for the Volga basin and three new phylogenetic lineages (candidates for new species) from six families were discovered in the Volga system: *Salmo trutta*, *Gobio caucasicus*, *Hemiculter leucisculus*, *Leucaspius* sp., *Leuciscus baicalensis*, *Phoxinus* sp., *Pseudorasbora parva*, *Cobitis faridpaki*, *Cobitis tanaitica*, and *Proterorhinus* sp. Some of these were identified by morphology and confirmed by *COI* barcodes (e.g. *Hemiculter leucisculus*, *Pseudorasbora parva*), while some others were detected primarily by *COI* barcodes (cryptic or pseudocryptic diversity).

New alien species

Hemiculter leucisculus (n=2; see photograph in File S7) was recorded on 23 June 2021 in the Lower Volga (Lagan channel – locality no. 201 in Fig. 1). It is considered one of the very dangerous alien species that quickly establish populations in new areas and may affect local ecosystems (Dong et al., 2020). Previously naturalized populations were detected in the neighboring water systems in the Caspian Sea basin in Azerbaijan as well as the Daghestan Region of Russia (Zardoya and Doadrio 1999; Mustafajev et al. 2015). Novel finding suggests an expansion of *H. leucisculus* in the system of Caspian Sea. Further monitoring of the expansion of this alien species is highly required in the Lower Volga. *Pseudorasbora parva* is another aggressive alien species that is quickly colonizing the Volga basin. One individual (see photograph in File S7) was recorded on 25 August 2024 in the Vershaut River, Middle Volga (locality no. 151 in Fig. 1), which shares watershed with the Don basin tributaries, where this species was known from the previous study (Karabanov et al. 2009). Another study recently reported finding the juvenile of *P. parva* (20 mm standard length) in the estuary of the Volga River although without morphological or genetic confirmation (Velikotskaya et al., 2024). Therefore, the colonization of the Volga by this aggressive alien species might occur via different routes - along the Caspian Sea to Lower Volga (*P. parva* was found recently in the rivers of the Dagestan Region, close to the estuary of the Volga River – Barkhalov et al. 2023) and via human-aided inter-basin fauna introductions in the Middle Volga / Don watershed. The alien species under consideration may rapidly expand their distribution within the Volga and Kama systems as well as colonize the Baltic Sea basin since the Volga serve as an invasive corridor from the Ponto-Caspian basin to the Baltic Sea basin (Dgebuadze, 2011).

New natural (and/or introduced?) species

While the record of new alien species is anticipated in the Caspian Sea basin (Dgebuadze 2011), the uncovering of a significant portion of novel natural diversity in the well-studied Volga basin is surprising. This deserves consideration of each newly recorded species for better understanding of the origin of this diversity. Whether we overlooked natural diversity or detected new species because of their range expansion.

Family Salmonidae

Genus *Salmo*

Apart from previously known resident populations of *S. caspius* that inhabit the Middle Volga and Kama (Red Book of Russian Federation 2021), we detected Atlantic lineage of *S. trutta* according to Bernatchez (2001) widely distributed in the neighboring Baltic and White Sea basins. Moreover, individuals of the *S. trutta* co-occurred with *S. caspius* in the Yamanyelga River in the Kama system (locality no. 84 in Fig. 1) and were different in color pattern (Fig. 6). In this locality, eight samples were identified as *S. caspius*, while three samples were identified as *S. trutta*. The genetic divergence between these two lineages is low as between most of *Salmo* spp. (e.g. Segherloo et al. 2021; Levin et al. 2022) but is enough to detect these species. It is unclear whether *S. trutta* is native to the Volga basin or was introduced and should be considered an invasive species. Another question is whether these two species hybridize in the zone of their secondary contact. Further genetic studies using nuclear markers are needed to clarify this issue. Since all *Salmo* populations in the Volga are threatened, clarifying their taxonomy and genetic distinctiveness is essential.

Figure 6. Resident forms of *Salmo trutta* (**A**) and *Salmo caspius* (**B**) cohabited the Yamanyalga River in the Kama basin. Scale bar: 10 mm. Photo by O. Artaev.

