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# Molecular phylogeny and taxonomy of roaches (*Rutilus*, Leuciscidae) in the southern part of the Caspian Sea

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## ABSTRACT

The aims of this study were to testify a phylogenetic hypothesis for the genus *Rutilus* in the Southern Caspian Sea using sequence variation of mitochondrial and nuclear genes and to define the taxonomic status of phylogenetic clades represented in this region. Phylogenetic analysis was based on the variability of mitochondrial genes cytochrome *b* and cytochrome *c* oxidase subunit I and nuclear recombination activating gene 1 and inter-photoreceptor retinoid-binding protein. Compared to previous studies, this phylogenetic analysis included significant material from the South Caspian, both from the collected samples and from GenBank data. As a result, only two species of *Rutilus* were confirmed in the ichthyofauna of the studied region. The first of them corresponds to *R. lacustris*, therefore, materials from the South Caspian can be included in genetically identified vouchers of this species to determine its morphological diagnostic features. The second species represented separate sister phylogenetic lineage for the Pontic populations of *R. frisii* sensu lato. Due to the low genetic distance between the Caspian and Pontic lineages and the strong overlap of their morphological features, but previously proven divergence in separate Pleistocene refugia, the Caspian populations are classified in this study as a subspecies *R. frisii kutum.*

**Keywords:** Ponto-Caspian region, Leuciscinae, *Rutilus lacustris*, *Rutilus frisii kutum*, Mitochondrial and Nuclear genes.
**Article type:** Research Article.

## INTRODUCTION

Fish species of the genus *Rutilus* Rafinesque, 1820 belong to the phylogenetic Leuciscine lineage, which is widespread across Eurasia and diverged from other leuciscine lineages approximately 21 MYA, according to the molecular clock (Perea *et al*. 2010). A total of 12 nominal taxa (species, subspecies and varieties) currently included in this genus have been historically described from the Caspian basin (see Fricke *et al*. 2022). Six of them were accepted as valid species or subspecies by Berg (1949): *Rutilus atropatenus* Derjavin, 1937, *R. rutilus schelkovnikovi* Derjavin, 1926, *R. rutilus fluviatilis* Yakovlev, 1873, *R. rutilus caspicus* Yakovlev, 1870 and *R. frisii kutum* (Kamensky, 1901). The latter author considered these populations from the Upper Volga drainage as nominotypical *R. rutilus rutilus* (Linnaeus, 1758),

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described from European lakes. "Using the phylogenetic species concept (PSC)" Kottelat (1997, p. 1) included the first three subspecies names in the synonyms of *R. rutilus,* while the last one in the synonyms of *R. frisii*. This decision was adopted by Bogutskaya (1998) for fishes of Russia. Later, Bogutskaya & Naseka (2004) recognized three species in Russian fresh and brackish waters: *Rutilus caspicus*, *R. frisii* (Nordmann, 1840) with Caspian subspecies, *R. frisii kutum*, and *R. rutilus* with Siberian subspecies *R. rutilus lacustris* (Pallas, 1814). Kottelat & Freyhof (2007) distinguished *R. caspicus* (vobla) from *R. rutilus* (roach) by terminal mouth, pectoral, pelvic and anal fins orange to red and iris from yellow to deep red in *R. rutilus* compared to sub-terminal to inferior mouth, hyaline to dark grey fins and whitish to pale yellow iris in *R. caspicus*. They separated *R. frisii* (both Caspian and Pontic populations combined) on the basis of an almost cylindrical body and 59-70 total lateral line scales, in contrast to compressed body and 41-46 scales in the other Black Sea and Caspian species. The identification key for Caspian *R. rutilus*, *R. kutum* and *R. caspicus* was further developed by Bogutskaya *et al.* (2013). An alternative taxonomic concept (Vasil'eva & Luzhnyak 2013; *Parin et al*. 2014) assumed the validity of the subspecies *R. frisii kutum* and the only species of roach, *R. rutilus* with such conspecific ecological forms as semi-anadromous Caspian vobla and Pontic taran - *R. caspicus* and *R. heckelii* (Nordmann, 1840) sensu Kottelat & Freyhof (2007). Earlier molecular studies on phylogeny and phylogeography of the genus *Rutilus* were focused on the western part of its range (Ketmaier *et al*. 2008; Kotlik *et al.* 2008; Larmuseau *et al*. 2009; Tsoumani *et al*. 2014). Nevertheless, a phylogeographic analysis of anadromous *R. frisii* sensu lato, based on sequence variation at two nuclear and one mitochondrial gene (Kotlik *et al*. 2008) suggested that the Black and Caspian seas have supported separate populations during the last glaciations. The latest gene migration from the Caspian Sea to the Black Sea occurred on average 250,000–450,000 years ago and migration in the opposite direction took place on average 270,000–480,000 years ago. The authors concluded that the two 'forms' maintained refugial populations in both the Black and Caspian seas and diverged despite periods of migration between them. These data together with some clear morphological differences gave reasons for considering *R. kutum* and *R. frisii* as distinct species (Naseka & Bogutskaya 2009), although the Caspian and Pontic populations were not reciprocally monophyletic at the analyzed loci, and the genetic results suggested a certain gene flow between them in recent times (Kotlik *et al*. 2008). However, Kuljanishvili *et al*. (2020) stated that since their own unpublished molecular data were unable to distinguish Caspian *R. kutum* from Black Sea *R. frisii*, they regarded them as synonyms. Further phylogenetic studies, which included sequences of *Rutilus* species, used single samples of the Caspian *R. frisii* and did not address the problems of its taxonomic status (Larmuseau *et al*. 2009; Levin *et al*. 2017; Schönhuth *et al*. 2018). Moreover, they unexpectedly discovered specimens phenotypically identified as *R. frisii*, but inserted into the clade formed by mitochondrial haplotypes of roaches, and vice versa; these events were explained by possible introgressive hybridization of mitochondria (Kotlik *et al*. 2008; Levin *et al*. 2017). In addition, Ketmaier *et al*. (2008) found very low mitochondrial genetic divergence between *R. caspicus* and Caspian *R. frisii*, which seemed puzzling to them.

