



Mitochondrial phylogeny, diversity, and ichthyogeography of gobies (Teleostei: Gobiidae) from the oldest and deepest Caspian sub-basin and tracing source and spread pattern of an introduced *Rhinogobius* species at the tricontinental crossroad

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Abstract Here, we combined archived mitochondrial sequences for Ponto-Caspian gobiids with new sequences from the south Caspian basin to assess and evaluate its gobioid diversity and taxonomy, and to provide a first mitochondrial-based phylogenetic and phylogeographic framework. We demonstrate that: (i) *Proterorhinus nasalis* is the tubenose goby taxon in the saline waters of the southern Caspian Sea, whereas the name *Pr. semipellucidus* for the Azov/northern Caspian Sea/Volga River populations is likely be

resurrected depending on the outcome of an integrative taxonomical approach; (ii) the deep-water goby *Ponticola bathybius* should be re-assigned to the genus *Neogobius*, as it is the sistergroup of *N. melanostomus*; (iii) specimens previously identified as *Po. cyrius* and *Po. iljini* from the south Caspian basin appear conspecific with *Po. iranicus* and *Po. gorlap*, respectively, and should be omitted from the checklist of Iranian and south Caspian freshwater fishes; (iv) the low stand of the Caspian Sea during the Tyurkynian regression is inferred to have led to the isolation and evolution of *Po. iranicus*; and (v) similarities in genetic background, and invasion history of *Rhinogobius* sp. and *Pseudorasbora parva* in Iran and Turkmenistan indicate that the initial introduction of both

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species into the region possibly originated from Japan in the 1980s.

Keywords Ponto-Caspian fishes · Molecular taxonomy · Evolutionary history · Biological invasion

Introduction

The Ponto-Caspian region is the stage of a highly diverse endemic evolution of two main lineages of Gobiidae sensu Gill & Mooi 2012: (i) the endemic Ponto-Caspian benthophiline gobies (Agorreta et al., 2013; referred to by Neilson and Stepien 2009a as a own subfamily, Benthophilinae Beling & Iljin, 1927), and (ii) a branch of the *Pomatoschistus*-lineage or sand gobies and related genera (Agorreta et al., 2013; Schwarzans et al., 2017).

Benthophiline gobies represent a monophyletic freshwater and brackish water radiation of Paratethyan gobies currently classified in nine genera and two major lineages subdivided in three tribes (Neilson & Stepien, 2009a): (1) the neogobiine-lineage with the two tribes Neogobiini (with the genus *Neogobius* Iljin, 1927), and Ponticolini (with the genera *Ponticola* Iljin, 1927, *Mesogobius* Bleeker, 1874, *Proterorhinus* Smitt, 1899, and *Babka* Iljin, 1927); and (2) the tadpole gobies lineage or Benthophilini (with the genera *Anatirostrum* Iljin, 1930, *Benthophilus* Eichwald, 1831, *Benthophiloides* Beling & Iljin, 1927, and *Caspiosoma* Iljin, 1927). Their adaptation to brackish and freshwaters most likely evolved during the process of isolation of the Paratethys from the Tethys in the Middle Miocene and the subsequent freshening of the Paratethys in the latest Miocene (Popov et al., 2004). In this diverse group, there are several real freshwater endemics, several species restricted to the brackish waters of the seas, and several euryhaline species, but no species has ever been recorded as inhabiting strictly marine conditions (Miller, 2003, 2004b; Kottelat & Freyhof, 2007; Freyhof, 2012). Its evolutionary history has been driven by the dynamic geologic and hydrologic evolution of the region (Rögl, 1999; Reid & Orlova, 2002; Neilson & Stepien, 2009a). Neilson and Stepien (2009a) provided the first comprehensive phylogenetic and biogeographic analysis of the group based on mitochondrial and nuclear markers, and addressed classification issues and phylogenetic

relationships within the group. They presented a revised taxonomy and nomenclature that does not, however, aptly fit with the current understanding of gobioid family and subfamily phylogenetics, as, e.g., presented in Agorreta et al. (2013), and recently confirmed by McCraney et al. (2020); nevertheless, their basic phylogenetic conclusions and tribe designations remain useful as they are not in conflict with any familial and subfamilial taxonomy.

The *Pomatoschistus*-sand goby lineage is primarily represented by several species of *Knipowitschia* Iljin, 1927 and the Caspian-endemic *Hyrceanogobius* Iljin, 1928 in the Ponto-Caspian basin. *Hyrceanogobius* Iljin, 1928 (type *H. bergi* Iljin, 1928) was originally distinguished as a separate genus because of the reduced condition of the head lateral-line canal system (Iljin, 1928), but later suggested as congeneric with *Knipowitschia* by Economidis and Miller (1990). However, following the last examination of actual material, Miller (2004c) would now agree that this genus warrants separation from *Knipowitschia*. Sand gobies have their highest diversity in the Mediterranean, but there are several endemics in the Black and Caspian Seas (Freyhof, 2012; Thacker et al., 2019).

Furthermore, there is an introduced *Rhinogobius* species in the Ponto-Caspian basin with established populations in the inland waters of Iran (e.g., in the Anzali Wetland and Zarivar Lake; Coad, 2016; Sadeghi et al., 2019), Turkmenistan (Kara-Kum Canal; Aliev et al., 1988), and the Caucasus (Epitashvili et al., 2020; Japoshvili et al., 2020), which has appeared under several scientific names over the last 2 decades [i.e., *Rhinogobius similis* Gill, 1859, *R. cheni* (Nichols, 1931), and currently *R. lindbergi* Berg, 1933]. There have been controversial debates about the taxonomic status of this introduced species to the Ponto-Caspian basin (Coad & Abdoli, 2000; Vasil'eva & Kuga, 2008; Sadeghi et al., 2019), and its invasion history, spread pattern, and genetic background remain unknown.

The Caspian Sea basin is ecologically split into three sub-basins (Naseka & Bogutskaya, 2009): (i) a northern shallow sub-basin (less than 10 m); (ii) a middle sub-basin with an average and maximum depth of 200 m and 790 m, respectively; and (iii) the southern and deepest sub-basin with a maximum depth of 980–1,025 m and an average of 325 m. It is a basin with high degree of species-level endemism up to 80% (Dumont 1998, 2000) as 99 out of 159 Caspian

Sea fish species (62%) are endemic and restricted to specific areas (Naseka & Bogutskaya, 2009). Gobiidae sensu Gill & Mooi 2012 is the second most species-rich fish family in the basin with 36 species in 11 genera, 31 (97.2%) of which are Caspian endemic (Miller, 2003, 2004b; Kottelat & Freyhof, 2007; Naseka & Bogutskaya, 2009). The high endemism of gobies in the Caspian Sea basin is highlighted by the fact that a large number of gobiid species have not been included yet in any large- or small-scale phylogenetic, biogeographic, or phylogeographic analysis (Neilson & Stepien, 2009a; Thacker & Roje, 2011; Agorreta et al., 2013; Medvedev et al., 2013; Thacker et al., 2019). Most importantly, gobiids of the southern and eastern parts of the Caspian Sea have not been included in any molecular study of gobiid systematics (Fig. S1, Online Resources), despite the fact that their documentation and analysis could provide in depth insights into yet unaddressed evolutionary biology questions. Field ichthyology remains to accomplish basic research in the Caspian Sea basin, as, e.g., the presence of a large number of gobiid species listed for the southern Caspian Sea basin has to be confirmed by any specimen (Miller, 2003, 2004b; Boldyrev & Bogutskaya, 2007; Kottelat & Freyhof, 2007; Esmaeili et al., 2018).

Nevertheless, several recently published sources of mitochondrial cytochrome *c* oxidase subunit 1 (COI) sequence information for several Ponto-Caspian gobiids (e.g., Neilson & Stepien, 2009a, b; Kesk n & Atar, 2013; Geiger et al., 2014; Kneibelsberger et al., 2015; Thalinger et al., 2016; Thacker et al., 2019) provide results of various recent efforts to obtain DNA barcodes for these taxa, which are useful for evolutionary analyses, species identification and application for conservation. The partial COI barcode fragment generally does not provide adequate phylogenetic resolution for large-scale analyses (for a review, see Rubinoff & Holland, 2005), but the taxonomic scope in this study is narrow enough that COI is useful as a first step for integrative taxonomy (see Thacker et al., 2019). Here, we combine archived COI data for Ponto-Caspian gobiids with new sequences from the southern Caspian Sea basin to assess its gobioid diversity, to provide a first mitochondrial-based phylogenetic and phylogeographic study, and to evaluate, from a mitochondrial viewpoint, the taxonomy of gobiid fishes in the deepest and oldest southern part of the Caspian Sea. The outcome will be a very first deep

insight into the southern Caspian Sea's gobiid diversity and evolution, and it will be important for the conservation and management programs, too.

Materials and methods

Study area, specimen collection, DNA extraction, amplification, and sequencing

During numerous field works between 2014 and 2020, we collected gobiid specimens from shallow and deep waters of the southern Caspian Sea and its associated waterbodies and river drainages (30 localities; Table 1) using beach seining, scuba diving, deep-water bottom beam trawls, and electrofishing devices. Information on sample codes, species, and sampling localities are given in Table 1, and a map of localities is shown in Fig. 1. We took photographs of live specimens, and after anesthesia with 1% clove oil solution or Quinaldine Sulfate (Ross & Ross, 2009), muscle tissue or the right pectoral fin of each individual was separated and fixed in 96% ethanol and subsequently kept at $-20\text{ }^{\circ}\text{C}$ until DNA extraction. Species-level identification of the specimens was carried out using major taxonomic keys and primary taxonomic literature (Miller, 2003, 2004b; Boldyrev & Bogutskaya, 2007; Kottelat & Freyhof, 2007; Vasil'eva et al., 2015). The specimens were fixed in 10% formalin, transferred to 70% ethanol, and deposited in the Zoological Museum of Shiraz University, Collection of Biology Department (ZM-CBSU) and in the SNSB-Bavarian State Collection of Zoology, Munich (SNSB-ZSM) as voucher specimens.