Family Gobionidae

Genus *Gobio*

One individual of gudgeon caught in the upper reach of a small river in the Middle Volga bordering with the Don River basin was identified as *G. caucasicus* having the same haplotype as gudgeons from the Don basin. The Don's gudgeon is considered an independent species *G. brevicirris* (Eschmeyer et al. 2025), but has no genetic difference from *G. caucasicus*, which was described earlier from the Caucasus. Therefore, we preliminarily consider *G. brevicirris* as a junior synonym of *G. caucasicus* but thorough revision is needed. Remarkably, the *G. caucasicus* was detected in the same river (locality no. 150 on Fig. 1) where alien species *Pseudorasbora parva* was (see above), what we consider as the case of repeated human-aided inter-basin introduction.

Family Leuciscidae

Genus *Leucaspius*

The genus is monotypic. The *L. delineatus* was described from the Danube basin in Austria. The Volga's samples are genetically distant to the Danubian samples (K2P distances are over 3%) and can be considered as separated species. Moreover, two lineages within Volga's samples were revealed with K2P distance exceeding 2% (File S8). Since both lineages cohabit throughout the Volga, the revealed cryptic divergence might mirror the past secondary contact as was already demonstrated for the *Rutilus rutilus* and *R. lacustris* within the Volga and Kama system (see Levin et al. 2017; Artaev et al. 2021). Further morphological and genetic studies need to revise the complexity of this genus.

Genus *Leuciscus*

Apart from three known species for the Volga (*L. aspius*, *L. idus*, and *L. leuciscus*), a fourth species – the Siberian dace *L. baicalensis* – was recorded in the Chusovaya River, Kama basin. The main range of this species is located in Siberian rivers drained to the Arctic Ocean. Although the Chusovaya River belongs to the Kama-Volga basin, its upper reaches are located in Siberia, east of the main Ural ridge and sharing a watershed with the Ob basin (locality no. 67 in Fig. 1). Its first finding in the Chusovaya River extends its range to the Volga-Kama system and further highlights the important role of the Chusovaya River as a waterway for fauna exchange between Europe and Siberia (Makhrov et al. 2021; Artaev et al. 2024)

Genus *Phoxinus*

For a long time, *P. phoxinus* was listed for the Volga basin. Recent revision showed that *P. isetensis* inhabits the Volga-Kama system in place of *P. phoxinus* (Artaev et al. 2024). This study revealed novel lineage in the upper reaches of the Oka basin (locality no. 136 in Fig. 1), which is highly divergent from the *P. isetensis* (5.9%). This cryptic diversity needs further investigation. We consider *Phoxinus* sp. from the Oka basin as a candidate species.

Family Cobitidae

Genus *Cobitis* Linnaeus, 1758

Two species of the genus *Cobitis* were known from the Volga – *C. taenia* and *C. melanoleuca* (Bogutskaya et al. 2013; Vasil'eva et al. 2020). In addition, this study revealed two new species for the Volga basin: i) *C. tanaitica* and ii) *C. faridpaki*.

Cobitis tanaitica is distributed in the basins of the Black Sea and Sea of Azov, but was found to be rather widely distributed in the Lower and Middle Volga as well as Kama systems by this study (Table S1). We suggest that this species might colonize the Volga basin during one of the pluvial post-glacial periods of Pleistocene-Holocene when connection between Volga and adjacent basins occurred. The routes of dispersal of the *C. tanaitica* are likely similar to those revealed for the *Eudontomyzon mariae* colonized Middle Volga from the Don basin and *Eudontomyzon* sp. 'Dnieper' colonized Middle Volga from the Dnieper basin (Levin et al. 2016). Likely, *C. tanaitica* also inhabits the part of the Western Dvina basin, Baltic Sea drainage (Vasil'ev and Vasil'eva 2022). The *C. tanaitica* is a species involved in the interspecific hybridization with other *Cobitis* spp. and generation of the diploid–polyploid hybrid complexes and the successive polyploidization with transitions from sexuality to asexuality experienced by triploid *Cobitis* hybrids (Vasil'ev et al. 1989; Choleva et al. 2014).