Significant results for assessing the status of nominal eastern *Rutilus* species were obtained in phylogenetic analysis based on extended volume of cytochrome *b* sequences from Eastern Europe to Eastern Siberia (Levin *et al*. 2017). Three major phylogenetic clades were detected: (i) *R. frisii*, (ii) *R. rutilus* s. stricto and (iii) group of six Ponto-Caspian nominal taxa: *R. caspicus*, *R. heckelii*, *R. rutilus aralensis* Berg, 1916, *R. rutilus lacustris* (Pallas, 1814), *R. schelkovnikovi* and *R. stoumboudae* Bianco & Ketmaier, 2014. The authors concluded that these taxa can be considered as the same species: *R. lacustris* according to the priority of description. This species is most widely distributed among others and covers the freshwaters from the Aegean Sea basin to the Laptev Sea tributaries, sympatrically found with *R. rutilus* s. stricto in the Black and Caspian seas basins, the Sea of Azov itself, and even in the White Sea system. The vastest zone of contact (about 1700 km) was discovered in the Volga basin (Levin *et al*. 2017). However, Caspian populations were poorly represented in all of the aforementioned phylogenetic studies with the complete absence of roach samples from the southern Caspian, where most of studies were devoted to the fisheries and aquaculture aspects (Rashidi *et al*. 2012; Vajargah *et al.* 2014, 2021; Sattari *et al*. 2019a, b, c; Sattari *et al*. 2020; Forouhar Vajargah *et al*. 2020a, b; Pourshabanan *et al*. 2020; Forouhar Vajargah *et al*. 2021; Pourshabanan *et al*. 2021a, b). The only phylogenetic studies in the southern Caspian (Gharibkhani *et al*. 2011; Chakmehdouz Ghasemi & Behmanesh 2015) showed a high genetic distance from the point view of the cytochrome *b* gene between *R. rutilus caspicus* and *R. frisii kutum* adopted according to Coad (1995). Most recent Iranian fish checklists included two species of the genus *Rutilus* for this area: *R. lacustris* and *R. kutum,* with comments on the need for further study on the

Caspian *Rutilus* populations (Esmaeili *et al*. 2018; Abbasi *et al*. 2019). Some authors (Jouladeh-Roudbar *et al*. 2020) added *R. rutilus* with the remark that its presence in Iran water needs confirmation. In addition, previous reviews recognized three species in Iran: *R. caspicus, R. rutilus* and *R. kutum* (Esmaeli *et al*. 2010; Jouladeh-Roudbar *et al*. 2015; Coad 2016); morphological and genetic variability were described for these species (Kavan *et al*. 2009; Abdolhay *et al*. 2010, 2012; Chakmehdouz Ghasemi *et al*. 2014; Coad 2016; Safari 2016; Kashiri *et al*. 2018; Ghojoghi *et al*. 2018). Most authors accepted *R. caspicus* as valid species or subspecies of *R. rutilus* (Chakmehdouz Ghasemi & Behmanesh 2015; Mirzajani *et al*. 2016; Keivani *et al*. 2016). Main goals of this study are: 1) to testify a phylogenetic hypothesis for the genus *Rutilus* in the southern Caspian Sea using sequence variation of both mitochondrial and nuclear genes and 2) to define the taxonomic status of phylogenetic clades represented in the southern part of the Caspian.