Genomic DNA samples were extracted from muscle tissues or fin clips according to the salt method protocol described by Bruford et al. (1992). Partial COI gene sequences were amplified using primer pairs FishF1 (5'-TCAACCAACCACAAAGACATTGGCAC-3') and FishR1 (5'-TAGACTTCTGGGTGGCCAAAGAATCA-3') (Ward et al., 2005). Amplification was performed on a Bioer XP Thermal Cycler (Bioer Technology Co. Ltd., Hangzhou, China), programmed as following: 94 $^{\circ}\text{C}$ for 1 min for initial denaturing, 35 cycles of 94 $^{\circ}\text{C}$ for 30 s, 52–56 $^{\circ}\text{C}$ for 45 s, and 72 $^{\circ}\text{C}$ for 45 s, followed by 72 $^{\circ}\text{C}$ for 5 min as the final extension. An alternative primer pair, FISH-BCL (5'-TCAACYAATCAYAAAGATATYGGCAC-3') and FISH-BCH (5'-

Table 1 Our sampling sites in southern Caspian Sea basin

Locality	Site ^a	SM ^b	Collection date/coordinates/habitat characteristics
Tajan River	1	A	16 February 2015 and 30 August 2019, 36.507191 N 53.084683 E, 75.3 m a.s.l., river width 87.8 m, depth to 0.8 m. Bottom composed mostly of gravel, cobbles and boulders
Polrud	2	A	11 July 2014 and 29 August 2019, 37.090728 N 50.374866 E, altitude 24.7 m b.s.l., river width 51 m, depth to 0.7 m. Bottom composed mostly of gravel, cobbles and boulders
Sefid Rud at Kucheshfahan	3	A	18 February 2015 and 28 August 2019, 37.246185 N 49.817643 E, 6.1 m b.s.l., river width 132 m, depth to 1 m. Bottom composed mostly of gravel and cobbles
Babolrud	4	A	13 July 2014, 17 February 2015 and 30 August 2019, 36.510467 N 52.679240 E, 5.2 m b.s.l., river width 26.6 m, depth to 0.6 m. Bottom composed mostly of clay
Nekarud	5	A	1 October 2019, 36.631168 N 53.340603 E, 68.9 m a.s.l., river width 43.6 m, depth to 0.8 m. Bottom composed mostly of gravel, cobbles and boulders
Ali Kiasoltan	6	A	29 September 2019, 36.620190 N 51.576503 E, 16.8 m b.s.l., river width 39.9 m, depth to 0.4 m. Bottom composed of gravel and cobbles
Siah Darvishan River	7	A	27 August 2019, 37.352679 N 49.421398 E, 21.1 m b.s.l., river width 12.5 m, depth to 0.7 m. Bottom composed of gravel and cobbles
Chalvand River	8	A	10 July 2014 and 26 August 2019, 38.291701 N 48.878390 E, 26.8 m b.s.l., river width 27.3 m, depth to 0.4 m. Bottom composed mostly of gravel and cobbles
Kaboudval Stream	9	A	15 February 2015 and 31 August 2019, 36.886378 N 54.893839 E, 194.8 m a.s.l., stream width 3.5 m, depth to 0.3 m. Bottom composed of clay, sand and gravel
Neka Beach, north of Tejen Lateh	10	B	15 November 2018, 36.827017 N 53.174082 E, depth to 10 m. Bottom composed of clay and sand
Qaleh Gardan River	11	A	12 July 2014, 36.993286 N 50.283624 E, 114.6 m a.s.l., river width 33 m, depth to 0.4 m. Bottom composed of sand, gravel, cobbles and boulders
Anzali Beach	12	B	18 July 2018, 37.478113 N 49.496917 E, depth to 10 m. Bottom composed of clay and sand
Sefid Rud Mouth	13	B	20 July 2018, 37.464139 N 49.938672 E, 27.7 m b.s.l., river width 181 m, depth to 1.5 m. Bottom composed of clay, sand, gravel and cobbles
Sefid Rud at Imamzadeh Hashem	14	A	7 July 2014 and 25 August 2019, 37.025493 N 49.635475 E, 80.5 m a.s.l., river width 96 m, depth to 1 m. Bottom composed of boulders, cobbles and gravel
Nowshahr River	15	A	29 September 2019, 36.636481 N 51.475448 E, 1.8 m a.s.l., river width 25.7 m, depth to 0.4 m. Bottom composed of boulders, cobbles and gravel
Shafa Rud	16	A	19 February 2015 and 21 July 2018, 37.527785 N 49.098831 E, 91.7 m a.s.l., river width 18.3 m, depth to 0.4 m. Bottom composed of boulders, cobbles and gravel
Babolsar Beach at Mirud	17	B	14 November 2018, 36.730474 N 52.752377 E, depth to 10 m. Bottom composed of clay and sand
Babolsar Beach near Karfun	18	B	14 November 2018, 36.739367 N 52.819817 E, depth to 10 m. Bottom composed of clay and sand
Kiashahr Beach	19	B	31 March 2020, 37.418377 N 50.009211 E, depth to 10 m. Bottom composed of clay and sand
Anzali Dredge	20	B-D	18 July 2018, 37.483182 N 49.462254 E, depth to 10 m. Bottom composed of clay and sand
Astara Dredge	21	B	19 July 2018, 38.406640 N 48.879821 E, depth to 10 m. Bottom composed of clay and sand
Miankaleh	22	B	17 November 2018, 36.865484 N 53.449398 E, depth to 10 m. Bottom composed mostly of clay and sand
Anzali Wetland	23	A	18 July 2018, 37.467990 N 49.354060 E, depth to 4 m. Bottom composed mostly of clay
Sefid Rud at Bojaq National Park	24	B	23 July 2018, 37.449382 N 49.930891 E, 28 m b.s.l., river width 108 m, depth to 1 m. Bottom composed of clay, sand and gravel
Shalmanrud Mouth	25	B	23 July 2018, 37.214029 N 50.269448 E, 26.5 m b.s.l., river width 68.6 m, depth to 1 m. Bottom composed of sand, cobbles and gravel
Oshmakrud Mouth	26	B	24 July 2018, 37.450751 N 49.867927 E, 27.4 m b.s.l., river width 62.7 m, depth to 1 m. Bottom composed of sand, cobbles and gravel
Zarivar Lake*	27	A	21 May 2017, 35.510100 N 46.137197 E, 1,292 m a.s.l., depth to 0.7 m. Bottom composed of clay

Table 1 continued

Locality	Site ^a	SM ^b	Collection date/coordinates/habitat characteristics
Massuleh River	28	A	27 August 2019, 37.396685 N 49.330899 E, 22.9 m b.s.l., river width 14.5 m, depth to 0.4 m. Bottom composed of clay, gravel and cobbles
Baham Bar River	29	A	27 August 2019, 37.462023 N 49.2497 E, 18.9 m b.s.l., river width 12.2 m, depth to 0.3 m. Bottom composed of gravel and cobbles
Pasikhan River	30	A	9 July 2019, 37.268707 N 49.474937 E, 9.4 m b.s.l., river width 35.8 m, depth to 0.7 m. Bottom composed of clay, gravel and cobbles

*Tigris River system

^aSite number refers to the location of each sampling site, as shown in Fig. 1

^bSampling method: A, electrofishing; B, beach seining; C, bottom trawling; D, scuba diving

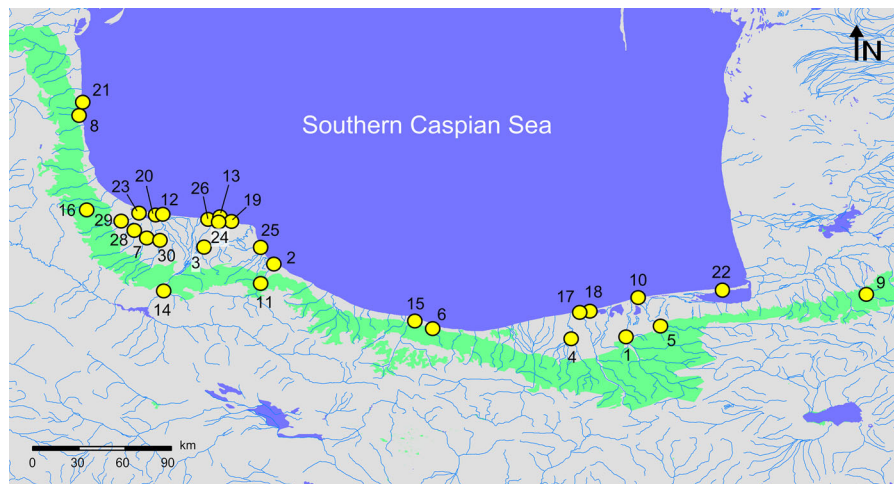


Fig. 1 A map of the collection sites in southern Caspian Sea. The location codes correspond to those in Table 1

TAAACTTCAGGGTGACCAAAAAATCA-3') (Baldwin et al., 2009), was used for *Knipowitschia caucasica*. The thermal cyclers program for PCR was: initial denaturation step of 94 °C for 5 min, 35 cycles of 94 °C for 1 min, 58.4 °C for 45 s, 72 °C for 1 min, and one cycle of 5 min at 72 °C. After purification of the PCR products with the ExoASP-IT® (usb) kit, they were sent out for Sanger sequencing with BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an ABI PRISM 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA) to the Faghihi Lab., Shiraz, Iran.

Phylogenetic, biogeographic, and species delimitation analyses

Sequence chromatograms were viewed and edited in BioEdit 7.0.4 (Hall, 1999). We generated COI sequence data (652 bp) from 68 representatives of the species *Ponticola gorlap* (Iljin, 1949), *Po. iranicus* Vasil'eva, Mousavi-Sabet & Vasil'ev, 2015, *Po. syrman* (Nordmann, 1840), *Po. bathybius* (Kessler, 1877), *Neogobius caspius* (Eichwald, 1831), *N. pallasi* (Berg, 1916), *N. melanostomus* (Pallas, 1814), *Benthophilus leobergius* Berg, 1949, *Protherorhinus nasalis* (De Filippi, 1963), *K. caucasica* (Berg, 1916), and *R. lindbergi* Berg, 1933 (Table 2). Sequences were tagged with species names following

Table 2 List of the gobiid specimens examined from the southern Caspian Sea, categorized by species

Taxa	Locality	Site	N ^a	H ^b	ZM-CBSU/SNSB-ZSM No. (haplotype)	GenBank No	
<i>Ponticola gorlap</i>	Tajan River	1	1	1	P2824 (H20)	MW393583	
	Polrud	2	1	1	P2823 (H19)	MW393584	
	Sefid Rud at Kuchesfahan	3	1	1	P2822 (H14)	MW393585	
	Babolrud	4	5	5	P2821 (H15), P2786 (H16), G2171 (H17), G2172 (H18), P2831 (H39)	MW393586–MW393590	
	Nekarud	5	1	1	P2787 (H9)	MW393591	
	Ali Kiasoltan	6	1	1	P2785 (H10)	MW393592	
	Siah Darvishan River	7	1	1	P2784 (H9)	MW393593	
	Chalvand River	8	2	1	P2783 (H11), M2830 (H11)	MW393594–MW393595	
	Kaboudval stream	9	3	1	P2776 (H12), P2777 (H12), P2778 (H12)	MW393596–MW393598	
	Neka Beach	10	1	1	M2516 (H13)	MW393599	
	Qaleh Gardan River	11	2	1	G2174 (H8), G2175 (H8)	MW393600–MW393601	
	Anzali Beach	12	1	1	GO1980 (H21)*	MW393602	
	<i>Ponticola iranica</i>	Sefid Rud Mouth	13	1	1	GO1988 (H22)*	MW393603
Massuleh River		28	1	1	P2832 (H38)	MW393604	
Siah Darvishan River		7	3	3	P2819 (H2), P2820 (H1), P2782 (H3)	MW393605–MW393607	
Sefid Rud at Imamzadeh Hashem		14	1	1	P2781 (H4)	MW393608	
Polrud		2	1	1	P2780 (H5)	MW393609	
Nowshahr River		15	1	1	P2779 (H6)	MW393610	
Shafa Rud		16	3	1	GO1997 (H7)*, GO1998 (H7)*, GO1999 (H7)*	MW393611–MW393613	
Massuleh River		28	1	1	P2788 (H42)	MW393614	
Baham Bar River		29	1	1	P2793 (H43)	MW393615	
Pasikhan River		30	1	1	P2794 (H43)	MW393616	
<i>Ponticola syrman</i>		Babolsar Beach at Mirud	17	1	1	P2789 (H23)	MW393617
<i>Ponticola bathybius</i>		Kiashahr beach	19	1	1	P2862 (H23)	MW393618
		Babolsar Beach at Karfun	18	2	1	M2848 (H24), M2114 (H24)	MW393619–MW393620
<i>Neogobius pallasii</i>	Kiashahr Beach	19	3	1	P2854 (H24), P2855 (H24), P2856 (H24)	MW393621–MW393623	
	Anzali Dredge	20	2	2	GO1972 (H29)*, GO1973 (H32)*	MW393624–MW393625	
	Astara Dredge	21	1	1	M2843 (H31)	MW393626	
<i>Neogobius caspius</i>	Miankaleh	22	1	1	M2844 (H30)	MW393627	
	Anzali Dredge	20	2	2	GO1978 (H28)*, M2839 (H25)	MW393628–MW393629	
<i>Neogobius melanostomus</i>	Miankaleh	22	3	3	M2837 (H25), M2838 (H26), M2840 (H27)	MW393630–MW393632	
	Anzali Dredge	20	2	2	GO1979 (H34)*, M2846 (H33)	MW393633–MW393634	