Cobitis faridpaki is a species inhabiting the rivers draining to the Southern Caspian Sea in Iran (Mousavi-Sabet et al. 2011). Our samples were identified as *C. faridpaki* via BLAST service (https://blast.ncbi.nlm.nih.gov/Blast.cgi) having a similarity of 99.69 % to the samples from the Iranian part of the Caspian basin – the Babol Rud, Karaj River, and Tajan River (MK506143; MK506158; KY646316). Our finding of this species in the estuary of the Volga River (locality no. 198 in Fig/ 1) greatly expands the range of this species. We suggest two explanations for the record of this species far from its known range. First, the species might be shipped with ballast waters from the Southern Caspian Sea to the Northern Caspian Sea, and therefore it is an alien species in the Volga system. Alternatively, this species may naturally have a wider distribution around the Caspian Sea than previously recognized. Given recently described *C. amphilekta* is widely distributed in Kazakhstan and Azerbaijan parts of the Caspian Sea basin (Vasil'eva and Vasil'ev 2012), one may suggest that *C. amphilekta* is conspecific to *C. faridpaki*, which is broadly distributed around the Caspian Sea. No DNA sequences of *C. amphilekta* are yet available to test this hypothesis.

Family Gobiidae

Genus *Proterorhinus*.

Apart from *Proterorinus nasalis*, widely distributed in basins of Caspian Sea and Sea of Azov (Vasil'eva et al. 2025), new unrecognized lineage *Proterorhinus* sp. not matching to any yet described valid species (*P. blennioides*, *P. semipellucidus*, *P. victori*, *P. marmoratus*, *P. semilunaris*) was discovered in Volga's estuary (locality nos. 186, 198 in Fig. 1). Genetically it is identical to candidate cryptic species *Proterorhinus* sp. recorded by Neilson and Stepien (2009) in the Chernozemelskiy canal (system of Manych-Don, Sea of Azov basin). The species status of this genetic lineage as well as its evolutionary history and routes of dispersal to the Volga basin is yet to be clarified.

Species with no barcoding gap

No barcoding gap between some species of the genera *Leuciscus* and *Rutilus* was detected. In particular, low K2P distance (0.9%) was revealed between the sister pair of *L. leuciscus* and *L. idus*. For this pair of species, hybridization and mitochondrial replacement have been reported (Hänfling and Brandl 2000; Zangl et al. 2022). Low levels of genetic divergence might also result from the past hybridization between ancestral lineages. The minute distance between *R. lacustris* and *R. caspicus* in mtDNA (0.2%) is not surprising since it was already reported based on cytochrome *b* sequences of mtDNA (Levin et al. 2017). The possible explanation for this is recent hybridization or recent speciation. All hypotheses explaining low mitochondrial divergence between phenotypically different species should be tested further using nuclear DNA data.

Deep intraspecific distance – cryptic diversity or traces of the past hybridization?

Five species showed relatively high intraspecies genetic distance. In particular, the Black and Caspian Sea sprat *Clupeonella cultriventris*, an alien species in the Volga basin, is genetically very diverse (2.21%). It points out i) the complex evolutionary history of the species, and/or ii) several donors of invasion to the Volga basin (see also Karabanov et al. 2025).

The belica '*Leucaspius delineatus*' inhabiting the Volga basin is greatly different from *L. delineatus* in the type basin, the Danube River (see above) and might be a separate species. But Volga hosts two divergent lineages of *Leucaspius* (2.38%), which, however, are not well grouped geographically (File S8). Most likely, the Volga basin is a zone of secondary contact of previously isolated lineages like fishes of the *Coregonus* and *Rutilus* spp. (Levin et al. 2017; Borovikova and Artamanova 2018). Alternatively, they might be the cryptic species. Both hypotheses should be tested further in morphological and genetic studies.

The ide *Leuciscus idus* showed a rather large intraspecific distance (1.42%) that is over the interspecific distance when compared to sister species *L. leuciscus* (see above). The possible reason is the past hybridization with *L. leuciscus* and secondary contact of half-isolated ancient lineages of *L. idus* sensu lato.

The Northern minnow *Phoxinus isetensis* has a moderately large intraspecific distance (1.26%). This species has the widest range among European *Phoxinus* minnows and likely geographically subdivided populations or sub-lineages (Artaev et al. 2024).