## MATERIALS AND METHODS

### Materials for genetic analysis

A total of 66 specimens of Leuciscine fishes of the genus *Rutilus* were collected using hand-nets in April 2018 from Aras reservoir (39°06'33"N, 45°22'33.9"E) and Anzali Wetland (37°27'19"N, 49°21'30.5"E) in Northern Iran (Fig. 1). A total of 22 specimens from Anzali Wetland with a total body length (TL) of 467–590 mm and a standard length (SL) of 407-511 mm possessed 49 (1 spec.), 50 (1 spec.) 51-62 total lateral line scales (LL; average 55.9). These fishes corresponded to *R. frisii kutum* sensu Berg, 1949 or *R. kutum* s. Bogutskaya *et al*. 2013 and Coad 2016. Another 22 fish samples from Anzali Wetland with 155-202 mm TL, 119-164 mm SL and LL = 41-46 (43.7 on average) were therefore identified as *R. rutilus* s. Berg, 1949 (including subspecies) and Coad 2016 and corresponded to the Ponto-Caspian group of *R. rutilus* – *R. caspicus* – *R. heckelii* s. Kottelat & Freyhof 2007 and Caspian *R. rutilus* – *R. caspicus* according to Bogutskaya *et al*. 2013, as well as 22 specimens from Aras Reservoir with 224-248 mm TL, 175-196 mm SL, and LL = 41-43 (42.1 on average).Fin clips from the collected specimens, stored in 99.7% ethanol and transferred to the genetic laboratory, were used as a source of DNA for genetic analysis. Voucher specimens were fixed in a 10% formaldehyde solution and deposited in the Zoological Museum, Ferdowsi University of Mashhad (ZMFUM), Mashhad, Iran.

### DNA extraction, PCR amplification and sequencing

DNA was extracted from pieces of fins according to the salt method protocols (Aljanabi & Martinez 1997). For molecular analysis, we used mitochondrial and nuclear markers, which showed their utility in previous genetic studies on *Rutilus* (Ketmaier *et al*. 2008; Perea *et al*. 2010; Levin *et al*. 2017; Ermakov 2017; Schönhuth *et al*. 2018). Mitochondrial genes included complete cytochrome *b* (CYTB, 1140 bp) and cytochrome *c* oxidase subunit I (COX1, 647 bp); nuclear genes comprised recombination activating gene 1 (RAG1, 1473 bp from exon 3) and interphotoreceptor retinoid-binding protein (IRBP, 950 bp). The polymerase chain reaction (PCR) was conducted under the conditions used in Perea *et al*. (2010) for CYTB, COX1 and RAG1, and from Chen *et al*. (2008) for IRBP.

### Data analysis

Obtained chromatograms were checked and gaps in the sequences were corrected for all genes. In addition, homologous regions for three coding genes were aligned based on the inferred amino acid sequence. Totally, we obtained DNA samples from 9 individuals: 3 from *R. kutum* of Anzali Wetland, along with 6 from *R. caspicus* of Aras Reservoir (n = 3) and Anzali Wetland (n = 3). All 36 obtained sequences were deposited in GenBank under the following accession numbers: MT755376-81 for CYTB, MT777458-63 for COX1, MT777467-72 for RAG1 and MT777476-81 for IRBP from *R. caspicus*, and MT755382-84 for CYTB, MT777464-66 for COX1, MT777473-75 for RAG1 and MT777482-84 for IRBP from *R. kutum*. In addition to our data, we included the analysis of 59 sequences obtained from GenBank, of which seven represented materials from the southern Caspian in Iran and 52 belonged to other parts of the Palearctic. The European chub *Squalius cephalus,* the common nase *Chondrostoma nasus* and the asp *Leuciscus aspius* were selected as outgroups (Fig. 1; Table 1). Inter- and intra-clade distances were calculated using ExcaliBAR software (Aliabadian *et al*. 2014). We determined the best-fit models of molecular evolution for the nucleotide alignment dataset using Jmodeltest v.2.1.10 (Darriba *et al*. 2012).