Table 2 continued

Taxa	Locality	Site	N^a	H^b	ZM-CBSU/SNSB-ZSM No. (haplotype)	GenBank No
<i>Benthophilus leobergius</i>	Anzali Dredge	20	4	2	GO1981 (H35)*, GO1982 (H35)*, GO1983 (H36)*, M2847 (H35)	MW393635– MW393638
<i>Proterorhinus nasalis</i>	Sefid Rud Mouth	13	2	1	GO1986 (H37)*, GO1987 (H37)*	MW393639– MW393640
	Sefid Rud at Bojaq National Park	24	1	1	M2834 (H37)	MW393641
	Shalmanrud Mouth	25	1	1	M2835 (H37)	MW393642
	Oshmakrud Mouth	26	1	1	M2836 (H37)	MW393643
<i>Knipowitschia caucasica</i>	Anzali Wetland	23	3	1	G2130, G2131, G2132	MW393644– MW393646
<i>Rhinogobius lindbergi</i>	Zarivar Lake/Tigris River	27	2	1	P2049, P2050	MW393647– MW393648
	Anzali Wetland	23	2	1	GO1970*, GO1971*	MW393649– MW393650

Asterisks indicate specimens deposited in the SNSB-Bavarian State Collection of Zoology, Munich (SNSB-ZSM)

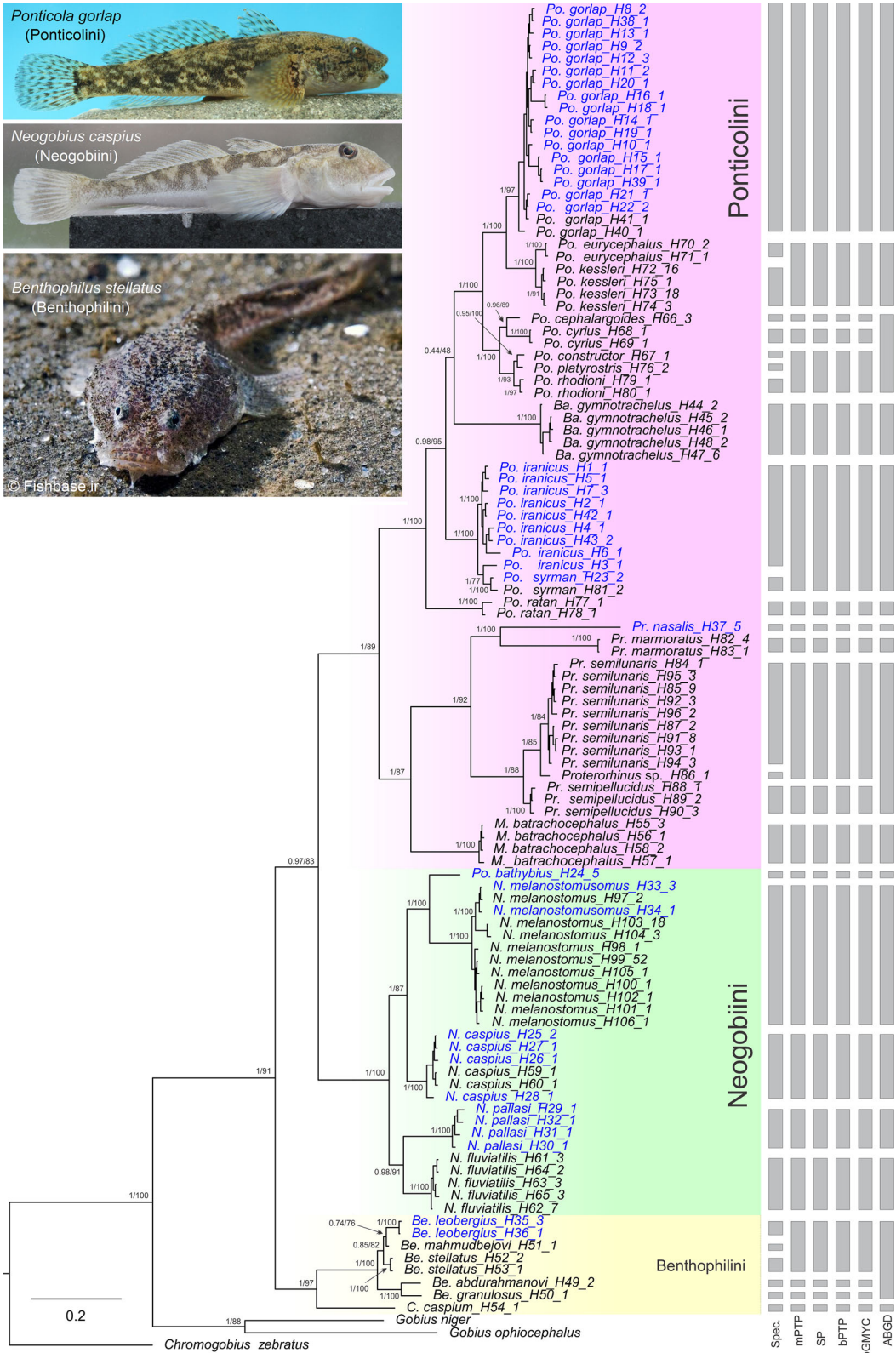
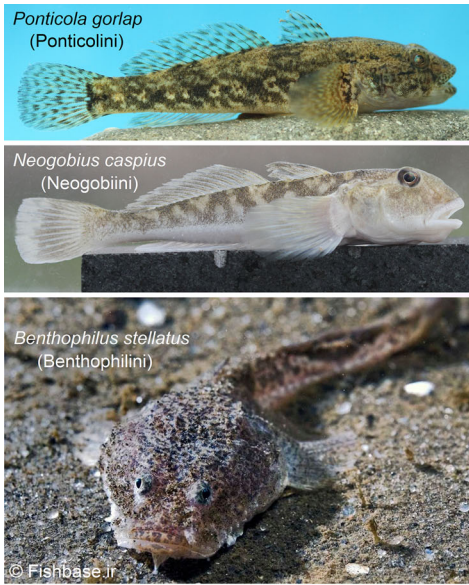
^aNumber of individuals

^bNumber of haplotypes

traditional taxonomic identification and submitted to GenBank (Table 2). In addition to the newly determined sequences from the southern Caspian Sea basin, we used 346 archived DNA barcode sequences (members of both benthophiline sublineages and *Knipowitschia*; Table S1, Online Resources) obtained from GenBank and largely derived from the works of Neilson and Stepien (2009a, b), Keskin and Atar (2013), Geiger et al. (2014), Kneibelsberger et al. (2015), Thalinger et al. (2016), and Thacker et al. (2019). Furthermore, we integrated 18 COI barcodes of *K. caucasica* obtained from digital catalogue of the Zoologisches Forschungsmuseum Alexander Koenig (ZFMK, www.collections.zfmk.de). GenBank numbers, ZFMK museum numbers, and geographical origins of these archived sequences are given in Table S1. Regarding the benthophilines, we assembled a total of 294 individual sequences (61 newly developed plus 233 archived) from 27 nominal Ponto-Caspian gobiid species, with sampling comprising the entire Ponto-Caspian region (except for the eastern parts of the Caspian Sea basin), and the European and North American invasive ranges. To assess the taxonomic status of the introduced freshwater goby of the genus *Rhinogobius* to Iran, 9 BOLD (derived from Japoshvili et al., 2020) and ZFMK-deposited COI barcodes of *R.*

lindbergi sampled from Georgia (Ozaani Stream) and Azerbaijan (Kura River basin), and 74 archived COI sequences of related species (Table S2) largely derived from the works of Yamasaki et al. (2015), Chang et al. (2017), Shen et al. (2016), Xia et al. (2018), and Chen et al. (2015) were included in analysis along with the four new sequences collected from the southern Caspian Sea basin (Anzali Wetland) and the Tigris River system (Zarivar Lake).

DNA sequences were aligned using ClustalW multiple alignment accessory application implemented in Mega 7 (Kumar et al., 2016). The best-fit nucleotide substitution models for the COI datasets were selected using the jModelTest 2.1.3 (Darriba et al., 2012) based on the Bayesian Information Criterion (BIC; Schwarz, 1978). PopART 1.7 (Leigh & Bryant, 2015) was used to depict the evolutionary relationships among haplotypes and evaluate the phylogeographic depth (Avise, 2000) based on the TCS method. Substitution saturation in the COI barcode region was examined with DAMBE 7 (Xia, 2018) using the Xia's et al. (2003) nucleotide substitution saturation test. For phylogenetic reconstruction, the Bayesian method (BI) was run based on four simultaneous runs of four Markov chains for 100,000,000 generations and a burn-in of 15% of the



◀ **Fig. 2** Bayesian phylogenetic hypothesis of the endemic Ponto-Caspian gobies (tribes Benthophilini, Neogobiini and Ponticolini), including 294 individuals (106 haplotypes) plus 3 outgroups. Each species names is followed by the designated haplotype and its frequency. The new haplotypes sampled from the southern Caspian Sea basin (61 individuals) are shown in blue. Support values are indicated beside the nodes (BI posterior probability/ML bootstrap)

initial trees in MrBayes 3.2.6 (Ronquist et al., 2012). Phylogeny of benthophiline gobies was rooted with *Gobius* (*G. niger*, FJ526837; and *G. ophiocephalus*, FJ526797) and *Chromogobius zebratus* (FJ526797). The *Rhinogobius* tree was rooted with *Awaous guamensis* (HQ639035). Resulting COI alignments were further analyzed phylogenetically using the Maximum Likelihood (ML) method in RAxML 7.2.5 (Stamatakis, 2006) with 10,000 bootstrap replicates. Phylogenetic hypothesis testing [Shimodaira–Hasegawa (SH) test; Shimodaira & Hasegawa, 1999; Goldman et al., 2000] was performed in CONSEL v.1.20 (Shimodaira & Hasegawa, 2001) to test for statistical significance of topological differences between the BI and ML trees.