The Koshewnikow's sculpin *Cottus koshewnikowi* in the Volga system has a rather large intraspecific distance (1.10%) that is geographically structured (Volga and Kama). The cryptic diversity within this species needs further investigation.

Biogeographical implications

Biogeography of the Volga's fishes requires a separate thorough study. In this paper, we highlight most important biogeographical issues revealed by this study. First, our results confirm the numerous inter-basin contacts and fish fauna exchange with the adjacent basins postulated previously (e.g., Marić et al. 2014; Levin et al. 2016; Makhrov et al. 2021). In particular, results of this study corroborate colonization of the Volga system by species from the following riverine basins: i) Arctic Ocean drainage via Siberian Ob' basin in the Kama-Chusovaya corridor – record of the Siberian dace *Leuciscus baicalensis*; ii) Black Sea and Sea of Azov via the Don and Dnieper watersheds – records of *Gobio caucasicus*, *Cobitis tanaitica*, *Phoxinus* sp. and likely *Proterorhinus* sp.); iii) Southern Caspian basin – record of *Cobitis faridpaki*. Based on newly obtained and published data (e.g., Levin et al. 2016, 2017; Makhrov et al. 2021; Artaev et al. 2024) on multiple and multidirectional past colonization of the Volga River by various species from adjacent riverine and brackish water systems, we consider the Volga a large-scale melting pot. Likely, the mixing of fish fauna also resulted in the rise of a permanent hybrid system of reticulate speciation with diploid-triploid-tetraploid in the genus *Cobitis* (Vasil'ev et al., 1989), or in homoploid hybridization in the genus *Rutilus* (Levin et al. in press) along with the genus *Coregonus* (Borovikova and Artamonova 2018). We ensure the upcoming studies will include new instances of interspecific hybridization in the Volga River basin.

Despite significant mixing of the fish fauna in the Volga basin caused by frequent inter-basin interactions during Pleistocene glaciations and deglaciations (Levin et al., 2017; Makhrov et al. 2021; this study), some indications of regional endemism were also found. In particular, the sculpins *Cottus* show a divergence of the Kama basin populations from the

Volga populations at a level supposedly sufficient for the delimitation of a new species, albeit this group is weakly variable in *COI* (e.g., Sideleva and Zhidkov 2024). Species of the genera *Barbatula*, *Phoxinus* and *Leucaspius* also demonstrate some geographical structure. The first two shows that their Kama's populations are semi-isolated (recorded in Kama and the part of the Middle Volga that is close to the Kama). More studies involving larger sets of the samples need to clarify issues of local endemism or delimitation of geographically unique populations within the Volga and Kama riverine systems. Generally, high endemism of fish fauna in the Kama riverine system revealed by this study is highlighted by the more ancient age of the Kama River (paleo-Kama) compared to the Volga River (Sokolov 1952). Consequently, the Kama River system might serve as both a corridor for fish fauna exchange with Arctic Ocean drainage and as a refuge for endemic lineages during Pleistocene glaciations.

Conclusions

This study highlights the utility of barcoding for species identification of fish and lamprey species inhabiting the Volga River system. The presence of a barcoding gap for over 95% of analyzed species confirmed the informativeness of the COI marker for species identification within the Volga basin. Therefore, generated COI sequences reference database can be successfully used in various disciplines ‑ taxonomy, phylogeography, evolutionary history, biogeography, conservation genetics, food control along with studies using different types of DNA – museum DNA, ancient DNA in zooarcheological studies, eDNA in studies of detection and monitoring of rare, endangered or alien species. In particular, this study using the COI sequences has discovered seven new species for the Volga basin not recorded previously and three novel evolutionary lineages, potentially new species for science. In addition, we showed divergent phylogeography patterns reflecting different evolutionary history of species and highlighted the biogeography of the large water system in Eastern Europe. The generated COI data set fills the large gap in the COI reference library of fishes inhabiting water bodies of Eastern Europe and will largely contribute to the subsequent studies of European fish fauna.

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Competing interests

The authors have declared that no competing interests exist.

Data Resources

All sequences were deposited to GenBank under Accession numbers PV840619-PV841769.

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