**Table 1.** List of RAG1, IRBP, CYTB and COX1 sequences of *Rutilus* species downloaded from NCBI GenBank.

| **Species** | **Locality** | **GenBank Acc. No** | | | | **Reference** |
|---|---|---|---|---|---|---|
| | | RAG1 | IRBP | CYTB | COX1 | |
| *Rutilus aula* | Zrmanja River, Adriatic Sea slope, Croatia | MG806207 | MG806540 | FJ824719 | MG806870 | Schönhuth *et al.* 2018 |
| *Rutilus aula* | Bascica lake, Croatia | HM998712 | | HM560157 | HM560322 | Perea *et al.* 2010 |
| *Rutilus basak* | Trebi at River, Neretva Drainage, Bosnia and Herzegovina | MG806208 | MG806541 | FJ824723 | MG806871 | Schönhuth *et al.* 2018 |
| *Rutilus basak* | Krupa River, Hutovo Blato, Neretva basin, Bosnia and Herzegovina | HM560438 | | HM560159 | HM560324 | Perea *et al.* 2010 |
| *Rutilus pigus* | Adda River, Po River Basin, Italy | | | HM560163 | HM560327 | |
| *Rutilus pigus* | Adda River, Po River Basin, Italy | | | HM560162 | HM560326 | |
| *Rutilus virgo* | Sava River, Danube Drainage, Black Sea slope, Croatia | MG806215 | MG806548 | FJ824730 | MG806878 | Schönhuth *et al.* 2018 |
| *Rutilus virgo* | Dyje River, Czech Republic | | | KU950742 | | Jurajda & Pavlov 2016 |
| *Rutilus rutilus* | Tech River, Ortafa, Tech Drainage, Mediterranean Sea slope, France | MG806214 | MG806547 | MG806695 | MG806877 | Schönhuth *et al.* 2018 |
| *Rutilus rutilus* | Sazava River, Elba basin, Czech Republic | HM560440 | | HM560167 | HM560329 | Perea *et al.* 2010 |
| *Rutilus rutilus* | Pazincica River, Istria, Croatia | | | HM560168 | HM560330 | |
| *Rutilus rutilus* | Nero Lake, Middle Volga, Russia | | | KX583865 | | Levin *et al.* 2017 |
| *Rutilus lacustris* | Struma River at Lithotopos, Aegean Sea slope, Greece | MG806210 | MG806543 | MG806693 | MG806873 | Schönhuth *et al.* 2018 |
| *Rutilus rutilus lacustris* | Ubse-Nur Lake, Yakutia, Russia | | | KX583994 | | Levin *et al.* 2017 |
| *Rutilus rutilus lacustris* | Lake Okunyovoe, Chara River, Tributary of Olyokma River, Russia | | | KX583987 | | |
| *Rutilus rutilus lacustris* | Irtysh River, China | | | | KT716365 | Yang *et al.* 2016 |
| *Rutilus rutilus caspicus* | Bandar Torkaman, Iran | | | KF056854 | | Chakmehdouz Ghasemi & Behmanesh 2015 |
| *Rutilus rutilus caspicus* | Lamir River, Iran | | | KF056855 | | |
| *Rutilus rutilus caspicus* | Caspian Sea, Azerbaijan | | | KX583920 | | Levin *et al.* 2017 |
| *Rutilus rutilus caspicus* | Caspian Sea, Aras Reservoir on the Aras River, Armenia | | | KX583930 | | |
| *Rutilus rutilus caspicus* | Ural River, Caspian Sea, Shalkar Lake, Kazakhstan | | | KX583940 | | |
| *Rutilus frisii* | Rezowska River, Black Sea slope, Bulgaria | MG806209 | MG806542 | MG806692 | MG806872 | Schönhuth *et al.* 2018 |
| *Rutilus frisii* | Rubas River, Dagestan, Russia | HM560581 | | HM560161 | | Perea *et al.* 2010 |
| *Rutilus frisii* | Ramsar, Iran | | | EU285048 | | |
| *Rutilus frisii* | Caspian Sea, Azerbaijan | | | KX583995 | | Levin *et al.* 2017 |
| *Rutilus frisii* | Iznik lake, Turkey | | | EU285042 | | Ketmaier *et al.* 2008 |
| *Rutilus frisii* | Mondsee Lake, Austria | | | EU285053 | | |
| *Rutilus frisii kutum* | Anzali Wetland, Iran | | | | JX266233 | Laloei *et al.* unp |
| *Rutilus frisii kutum* | Shirud River, Iran | | | KF056856 | | Chakmehdouz Ghasemi & Behmanesh 2015 |
| *Rutilus frisii kutum* | Sefid-rud River, Iran | | | KF056857 | | |
| *Rutilus frisii kutum* | Lamir River, Iran | | | KF056858 | | |
| **Outgroups** | | | | | | |
| *Squalius cephalus* | No information | MG806220 | MG806553 | MG806700 | MG806883 | Schönhuth *et al.* 2018 |
| *Chondrostoma nasus* | Diina River, Morava River, Danube Drainage, Serbia | MG806169 | MG806497 | MG806657 | MG806833 | |
| *Leuciscus aspius* | Strymon River, Aegean Sea slope, Greece | MG806177 | MG806507 | MG806666 | MG806842 | |