Species delimitation analyses estimate the number of lineages (i.e., putative species) supported by molecular sequences or gene trees without a priori assignment of individuals to species, and to generate a preliminary hypothesis of species limits (Carstens et al., 2013). Five single-locus species delimitation analyses were performed on the benthophiline COI dataset: Automatic Barcode Gap Discovery (ABGD; Puillandre et al., 2012), reversed Statistical Parsimony (SP; Hart & Sunday, 2007), Bayesian Poisson Tree Process (bPTP; Zhang et al., 2013), multiple rate PTP (mPTP; Kapli et al., 2017), and Bayesian General Mixed Yule-Coalescent (bGMYC; Reid & Carstens, 2012). COI data set was tested on the ABGD webserver (<https://bioinfo.mnhn.fr/abi/public/abgd/>) with combinations of ABGD settings within the parameter range of $P_{\min} = 0.001$, $P_{\max} = 0.1$, all for a total of 10 steps and applying a K2P-corrected genetic distance matrix calculated in Mega. TCS 1.21 (Clement et al., 2000) was used to calculate a Statistical Parsimony network, using a 95% connection probability threshold to delineate putative species. The bPTP server (<http://species.h-its.org/>) was used with a Bayesian tree produced in MrBayes as input tree and analyses were run under default settings. Convergence

was visualized on the MCMC interactions plots vs. log-likelihood (Fig. S2). The mPTP analysis was run using the online server (<http://mptp.h-its.org>), under the same parameters as for bPTP. The bGMYC method is conceptually similar to bPTP and mPTP, and uses a tree topology to infer species hypotheses, but unlike these methods, it applies an ultrametric tree as an input topology. This analysis was performed using the bGMYC package (Reid & Carstens, 2012) for R ver. 3.6.1 (R Core Team, 2013) on a time-calibrated tree produced in BEAST ver. 1.8.2 (Drummond et al., 2012) as an input file. To achieve a first and preliminary time calibration of the benthophiline tree, we reduced the data set to limited haplotypes of each species to avoid loss of computational efficiency due to the intraspecific polytomies. We reconstructed a Bayesian phylogeny as outlined above and performed the calibration analysis on a matrix of 56 haplotypes (including the 3 outgroup exemplars) with BEAST, run with an uncorrelated lognormal relaxed clock model and a birth–death speciation prior. Although not yet fully scrutinized, we followed Neilson and Stepien (2009a) and set the age for the most recent common “neogobiin” ancestor to 10 Mya, at date derived from fossil otoliths of *Neogobius* inferred to be of Late Miocene–Early Pliocene origin (Rückert-Ülkümen, 2006). We assigned a secondary calibration of 6.25 Mya to the node subtending *Proterorhinus* + *Mesogobius* (derived from the analysis of Neilson & Stepien, 2009a) and ran the BEAST analysis in four independent runs of 100,000,000 generations, with trees sampled every 1,000 generations; the first 10% were discarded as burn-in. At the end, convergence and sufficient effective sampling sizes (ESS; $ESS > 200$) were confirmed using the Tracer ver. 1.6 (Rambaut et al., 2014), and a maximum clade credibility consensus tree was constructed in Tree Annotator 1.8.2 (Drummond et al., 2012). Phylogenetic trees were edited in FigTree 1.4.4 (Rambaut & Drummond, 2012), and the species delimitation results were depicted as grey bars on the tree.

Results

JModelTest determined GTR + I + G as the best-fitting substitution model for the benthophiline data set (294 individuals, 652 bp length). The nucleotide

substitution pattern showed that the benthophiline sequences have not reached substitution saturation and are, therefore, well suitable for phylogenetic analyses ($Iss < Iss.c$ S/Iss.c A; Table S3). Sequence analysis of this dataset detected 253 variable nucleotide sites (13 singleton variable and 240 parsimony informative sites), which allowed the definition of 106 haplotypes (H1–H106; see Tables 2, S1). Phylogenies inferred from the BI and ML (Fig. S3) analyses of benthophilines were not significantly different (SH test, $P = 0.43$), and the BI tree is presented in Fig. 2 for clarity. In total, the resulting benthophiline phylogenetic hypothesis includes novel sequences for 61 individuals from southern Caspian Sea basin combined with 233 archived sequences, defining 106 haplotypes, and three outgroup individuals (Fig. 2). Most genera and nominal species are highly supported, and three clades correlating with previously identified sublineages within the benthophilines are highly resolved.

(i) The first basally diverging clade includes the tadpole gobies (*Benthophilus* and *Caspiosoma*) (97% bootstrap support, 1.00 PP), comprising the tribe Benthophilini. The deepest split within Benthophilini is between *C. caspium* and the remainder of the species. The two tadpole goby species, *Be. granulatus* and *Be. abdurahmanovi* comprise a group (100% bootstrap support, 1.00 PP) constituting the sister clade to the clade containing *Be. stellatus*, *Be. mahmudbejovi*, and *Be. leobergius* sampled from southern Caspian Sea (100% bootstrap support, 1.00 PP).

(ii) A clade corresponding to the tribe Neogobiini (100% bootstrap support, 1.00 PP) includes a now-restricted *Neogobius* (*N. fluviatilis*, *N. pallasi*, *N. caspius*, and *N. melanostomus*) and *Po. bathybius*. *Neogobius fluviatilis* and *N. pallasi* are sister species (91% bootstrap support, 0.98 PP), forming a clade sister to the remainder of the species (100% bootstrap support, 1.00 PP). The mitochondrial haplotype of *Po. bathybius*, sampled from southern Caspian Sea, is closely related to *N. melanostomus* with robust statistical support (100% bootstrap support, 1.00 PP).

(iii) A larger clade comprising the tribe Ponticolini (89% bootstrap support, 1.00 PP) contains the genera *Proterorhinus*, *Mesogobius*, *Babka*, and *Ponticola*. *Mesogobius* and *Proterorhinus* are strongly supported as sister groups (87% bootstrap, 1.00 PP), and the *Mesogobius* + *Proterorhinus* clade is then the sister

to the clade containing *Babka* and *Ponticola* (except for *Po. bathybius*) (89% bootstrap support, 1.00 PP). Our trees show that *Ponticola* (comprising *Po. cephalargoides*, *Po. constructor*, *Po. cyrius*, *Po. eurycephalus*, *Po. gorlap*, *Po. kessleri*, *Po. platyrostris*, *Po. ratan*, *Po. rhodioni*, *Po. syrman*, and *Po. iranicus*) is strongly supported as a separate clade (100% bootstrap support, 1.00 PP), which includes, however, *Babka gymnotrachelus* (with low support; 48% bootstrap, 0.44 PP). Interestingly, the combined analysis of a concatenated alignment of two mitochondrial and two nuclear gene fragments analysis of Neilson and Stepien (2009a) strongly supported a *Babka*–*Ponticola* sister group relationship, each with strongly supported clades clearly divergent from the other genera, thus supporting the recognition of *Babka* and *Ponticola* as distinct genera. Four clades are highly resolved within *Ponticola*. The deepest split is between *Po. ratan* and the remainder of the species (100% bootstrap support, 1.00 PP). The second clade contains *Po. syrman* and *Po. iranicus* with shallow divergence, in which the placement of one *Po. iranicus* haplotype from Siah Darvishan with the *Po. syrman* haplotypes renders *Po. iranicus* paraphyletic. The other two clades comprise the “*platyrostris*” (*Po. cephalargoides*, *Po. cyrius*, *Po. constructor*, *Po. platyrostris*, and *Po. rhodioni*), and the “*kessleri*” (*Po. eurycephalus*, *Po. gorlap*, and *Po. kessleri*) species groups, respectively. Furthermore, two reciprocally monophyletic clades with deep divergence are strongly supported within *Proterorhinus*: a freshwater *Proterorhinus* clade including *Pr. semilunaris*, *Pr. semipellucidus* and *Proterorhinus* sp., and a marine/brackish water *Proterorhinus* clade including *Pr. marmoratus* and *Pr. nasalis*. Neogobiini are strongly supported (83% bootstrap, 0.97 PP) as the sister clade of Ponticolini.

Our hypothesis based on a single mitochondrial locus is very similar to the results derived from combined four gene analysis of Neilson and Stepien (2009a), except for the slightly different placement of *Babka*, and with regard to the sister group relationship of Ponticolini with Neogobiini rather than with Benthophilini; this grouping is, although, not robustly supported in the study of Neilson and Stepien (2009a).

The species delimitation analysis was performed using one distance-based (ABGD), one network-based (SP) and three tree/topology-based species delimitation methods (mPTP, bPTP, and bGMYC). Results are

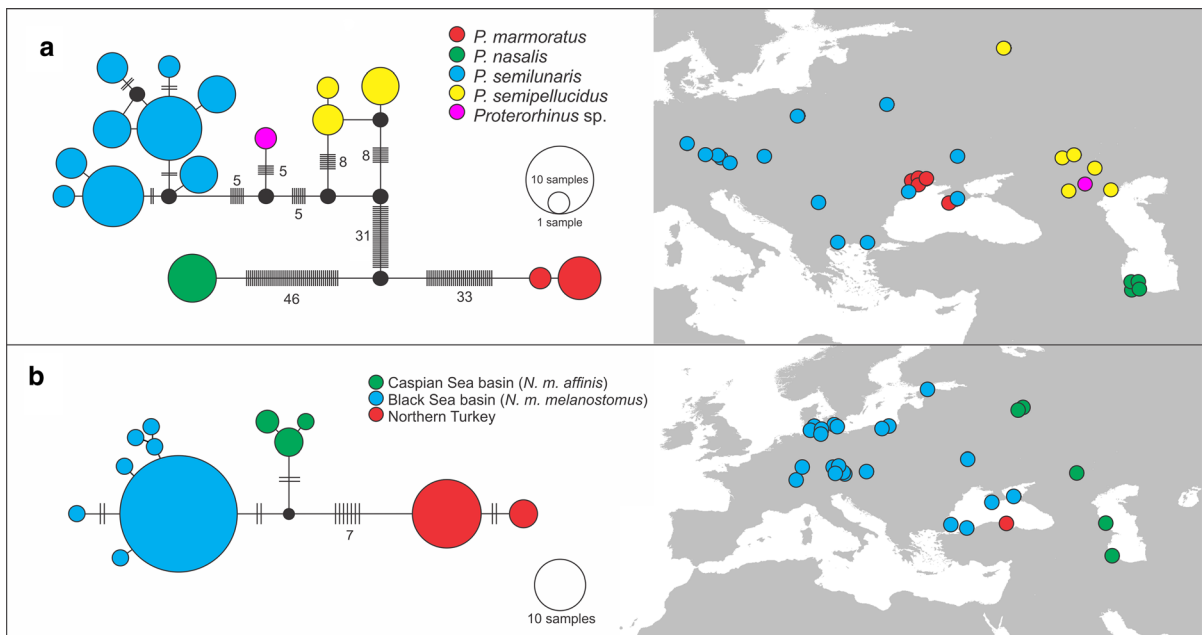


Fig. 3 TCS haplotype networks and maternal phylogeographies of 16 haplotypes (49 individuals) in *Proterorhinus* spp. (a), and 12 haplotypes (85 individuals) in *Neogobius melanostomus* (b). Each line without hatch marks/number of mutations between two neighboring haplotypes represents one mutational

step. Circle sizes depict proportions of haplotypes; the smallest corresponds to one. Small black circles correspond to missing/hypothetical haplotypes. Distribution of *Pr. semilunaris* and the Black Sea lineage of *N. melanostomus* in their North American invasive ranges are not depicted

depicted as grey bars in Fig. 2. ABGD was the most conservative method, since it found support for 12 out of 27 nominal species included here, and lumped *Po. eurycephalus* and *Po. kessleri*, the “*platyrostris*” group elements (*Po. cephalargoides*, *Po. cyrius*, *Po. constructor*, *Po. platyrostris*, and *Po. rhodioni*), *Po. syrman* and *Po. iranicus*, the freshwater *Proterorhinus* species (*Pr. semilunaris*, *Pr. semipellucidus*, and *Proterorhinus* sp.), and *Benthophilus* taxa (*Be. granulatus*, *Be. abdurahmanovi*, *Be. stellatus*, *Be. Mahmudbejovi*, and *Be. leobergius*) into single entities. The other four methods retrieved similar results, providing robust supports for 18 out of 27 nominal species, and lumped *Po. eurycephalus* and *Po. kessleri*, *Po. constructor*, *Po. platyrostris* and *Po. rhodioni*, *Po. syrman* and *Po. iranicus*, and *Be. stellatus*, *Be. mahmudbejovi*, and *Be. leobergius* into single entities.

Using samples from the southern Caspian Sea basin for the first time enables us to reveal refined phylogeographic patterns of several Ponto-Caspian gobiid species. Sequence analysis of 49 marine and freshwater *Proterorhinus* specimens, spanning its native and invasive ranges, detected 126 variable nucleotide sites (4 singleton, 122 parsimony informative), leading to

the definition of 16 haplotypes in 2 independent evolutionary clades with marked phylogenetic divergence (92% bootstrap, 1.00 PP; 5.4 Mya; Figs. 2, 3, 4), the marine and freshwater *Proterorhinus* clade. The marine clade comprises 2 deeply divergent lineages (100% bootstrap, 1.00 PP; 4.6 Mya) separated by 79 fixed substitutions: one from the Caspian Sea basin corresponding to *Pr. nasalis* (collected from the southern Caspian Sea basin); and the second from Black Sea basin corresponding to *Pr. marmoratus*. The freshwater clade comprises three primary lineages: one from the freshwater Caspian Sea basin corresponding to *Pr. semipellucidus* (Fig. 3a); the second from freshwater Black Sea basin (native range), Central Europe and the North American Great Lakes locations (invasive range) corresponding to *Pr. semilunaris*; and the third lineage with a single specimen corresponding to *Proterorhinus* sp. (Neilson & Stepien, 2009a) from the Kuma-Manych Depression. Among basin differences (Black Sea, Caspian Sea, Kuma-Manych Depression) within the freshwater clade showed very high supports in phylogenetic analyses (> 80% bootstrap, 1.00 PP; Fig. 2). ABGD lumped these freshwater lineages into a single putative

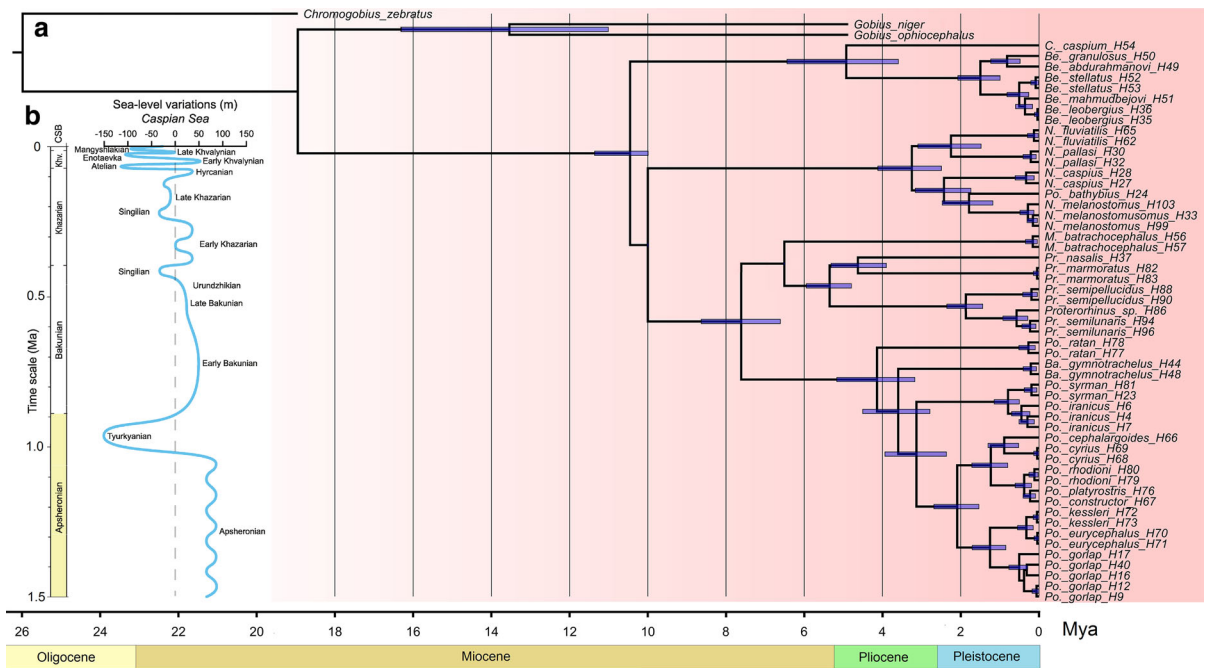


Fig. 4 a Phylogeny of benthophiline gobies, based on a reduced data set of 53 haplotypes plus 3 outgroups. The phylogeny is calibrated with a legacy date of 6.25 Mya at the base of *Proterorhinus* + *Mesogobius* (from Neilson & Stepien, 2009a), and fossil calibration for the origin of “neogobiin” (10 Mya)

derived from Rückert-Ülkümen (2006). Error bars indicate 95% highest posterior density. b Schematic reconstruction of the Caspian Sea water-level curve during the Pleistocene to Holocene

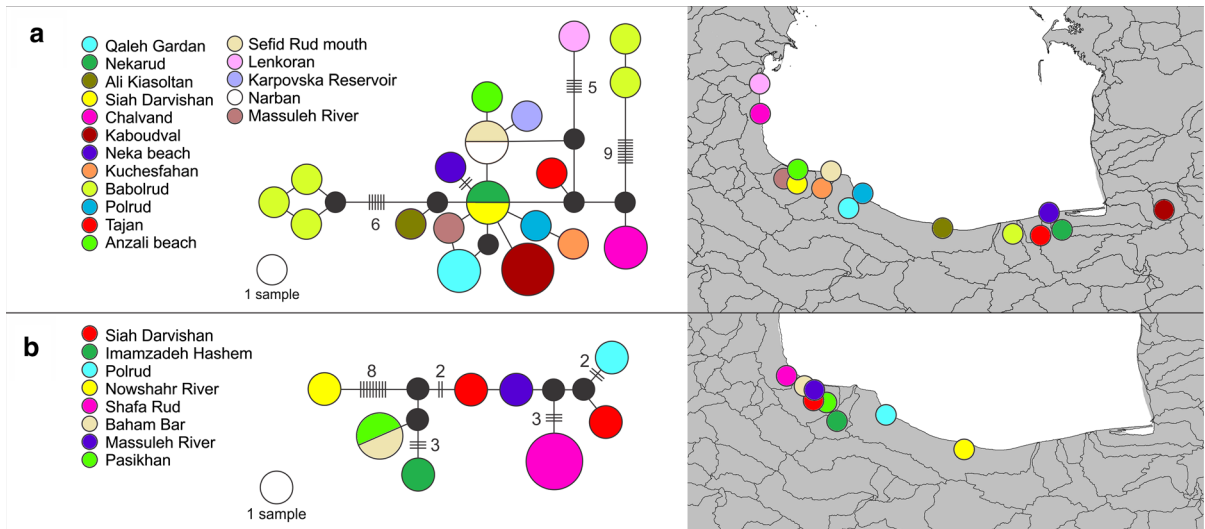


Fig. 5 TCS haplotype networks and maternal phylogeographies of 19 haplotypes (25 individuals) in *Ponticola gorlap* (a), and eight mitochondrial haplotypes (12 individuals) in *Po. iranicus* (b). Each line without hatch marks/number of mutations between two neighboring haplotypes represents one

mutational step. Circle sizes depict proportions of haplotypes; the smallest corresponds to one. Small black circles correspond to missing/hypothetical haplotypes. The geographic locations of Narban (middle Caspian Sea) and Karpovska Reservoir (northern Caspian Sea) are not depicted on the map

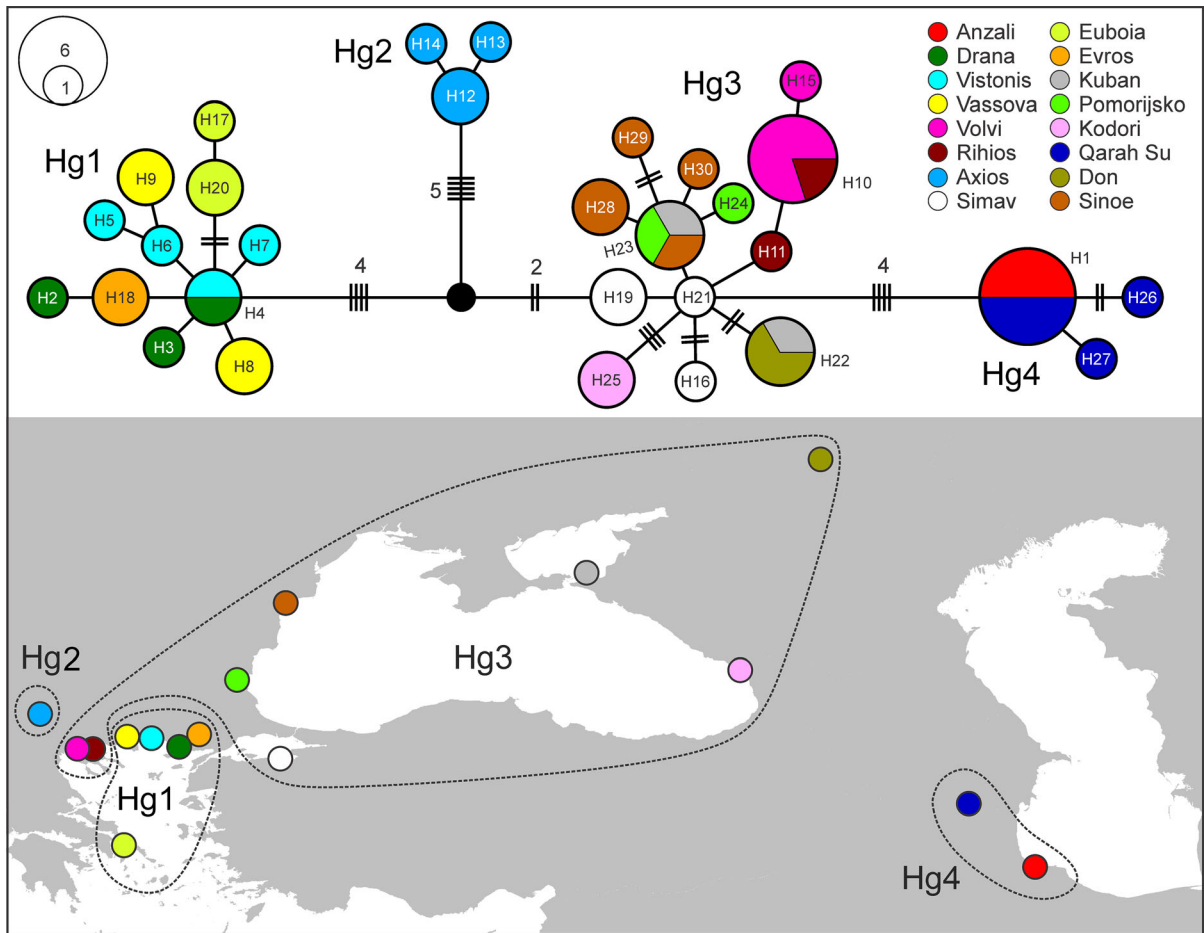


Fig. 6 TCS haplotype network and maternal phylogeography of 30 mitochondrial haplotypes (52 individuals) in *Knipowitschia caucasica*. Hg1–Hg4 represent major lineages. Each line without hatch marks/number of mutations between two

neighboring haplotypes represents one mutational step. Circle sizes depict proportions of haplotypes; the smallest corresponds to one. Small black circles correspond to missing/hypothetical haplotypes

species; however, the four other delimitation methods considered *Pr. semilunaris* + *Proterorhinus* sp., and *Pr. semipellucidus* as separate entities (Fig. 2). In addition, if the K2P distance species threshold value is accepted (> 2%: Ward, 2009; Kartavtsev, 2011), *Pr. semilunaris* and *Pr. semipellucidus* (3.1% sequence divergence) would represent separate species (Table S4).