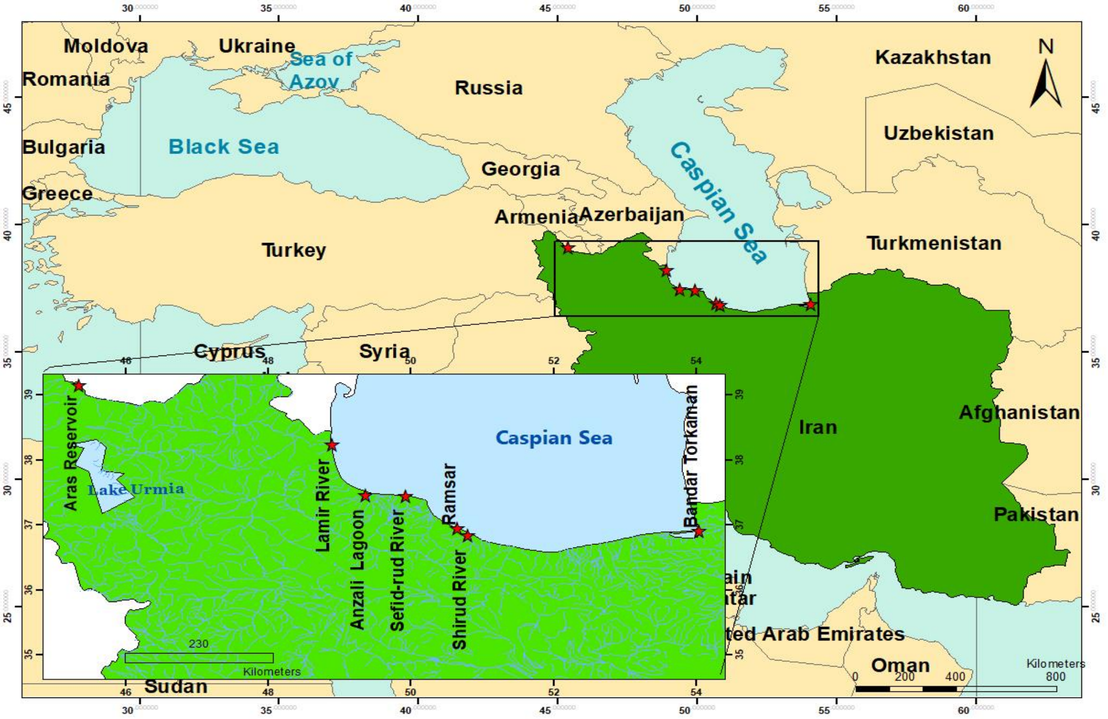

**Fig. 1.** Ponto-Caspian region with sampling sites (Aras Reservoir and Anzali Wetland) and samples from the southern Caspian Sea, Iran, represented in this study by sequences from GenBank (Lamir River, Anzali Wetland, Sefid-rud River, Ramsar, Shirud River and Bandar Torkaman) marked with stars.

Phylogenetic analyses were performed using Maximum Likelihood (ML) and Bayesian Inference (BI) with RAxML v.8.2.12 (Stamatakis 2006) and MrBayes v.3.2.7 (Huelsenbeck & Ronquist 2001) on the CIPRES Science Gateway platform (Miller *et al*. 2010), respectively. Since the four markers used were available only for some samples from GenBank, and for most samples from GenBank only CYTB gene sequences were presented, phylogenetic trees for this marker and the combined dataset (CYTB+COX1+RAG1+IRBP: 4,210 bp) were considered separately. Evaluation of ML trees using bootstrap analysis per 1000 replications and BI analysis were evaluated with 40,000,000 generations using TrN+I and TPM1+I+G models (Bayesian Information Criterion-BIC) for CYTB gene and combined dataset, respectively. The first 25% (10 million) generations were excluded as burn-in, and the remaining trees were considered to compute in each BI analysis. The phylogenetic trees resulting in ML and BI analyses were visualized and edited using FigTree, v.1.4.4.

## RESULTS

The results of phylogenetic analysis are presented on Figs. 2 and 3. Phylogenetic reconstruction based on combined dataset on the four markers (Fig. 2) and CYTB gene sequences (Fig. 3) showed consistent and well supported topologies by both Bayesian (BI) and Maximum likelihood (ML) analyses. The sequences of the analyzed *Rutilus* taxa were organized into three major clades: 1) clade A included the only species *R. pigus* (Lacepède, 1803), found in the lakes of southern Switzerland and Italy; 2) clade B was represented by two subclades, the first involved Western European monophyletic lineages with high support (*R. virgo*, *R. basak* and *R. aula*), and the second consisted of sequences of *R. frisii* sensu lato; 3) clade C incorporated sequences of European and Ponto-Caspian populations of *R. rutilus* sensu Berg (1949), which were divided into two subclades with a high degree of support. The subclade *R. frisii* were divided into two phylogenetic lineages: the first lineage combined sequences of specimens from the Caspian Sea basin (in Iran, Azerbaijan and Russia) and the second included samples from the Black Sea basin (in Turkey, Bulgaria and Austria). The sequences of specimens collected from Anzali Wetland and identified in this study as *R. kutum* were clustered within the first lineage (Figs. 2-3).