We analyzed 85 individuals of *N. melanostomus* and recovered 12 haplotypes in 3 lineages with shallow genealogical separations (Fig. 3b): 1 from the Caspian Sea basin corresponding to described subspecies *N. m. affinis*; one from the Black Sea basin (native range), European and North American locations (invasive ranges) corresponding to *N. m.*

melanostomus; and one undescribed lineage confined to the southern Black Sea basin at Sinop, northern Turkey. All delimitation methods lumped these lineages into a single entity (Fig. 2).

Phylogeographic analysis of 25 individuals of *Po. gorlap* from 17 localities (15 in the southern Caspian Sea basin) detected 30 variable nucleotide sites (10 singleton, 23 parsimony informative), defining 19 haplotypes in 4 lineages with shallow separations (5–9 mutational steps; Fig. 5a): 1 from Lenkoran (brackish water); 2 sympatric lineages in the Babolrud (freshwater); and the most common and diverse lineage (17 individuals, 12 haplotypes) distributed across the southern Caspian Sea basin (fresh and brackish

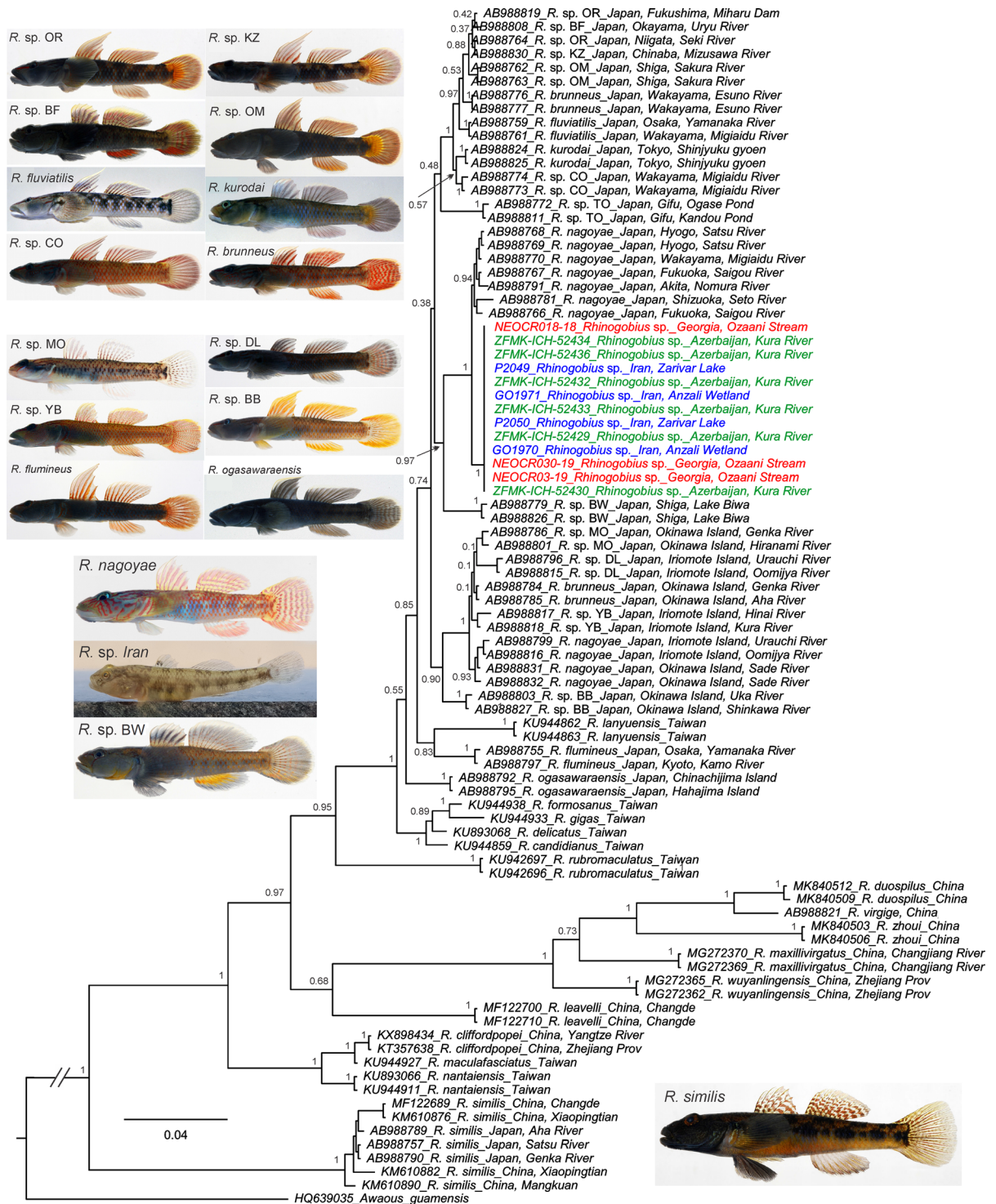


Fig. 7 Bayesian tree of the genus *Rhinogobius*, including 87 individuals plus one outgroup, showing the phylogenetic placement of Iranian (shown in blue), Georgian (red), and

Azerbaijani (green) samples. Posterior probability (PP) values are indicated beside the nodes

waters). All delimitation methods lumped these lineages into a single entity (Fig. 2).

A preliminary phylogeographic analysis of *Po. iranicus* is shown in Fig. 5b [ZM-CBSU P2782 (haplotype H3; see Fig. 2) was excluded from this analysis due to the possibility of mitochondrial introgression from *Po. syrman*]. Within its entire range in the southern Caspian Sea basin, 22 variable nucleotide sites (13 singletons, 9 parsimony informative) were detected among 11 *Po. iranicus* individuals, leading to the definition of eight mitochondrial haplotypes in 2 main lineages (1 with additional substructure) with shallow separation (8 mutational steps; Fig. 5b): one lineage confined to the Nowshahr River, the most distant locality; and a larger lineage (7 haplotypes) with further phylogeographic substructure in the study area. All delimitation methods lumped these two lineages into a single entity (Fig. 2).

Sequence analysis of *K. caucasica* detected 45 variable nucleotide sites (15 singleton and 30 parsimony informative) among 52 individuals, leading to the definition of 30 haplotypes in four main lineages (Hg1–Hg4) with shallow genealogical separations (Fig. 6): a diverse lineage (Hg1) in the Aegean Sea basin (Greece) with little substructure; a second lineage (Hg2) confined to Axios (Greece); a third lineage (Hg3) found in Rihios and Volvi (Greece), and other localities in the Black Sea basin [i.e., Simav (Turkey), Pomorijsko (Bulgaria), Sinoe (Romania), Kuban (Russia), Kodori (Georgia), and Don River (Russia)]; and a fourth lineage (Hg4) in the southern Caspian Sea basin (Anzali and Qarah Su). The mean K2P genetic distance between these four lineages varied between 0.01 (Hg3/Hg4) and 0.022 (Hg2/Hg4) (Table S5).

For the *Rhinogobius* data set (87 sequences, 669 bp length), GTR + I + G was detected as the best-fitting substitution model. The saturation test of Xia's et al. (2003) showed that the *Rhinogobius* sequences have not reached substitution saturation ($Iss < Iss.c$ S/Iss.c A; Table S3), and their BI and ML phylogenies were not significantly different (SH test, $P = 0.14$). The BI phylogeny is presented in Fig. 7, and it includes novel sequences for 4 *Rhinogobius* individuals from Iran combined with 83 archived sequences (GenBank, ZFMK and BOLD-deposited) including 9 *Rhinogobius* individuals from Azerbaijan and Georgia. Several major clades are highly supported as being distinct

within this phylogeny. *Rhinogobius similis* is the most distant species within the genus (100% bootstrap, 1.00 PP). Within the largest and most diverse clade, the four COI sequences developed for the *Rhinogobius* individuals collected from the Anzali Wetland (southern Caspian Sea basin) and the Zarivar Lake (Tigris River system) along with all *Rhinogobius* samples from Azerbaijan and Georgia defined one haplotype, closely related to the archived *R. nagoyae* haplotypes from Japan main islands, Honshuu (Nomura River at Akita, Seto River at Shizuoka, Migiaidu River at Wakayama, and Satsu River at Hyogo) and Kyuushuu (Saigou River at Fukuoka) (100% bootstrap, 1.00 PP). The *R. nagoyae* haplotypes from Okinawa (Okinawa and Iriomote islands) are placed in a separate clade with *R. brunneus* from Okinawa Island and the three other undescribed species (see Akihito et al., 2013; Yamasaki et al., 2015; Suzuki et al., 2020): *Rhinogobius* sp. YB (Kibara-Yoshinobori or yellow belly medium-egg type, distributed in Ryukyu Archipelago), *Rhinogobius* sp. DL (Hira-Yoshinobori, distributed in Yakushima-Iriomote-jima Island), and *Rhinogobius* sp. MO (Aya-Yoshinobori, distributed in Amamioshima-Kume-jima Island).

Discussion

Diversity, systematics, and ichthyogeography of tubenose gobies in the Ponto-Caspian basin

The expanded geographical area of molecular genetic investigation by adding tubenose goby samples from estuarine habitats of the southern Caspian Sea for the first time, as well as including modern statistical, phylogenetic, and species delimitation analyses allowed us to reassess hypotheses on the taxonomy, zoogeography, and evolution of tubenose gobies.

All populations of *Proterorhinus* were once classified as *Pr. marmoratus* (Pallas, 1814), originally described from Sevastopol, Ukraine (Kottelat, 1997; Pinchuk et al., 2004). Stepien and Tumeo (2006) used cytochrome *b* (*Cyt b*) data and considered two species in the genus, *Pr. marmoratus* as the marine species in the Black Sea and *Pr. semilunaris* (Heckel, 1837), as the freshwater species in other Ponto-Caspian habitats; however, Caspian Sea samples were not included in their study. Freyhof and Naseka (2007) restricted *Pr. marmoratus* to brackish waters in Sevastopol, and,

based on samples primarily from marine regions of northern and middle Caspian Sea basin, concluded that the Caspian Sea basin specimens likely constitute another separate species, suggesting resurrection of *Pr. nasalis* (De Filippi, 1863), the oldest available name for the tubenose gobies in the Caspian Sea basin, originally described from near Baku, Azerbaijan. Neilson and Stepien (2009b) examined a concatenated nuclear and mitochondrial dataset and inferred the presence of at least three separate species, *Pr. marmoratus* from marine and estuarine habitats of the Black Sea; *Pr. semilunaris* from the freshwater Black Sea basin (also introduced to the North American Great Lakes); and they tentatively advocated for the name *Pr. cf. semipellucidus* as available taxon for another freshwater species inhabiting the Caspian Sea/Volga River basins. They further predicted *Pr. nasalis* to be the taxon distributed in the more saline waters of southern Caspian Sea. Sorokin et al. (2011) used *Cyt b* data and consider *Pr. nasalis* as the taxon widely distributed in the Caspian/Azov Sea basins, with *Pr. semipellucidus* as its synonym.

We found two primary *Proterorhinus* clades with marked genetic divergence, i.e., the marine and the freshwater clade. The marine clade comprises two independent evolutionary lineages with deep divergence: *Pr. marmoratus* in marine and estuarine habitats of the Black Sea basin, and *Pr. nasalis* in marine and estuarine habitats of the southern Caspian Sea. The freshwater clade comprises three lineages: *Pr. semilunaris* in the freshwater Black Sea basin (also introduced to the European rivers and North American Great Lakes); another freshwater lineage inhabiting the Sea of Azov and northern Caspian Sea/Volga River basins, which Neilson and Stepien (2009b) tentatively identified as *Pr. cf. semipellucidus*; and the *Proterorhinus* sp. lineage from the Kuma-Manych Depression. The species delimitation analyses including SP, mPTP, bPTP, bGMYC, and the K2P distance



Fig. 8 Live specimen of *Neogobius bathybius* sampled from Babolsar Beach, Iran (ZM-CBSU M2114)

species threshold value of $> 2\%$ congruently inferred *Pr. semilunaris* + *Proterorhinus* sp., and *Pr. semipellucidus* as two separate species. The pronounced phylogenetic distinction between *Pr. semilunaris* and *Pr. semipellucidus* is also registered in the sequence of mitochondrial *Cyt b*, and nuclear recombination activating gene 1 (RAG1) and S7 ribosomal protein gene (Neilson & Stepien, 2009b; Sorokin et al., 2011). Accordingly, we hypothesize *Pr. nasalis* to be the taxon in the more saline waters of the southern Caspian Sea, and, we hypothesize that the name *Pr. semipellucidus* for the Azov/northern Caspian Sea/Volga River basin populations will be resurrected depending on the outcome of additional morphological and molecular data in an integrative taxonomical approach. Morphological divergence among tubenose goby lineages appears not as marked as their genetic divergence (Freyhof & Naseka, 2007; Neilson & Stepien, 2009b), but pending additional molecular and/or morphological data, we would support taxonomic distinction of morphologically similar *Proterorhinus* species separated by deep molecular differences, since significant genetic divergence among morphologically cryptic species is not rare in Gobiidae (e.g., Lima et al., 2005; Victor, 2010, 2014; Hashimoto et al., 2014).

Based on mixed nature of samples at several localities, Sorokin et al. (2011) rejected the freshwater/marine Black Sea *Proterorhinus* species hypothesis erected by Neilson and Stepien (2009b). They suggested that *Pr. semilunaris* and *Pr. marmoratus* are two euryhaline species evolved in northwestern vs. northeastern part of the Black Sea basin, followed by recent expansion and secondary contact. Our results not only support the Neilson and Stepien (2009b) scenario, but also extend it to the Caspian Sea basin, because: (i) Sorokin's et al. (2011) hypothesis entails a sister relationship between *Pr. marmoratus* and *Pr. semilunaris*, which our phylogeny does not support; (ii) a freshwater/marine Caspian Sea *Proterorhinus* species pattern is also present; (iii) the observed northwestern vs. northeastern distribution of *Pr. semilunaris* and *Pr. marmoratus* in the Black Sea basin might have resulted from unbalanced sampling, as samples from many parts of the Black Sea basin have not yet investigated in any genetic study; and (iv) only a freshwater/marine species hypothesis could better explain the successful invasion of freshwater preadapted *Pr. semilunaris* and the wider

distributional range of *Pr. semipellucidus*, whereas *Pr. marmoratus* and *Pr. nasalis*, adapted to higher salinities would explain their predominant restriction to the marine and estuarine habitats of the Black Sea and southern Caspian Sea.

Based on our extended taxon sampling and preliminary time tree analysis, a revised hypothesis for the evolutionary and diversification history for tubenose gobies is possible. According to our preliminary node age estimates, *Proterorhinus* and *Mesogobius* separated approximately 6.3 Mya in the Pontian Lake-Sea (Neilson & Stepien, 2009b). In Late Miocene (Pontian) times, the Caspian and Black Sea basins were still connected (Popov et al., 2004; Krijgsman et al., 2010). About 5.4 Mya (Fig. 4), the first major division occurred, and due to decrease in salinity of the Late-Pontic and Kimmerian Lake-Seas (proto-Black Sea basin), the marine and freshwater tubenose goby clades were separated (Zaitsev & Mamaev, 1997; Reid & Orlova, 2002; Neilson & Stepien, 2009b). In the earliest Pliocene, the two basins became isolated after a major drop in water level in the far southward retreat of lacustrine environments (Van Baak et al., 2016), leading to a division between the Black and Caspian Sea marine tubenose goby lineages approximately 4.6 Mya (Fig. 4). The Black Sea and Caspian Sea freshwater tubenose goby lineages were separated about 1.9 Mya (early Pleistocene; Fig. 4) when freshwater tubenose gobies migrated during the Apsheonian transgression from the Gurian Lake-Sea into the Apsheon Lake-Sea across the Kuma-Manych Depression (Reid & Orlova, 2002; Cristescu et al., 2003; Neilson & Stepien, 2009b).

Generic assignment and systematics of the Caspian deep-water goby

The Caspian deep-water goby (Fig. 8) was originally described as *Gobius bathybius* by Kessler (1877) from Svinoi Island, south of Baku, Caspian Sea, Azerbaijan. The genus name *Chasar* appears in print for the first time in Berg (1949) as a subgenus of *Neogobius* to accommodate *G. bathybius* Kessler, 1877. Berg (1949) attributed this name to Iljin but without reference to any publication by the latter author. Berg (1949) provided a brief description of the species as *Neogobius (Chasar) bathybius*, but did not define the genus-group category. Although Vasil'eva (1996) stated that Iljin (1927, 1930) had used this subgeneric

name for the classification of *bathybius*, a search of the latter publication by Miller (2004a) found this species mentioned only as being “incertae sedis” but without reference to any previous use of the name *Chasar* or to a definition by Iljin. Both Iljin (1956) and Ragimov (1967) used the name at a subgeneric level, but again provided no diagnosis. Pinchuk and Ragimov (1985), in their redescription of *bathybius*, placed this species in *Neogobius* without comment about a possible subgeneric location. The first generic diagnosis of *Chasar*, with indication of the type and only species, thus appears to be that by Vasil'eva (1996). The monotypic genus *Chasar* was recognized as a valid taxon by Miller (2004a) on the basis of the head sensory papillae patterns noted by Pinchuk and Ragimov (1985) and Vasil'eva (1996). The resulting paraphyly of *Neogobius* sensu lato (Berg, 1949) was changed in Neilson and Stepien's (2009a) revised classification, by elevating two of Iljin's (1927) subgenera to genus rank, i.e., *Babka* and *Ponticola* for the remainder of the ‘neogobiin’ species. The new classification thus recognized five genera, *Proterorhinus*, *Mesogobius*, *Neogobius*, *Babka*, and *Ponticola*. Neilson and Stepien (2009a) included *bathybius* in *Ponticola* in their molecular study without further justification, since they had not included any specimen of *bathybius* nor any discussion of the nominal genus. Here, for the first time, we presented sequence data from five specimens of *bathybius* from southern Caspian Sea deep waters, allowing us to suggest a revised generic assignment hypothesis, which should be tested with additional morphological and/or nuclear DNA data. The five *bathybius* sequences defined one haplotype, and in our extended phylogeny, the clade corresponding to the tribe Neogobiini (100% bootstrap support, 1.00 PP) comprises a now-restricted *Neogobius* (*N. fluviatilis*, *N. pallasi*, *N. caspius*, and *N. melanostomus*) and *bathybius*, as a distant sister species of *N. melanostomus* (100% bootstrap support, 1.00 PP). Therefore, *bathybius* is likely to be assigned to the genus *Neogobius* sensu stricto (Neilson & Stepien, 2009a) as the fifth species, and the species would achieve a new combination as *N. bathybius* (Kessler, 1877). *Neogobius caspius*, *N. pallasi*, and *N. bathybius* are Caspian endemics, *N. fluviatilis* in the Black Sea is a sister species of *N. pallasi*, and *N. melanostomus* is native to both basins. *Neogobius bathybius* differs from other *Neogobius* species in their cheek sensory papillae pattern by featuring one

Fig. 9 Live specimens of *Ponticola gorlap* sampled from **a** Sefid Rud at Kucheshfahan (ZM-CBSU P2822), **b** Siah Darvishan River (ZM-CBSU P2784), **c** Massuleh River (ZM-CBSU P2832), and **d** Qaleh Gardan River (ZM-CBSU G2174)

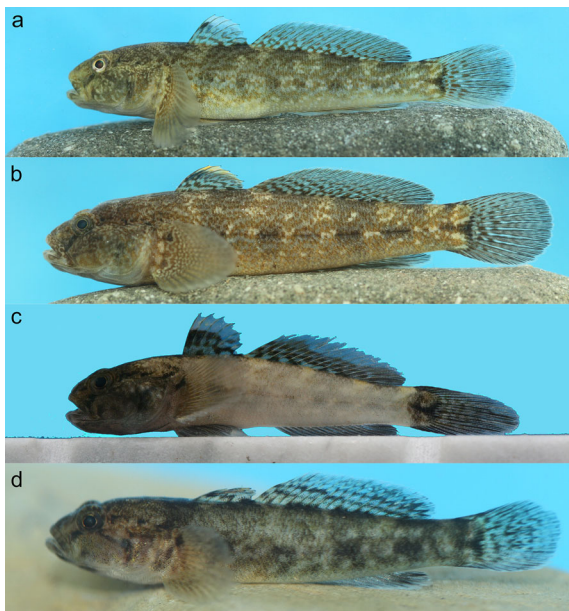
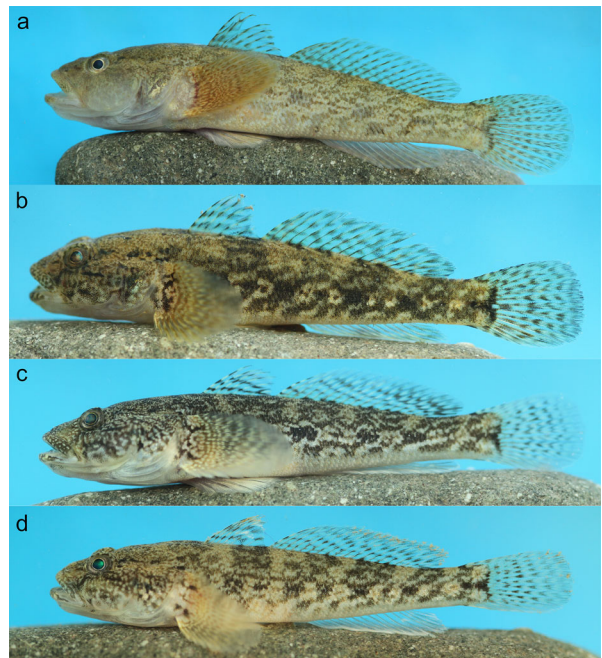


Fig. 10 Live specimens of *Ponticola iranica* sampled from **a** Baham Bar River (ZM-CBSU P2793), **b** Massuleh River (ZM-CBSU P2788), **c** Siah Darvishan River (ZM-CBSU P2819), and **d** Pasikhan River (ZM-CBSU P2794)

additional transverse row before row *b* (Pinchuk & Ragimov, 1985). The presence of five transverse infraorbital rows before row *b* might be interpreted as a synapomorphy with *Mesogobius*, but *N. bathybius*

does not have more than two such rows below row *b*, a plesiomorphic feature shared with the *Neogobius* and *Ponticola* (except for *Po. syrman*) but not with *Mesogobius*, which has three. As an alternative to our mitochondrial DNA (mtDNA)-based hypothesis, an ancient hybridization scenario with *N. melanostomus* as one partner and either a *Ponticola* or *Mesogobius* as another lineage might have led to the same mitochondrial clade phylogenetic pattern as a sister group relationship of *bathybius* with *N. melanostomus*, hereby highlighting the necessity of an integrative taxonomic approach to revise the generic classification of Neogobini and Ponticolini tribes. In the meantime, assignment of *bathybius* to *Neogobius* is nevertheless the most parsimonious solution, given the first phylogenetic evidence provided here.