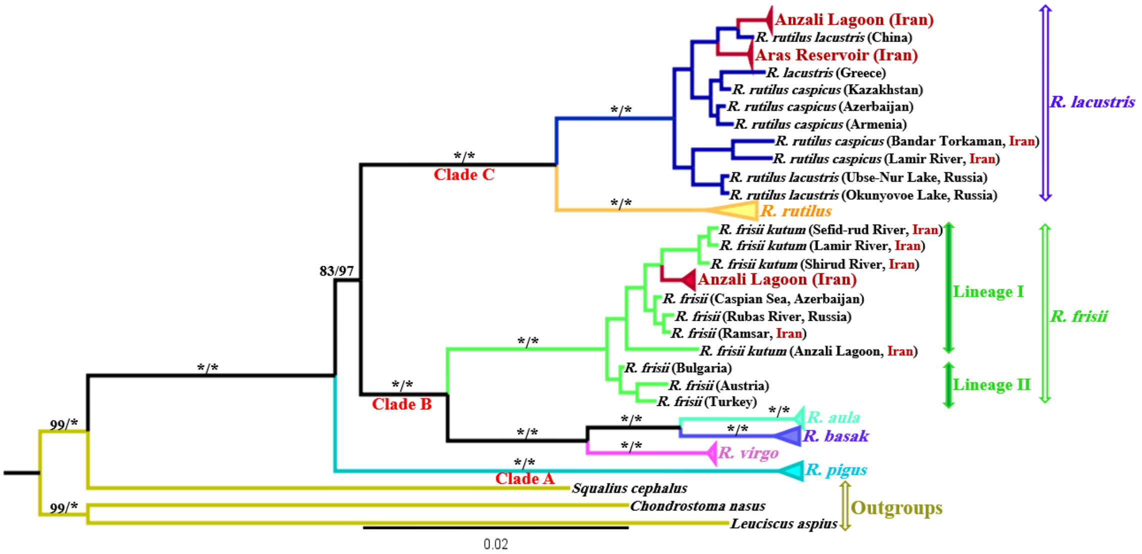

**Fig. 2.** BI estimation of the phylogenetic relationships based on the combined dataset (CYTB+COX1+RAG1+IRBP) and TPM1 + I + G model with values on branches corresponding to Bayesian posterior probabilities, a star denotes highest possible PP = 1.

The genetic distance based on CYTB sequences between the phylogenetic lineages of *R. frisii* and *R. kutum* was very low (K2p distance of 0.6%), slightly higher than the interlineage distances, and significantly lower than intergroup genetic distances, obtained from different species of *Rutilus* (Table 2). The first subclade of the major clade C included sequences from GenBank representing roach populations from the Tech River drainage (Mediterranean Sea slope), Elba River basin, Pazincica River (Istria), and Nero Lake (Middle Volga). The last sample consisted of haplotypes as a part of the phylogenetic lineage *R. rutilus* in the consensus tree of relations within the genus *Rutilus* based on CYTB, from Levin *et al*. 2017. The second subclade comprised samples from the Caspian Sea basin, Siberia, China and the Struma River in Greece registered in GenBank; most included haplotypes were involved in phylogenetic lineage *R. lacustris* in the tree from Levin *et al*. 2017. All sequences of specimens collected from Anzali Wetland and Aras reservoir and identified in this study as *R. caspicus*, were clustered within the second subclade (Figs. 2-3). The genetic distance based on CYTB sequences between indicated subclades was at the same level (K2p distance of 5.1%) as interspecific distance between West European roach species (K2p distance of 3.3-8.7%; Table 2).

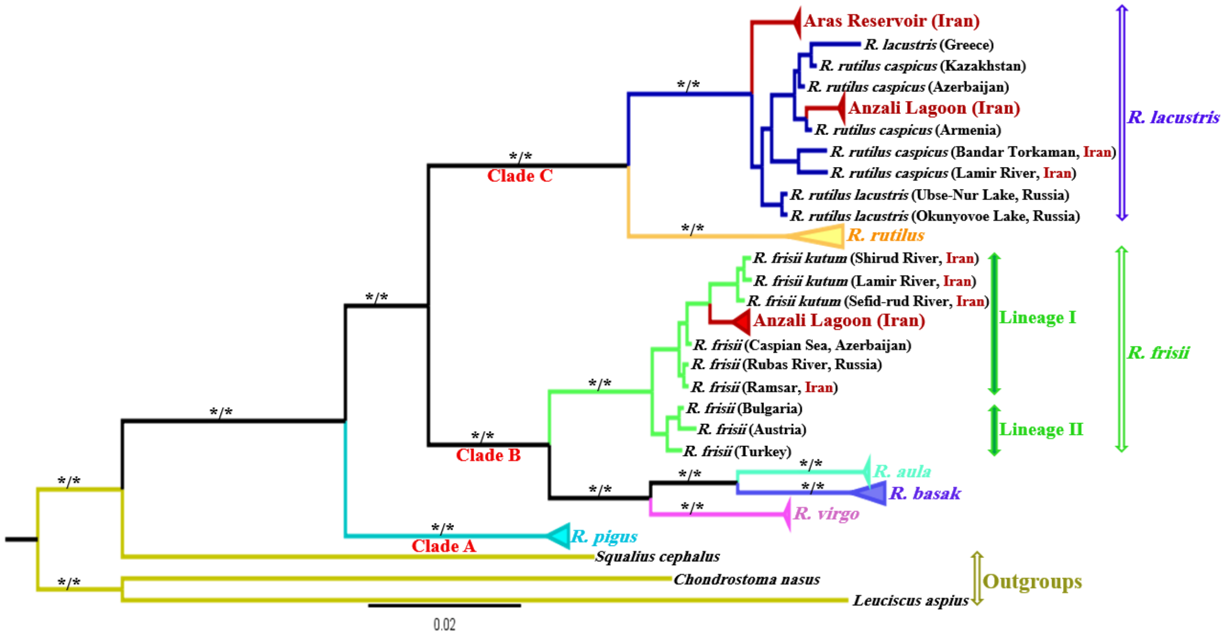

**Fig. 3.** BI estimation of the phylogenetic relationships based on the mitochondrial cytochrome *b* (CYTB) marker and TrN+I model with values on branches corresponding to Bayesian posterior probabilities, a star denotes highest possible PP = 1.

**Table 2.** K2p genetic distances for sequences of CYTB gene for groups (below the diagonal), %. Values on the diagonal correspond to average intra-group distances.

| **Group** | *R. aula* | *R. basak* | *R. pigus* | *R. virgo* | *R. rutilus* | *R. lacustris* | *R. frisii* | *R. kutum* |
|---|---|---|---|---|---|---|---|---|
| *R. aula* | **0** | | | | | | | |
| *R. basak* | 3.4 | **0.5** | | | | | | |
| *R. pigus* | 8.7 | 7.9 | **0.3** | | | | | |
| *R. virgo* | 4.2 | 4.3 | 7.8 | **0** | | | | |
| *R. rutilus* | 9.5 | 9.4 | 8.2 | 8.8 | **0.7** | | | |
| *R. lacustris* | 8.4 | 9.1 | 8.1 | 8.3 | 5.0 | **0.8** | | |
| *R. frisii* | 5.1 | 5.4 | 6.5 | 4.5 | 7.6 | 7.4 | **0.4** | |
| *R. kutum* | 5.3 | 5.5 | 7.1 | 5.0 | 7.6 | 7.7 | 0.6 | **0.2** |

## DISCUSSION

This study focused mainly on the determination of the phylogenetic structure of the genus *Rutilus* in the southern part of the Caspian region with the involvement of the majority of the West Palearctic lineages, which made it possible to more accurately determine the phylogenetic structure of the genus in the Ponto-Caspian basin. In general, the topology of our phylogenetic trees highly supported previous studies (Ketmaier *et al.* 2008; Larmuseau *et al.* 2009; Perea *et al.* 2010; Tsoumani *et al.* 2014; Levin *et al.* 2017; Schönhuth *et al.* 2018; Jorfipour *et al.* 2022; Abdullah *et al.* 2022), which showed the presence of three main phylogenetic clades associated with (i) *Rutilus pigus*, (ii) West European species in combination with *R. frisii* group, and (iii) the association of *R. rutilus* s. str. and the “Ponto-Caspian” lineage widely distributed in Eurasia (from the Aegean Sea basin to Laptev Sea tributaries) and identified as *R. lacustris*. These phylogenetic relationships were confirmed in the present study both on the basis of mitochondrial cytochrome *b* variability and on the basis of a combined data set on mitochondrial and nuclear genes (CYTB+COX1+RAG1+IRBP). Compared to previous studies, recent phylogenetic analysis included significant material from the southern Caspian Sea, both from collected samples and from data stored in GenBank. All involved sequences were grouped in the subclades of *R. frisii* or *R. lacustris* (Figs. 2-3). Thus, in the studied region, only two species of *Rutilus* were confirmed, in contrast to previous reviews, which recorded *R. rutilus* in the ichthyofauna of Iran (Coad 1980, 2016; Esmaeili *et al.* 2010; Jouladeh-Roudbar *et al.* 2015). Naseka & Bogutskaya (2009) and Bogutskaya *et al.* (2013) indicated *R. rutilus* only for the north Caspian Sea. However, their conclusions were based on morphological identification developed for genetically unexplored specimens of mixed origin. Indeed, *R. rutilus* sensu Kottelat & Freyhof (2007) combined numerous non-conspecific populations north of the Iberian Peninsula and the Alps, east to the Ural and Eya drainages in Europe, as well as in Anatolia and Siberia in Asia. Thus, this conglomerate combined two genetically confirmed species, namely *R. rutilus* s. stricto and *R. lacustris*. At the same time, some conspecific populations of the latter species were isolated as independent taxa (*R. caspicus* and *R. heckelii*) with artificially constructed diagnostic features. These artificial diagnoses were accepted and used by the following authors: Naseka &

Bogutskaya 2009; Bogutskaya *et al.* 2013; Jouladeh-Roudbar *et al.* 2015; Coad 2016). As a result, *R. rutilus* was recorded not only for the Northern Caspian (including Upper, Middle, and most of Lower Volga), but also for Iran. However, according to genetic data (Levin *et al.* 2017), this species preoccupies only the Upper Volga, while the Middle and Lower Volga are predominantly inhabited by *R. lacustris*, the only roach species confirmed for the Caspian Sea, also in the present study. It should be emphasized that the diagnostic characters developed for *R. rutilus lacustris* and other subspecies of *R. rutilus* adopted by Berg (1949) are also not suitable for the diagnoses of *R. rutilus* s. stricto and *R. lacustris* in a modern interpretation, since *R. rutilus lacustris* sensu Berg was limited only to Siberian populations. Therefore, the remark of Ermakov *et al.* (2017) that the morphological identification of *R. rutilus* and *R. lacustris*, "especially sampled in the sympatric zone, is very complicated" (p. 112), is not surprising (of course, in the non-overlapping parts of the range there are no problems with the identification of the only species of the genus). Accordingly, test systems using multiplex PCR and restriction analysis were designed to easily and quickly identify the aforementioned species (Ermakov *et al.* 2017). However, the development of morphological keys remains relevant. For this purpose, genetically identified samples are needed for both species, and, as shown by the present studies, materials from the southern Caspian can be included in voucher samples of *R. lacustris* to determine the diagnostic features of this species. The only previous phylogenetic studies of the genus *Rutilus* from the Southern Caspian Sea showed a high genetic distance between the two species identified by the authors (Gharibkhani *et al.* 2011; Chakmehdouz Ghasemi & Behmanesh 2015) as *Rutilus frisii kutum* and *Rutilus rutilus caspicus* according to Coad (1995). In this regard, it can be assumed that the sequence AF095610 from the specimen collected in Samur River, identified as *R. caspicus* and showed a very low genetic divergence in mt cyt *b* gene from *R. frisii* according to Ketmaier *et al.* (2008), really should belong to the hybrid fish. Such hybrids were recorded by Berg (1949) from Kumbashi River in Azerbaijan. Our results confirmed that Caspian *R. lacustris* and *R. frisii* are members of different phylogenetic clades, as was shown in previous studies (Ketmaier *et al.* 2008; Larmuseau *et al.* 2009; Perea *et al.* 2010; Levin *et al.* 2017). Moreover, the involvement of additional materials from the South Caspian samples of *R. frisii*, unlike the only Iranian GenBank sequence EU285048 in the aforementioned studies, allowed us to analyze the phylogenetic relationships in the *R. frisii* group. Our phylogenetic trees showed that the subclade of *R. frisii* was divided into two highly supported phylogenetic lineages: (i) populations from the Caspian Sea (Iran, Azerbaijan, Russia) and (ii) populations from the Black Sea and Danube River basin (Turkey, Bulgaria, Austria; Figs. 2-3). However, we found a low genetic distance between these lineages, with only 1.5-3.0 fold difference from the K2p genetic distances in these lineages. Moreover, its value was significantly lower than intraspecific distances in *R. rutilus* and *R. lacustris*. For comparison, interspecific K2p genetic distance between the last two species reached 5.0% and was 6.25 - 7.1 times greater than their intraspecific distances. This low genetic distance between the Caspian and Pontic lineages of *R. frisii* corresponds to the hypothesis of their Pleistocene divergence followed by substantial gene flow (Kotlik *et al.* 2008). Two separate populations were supported during the last glaciation, but their isolation was not complete, and they continued to exchange genes in both directions. The long-term gene exchange between the Caspian and Pontic populations of *R. frisii* can explain the significant overlap and variability of their morphological features observed when comparing different studies (Berg 1949; Abdurakhmanov 1962; Abbasi *et al.* 1999; Kottelat & Freyhof 2007; Abdoli & Naderi 2009; Vasil'eva & Luzhnyak 2013; Bogutskaya *et al.* 2013; Coad 2016). A number of morphological studies (Abdolhay *et al.* 2010; Rashidi *et al*. 2012; Vajargah *et al.* 2014, 2021; Ghojoghi *et al.* 2018; Sattari *et al*. 2019a, b, c; Sattari *et al*. 2020; Forouhar Vajargah *et al*. 2020a, b; Pourshabanan *et al*. 2020; Forouhar Vajargah *et al*. 2021; Pourshabanan *et al*. 2021a, b). and molecular analyses of the genetic diversity and genetic structure of populations (Kavan *et al.* 2009; Abdolhay *et al.* 2012; Chakmehdouz Ghasemi *et al.* 2014; Safari 2016; Kashiri *et al.* 2018) revealed phenotypic intra-population variability in different parts of the southern Caspian Sea in Iran. According to the available data, the main diagnostic character separating the Caspian and Black Sea populations of *R. frisii* demonstrates a strong overlap: 53 - 68 (most often 60 - 64) total lateral line scales in specimens from the Black Sea basin and 47 - 68 (most often 55 - 58) in specimens from the Caspian Sea (Berg 1949; Abdurakhmanov 1962; Bogutskaya *et al.* 2013; Coad 2016). Despite the low morphological and genetic divergence (revealed for the analyzed genetic markers), gene-tree reconstructions showed a clear geographical division between the Black Sea and Caspian Sea populations (Kotlík *et al.* 2008; this study). Both phylogenetic lineages were monophyletic in CYTB and in the combined dataset (CYTB+COX1+RAG1+IRBP; Figs. 2-3) and their divergence in separate Pleistocene Ponto-Caspian refugia was strongly supported by Kotlik *et al.* (2008). That is why in this study we join the previous taxonomic concept, according to which populations from the Caspian Sea have been classified as a separate subspecies, *R. frisii kutum* (Berg 1949; Bogutskaya & Naseka 2004; Vasil'eva & Luzhnyak 2013; Parin *et al.* 2014), in order to reflect stable (but low) morphological differences and geographic separation from the Black Sea population.

## CONCLUSION

Phylogenetic analysis of *Rutilus* samples from the South Caspian based on mitochondrial and nuclear genes proves the presence of only two species in this area. The first corresponds to *R. lacustris,* and the second is classified as the Caspian subspecies of *R. frisii*, i.e., *R. frisii kutum.* The populations of *R. lacustris* from the South Caspian should be used as genetically identified vouchers of this species to develop its morphological key features.

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