South Caspian riverine species of *Ponticola* and the evolutionary history of *Po. iranica*

The taxonomic composition in the genus *Ponticola* has been variable, unstable, and frequently uncertain especially in the group *Po. cephalarges* (Pallas, 1811), *Po. platyrostris* (Pallas, 1811), and *Po. kessleri* (Günther, 1861); this is not only because of slight morphological and genetic differences and a mosaic pattern of morphological and karyological features,

but also because of distinctive migratory and resident populations in some species. Thus, the presence of two *Ponticola* species in the southern Caspian Sea basin had become dubious. Ahnelt and Holcik (1996) collected gobies from four rivers, Massuleh, Siah Darvishan, Pasikhan, and Baham Bar, forming a part of watershed of the Anzali Wetland. They reported *Po. cyrius* from Massuleh and Pasikhan, and *Po. iljini* Vasil'eva and Vasil'ev, 1996 from Siah Darvishan and Baham Bar as new for Iran and the southern Caspian Sea, as well. Coad (1998) placed both species in context with the Iranian ichthyofauna. Neilson and Stepien (2009a) placed *Po. iljini* as a synonym of *Po. gorlap* in their revised classification, but they had not studied any material of this species. Vasil'eva et al. (2016) reestablished the validity of *Po. iljini* based on karyological data, but also limited its distribution to the coast of the Mangyshlak Peninsula, western Kazakhstan. Morphological as well as karyological analyses of specimens collected by Vasil'eva et al. (2015) from localities in Sefidrud and Gisum River revealed noticeable differences between those fishes and other known *Ponticola* species including *Po. cyrius*, leading to the description of *Po. iranicus* as the only endemic riverine gobiid species in the southern Caspian Sea basin. Esmaeili et al. (2010, 2014, 2017, 2018) followed Kottelat (1997) and Neilson and Stepien (2009a) in placing *Po. iljini* as a synonym of *Po. gorlap*, but listed *Po. cyrius* as a member of the Iranian and southern Caspian Sea freshwater fish fauna. Our extensive fieldwork between 2014 and 2020 and the phylogenetic results presented here can only support the presence of two species *Po. gorlap* and *Po. iranicus* in the riverine system and reservoirs of the southern Caspian Sea. In the rivers of the Anzali Wetland watershed that were investigated by Ahnelt and Holcik (1996), we only collected *Po. gorlap* and *Po. iranicus* from Siah Darvishan, *Po. gorlap* and *Po. iranicus* from Massuleh, and *Po. iranicus* from Baham Bar and Pasikhan (Figs. 9, 10). Here, we infer accordingly that fishes previously identified by Ahnelt and Holcik (1996) as *Po. cyrius* and *Po. iljini* from the Anzali Wetland watershed are conspecific with *Po. iranicus* and *Po. gorlap*, respectively; thus, *P. iljini* and *P. cyrius* should thus be omitted from the checklist of Iranian freshwater fish and the southern Caspian Sea.

The placement of one *Po. iranicus* haplotype from Siah Darvishan within the *Po. syrman* clade suggests

possible introgression. *Ponticola syrman* differs noticeably from *Po. iranicus* in distribution, ecological requirements, coloration. Its morphological identification is unambiguously possible, especially with regard to differentiation from *Po. syrman* due to the presence of ctenoid scales on the nape (vs. nape scaled completely with cycloid scales), narrow upper lip of uniform width, never swollen (vs. upper lip expanding in middle and slightly swollen, with sharpened end), head depth usually greater than head width (vs. head width slightly larger than depth), anterior membrane of pelvic disc with very shallow, rounded lateral lobes (vs. anterior pelvic membrane with clearly pointed, but short lateral lobes), and three transverse infraorbital papillae rows below longitudinal hyomandibular row *b* (vs. two rows) (Miller, 2004b; Vasil'eva et al., 2015). In the southern Caspian Sea, *Po. syrman* has never been recorded from freshwaters, but it is found in inshore habitats, and less saline estuaries of Sefidrud, Shalmanrud, and Anzali Wetland, where *Po. iranicus* is also present (Abbasi, 2017). According to our preliminary node age estimate, *Ponticola iranicus* and *Po. syrman* separated at about 0.9 Mya (Fig. 4), a date almost coinciding with the Turkynian regression at the end of the Pleistocene Apsheronian (Svitoch, 2012; Krijgsman et al., 2019). This low stand, around 150 m below sea level and with a temporal extent of ca. 100 ka, was one of the most severe regressions in the history of the Caspian Sea. We hypothesize that during this low stand, ancestors of contemporary *Po. iranicus* were isolated in freshwaters of the Anzali Wetland watershed, and, because of evolution under isolation, a high degree of morphological and ecological divergence was achieved rather quickly. Following the early Bakunian transgression, *Po. iranicus* has remained confined to the rivers of Anzali Wetland watershed. Therefore, it is possible that original reproductive barriers formed during speciation secondarily broke down during secondary contact in the estuaries after the early Bakunian transgression, allowing mitochondrial introgression. However, our result could simply be a result of incomplete lineage sorting (Pollard et al., 2006), although multilocus data will be required to test this hypothesis (for a review, see Rubinoff & Holland, 2005).

Taxonomic status, source, and dispersal pattern of the introduced *Rhinogobius* species into Iran, Turkmenistan and the Caucasus

The genus *Rhinogobius* with more than 86 species is the largest genus of freshwater gobies (Yamasaki et al., 2015; Fricke et al., 2020) widely distributed from East to Southeast Asia. The large variations in their life history and egg size resulted in radiation via colonization of novel habitats associated with the ecology of migration. An introduced *Rhinogobius* species has been reported from Iranian inland waters from the Kashaf and Hari Rivers in the Hari River basin (Feb 1996: Abdoli et al., 2000; Coad & Abdoli, 2000), the Anzali Wetland in the Caspian Sea basin [July 2007: K. Abbasi's unpub. data in Coad (2016)], the Zarrineh River in Urmia Lake basin (July 2013: Eagderi & Moradi, 2017), the Jajrud River in the Namak Lake basin (2016: Eagderi et al., 2017), the Zarivar Lake (May 2017: Sadeghi et al., 2019), the Gaveh River (Sep 2016: Eagderi et al., 2018), the Eivashan River (Oct 2018: Eagderi et al., 2018), and the Tange-Hamam River (May 2018; Mousavi-Sabet et al., 2019), all in the Tigris River basin; and, most recently, from the Aras River in the Caspian Sea basin (Jouladeh-Roudbar et al., 2020). Aliev et al. (1988), Shakirova and Sukhanova (1994), and Sal'nikov (1995) refer to a *Rhinogobius* species from the Kara-Kum Canal in Turkmenistan without confirming the species identity. There have been controversial debates on the correct species identification of introduced *Rhinogobius* populations into Iran and Central Asia. Abdoli et al. (2000), and Coad and Abdoli (2000) provisionally identified the Hari River basin samples as *R. similis* Gill, 1859 and considered them conspecific with the species found in the Kara-Kum Canal. Based on this identification, these authors considered it an accidental introduction from the Amur River basin in eastern Asia penetrated into Iran via the Tedzhen/Hari River, although *R. similis* is more widely distributed (Suzuki et al., 2016). Vasil'eva and Kuga (2008) consider the *Rhinogobius* introduced to Central Asia as *R. cheni* (Nichols, 1931), a Chinese species of the Yangtze River drainage. The taxonomic work of Sadeghi et al. (2019) following a redescription of *R. similis* by Suzuki et al. (2016) show that the Iranian populations differ from both *R. cheni* and *R. similis*, and rather identified it *R. lindbergi*, the

northernmost species of the genus described from Russia (Amur and Ussuri Rivers).

Rhinogobius taxonomy is plagued with considerable confusion; and molecular and morphological phylogenetic studies have targeted only a small set of species and have yet failed to obtain a robust phylogenetic hypothesis. Despite its limitations, the phylogeny presented here contains significant information about the Iranian and Caucasian *Rhinogobius* populations; (i) they belong to the same species and mitochondrial haplotype; (ii) it supports the taxonomic recognition by Sadeghi et al. (2019), indicating that the Iranian samples do not belong to *R. similis*; and (iii) Iranian and Caucasian samples are placed within in a particular clade (the *R. brunneus* species complex) with most, but not all, species being endemic to Japan. Within this clade, the Iranian and Caucasian samples are closely related to the *R. nagoyae* haplotypes collected by Yamasaki et al. (2015) from the mainland Japan. A total of 18 species of *Rhinogobius* are known from Japanese waters (all included here), 15 of which are endemic to Japan (Suzuki & Chen, 2011; Akihito et al., 2013; Yamasaki et al., 2015). In spite of the fact that their species status has been strongly supported by morphological, ecological, and genetic studies, scientific names of more than half of the species have not yet been published (Mizuno, 2001; Suzuki et al., 2011); rather, species codes consisting of two alphabet characters have been commonly used for such species (Fig. 6) (e.g., BB, YB, DL, MO, BW, TO, CO, OM, BF, KZ, and OR; Mizuno, 2001; Akihito et al., 2013). We were not able to include autochthonous *R. lindbergi* in our analysis; however, allozyme comparisons with seven Japanese congeners by Sakai et al. (2000) indicate that *R. lindbergi* is genetically only distantly related to these Japanese congeners. Therefore, the species identity of Iranian and Central Asian populations as *R. lindbergi* is ambiguous and must remain provisional.

Based on our results, an alternative hypothesis for the origin and colonisation of this *Rhinogobius* species in the Iranian, Central Asian, and Caucasian waters is possible, and strong support may come from another non-native fish in Iranian waters, the topmouth gudgeon *Pseudorasbora parva* (Temminck & Schlegel, 1846). *Pseudorasbora parva* (Teleostei: Gobionidae), native to East Asia, is one of the most successful invasive fish species in the world. The invasion history, historical records, and genetic

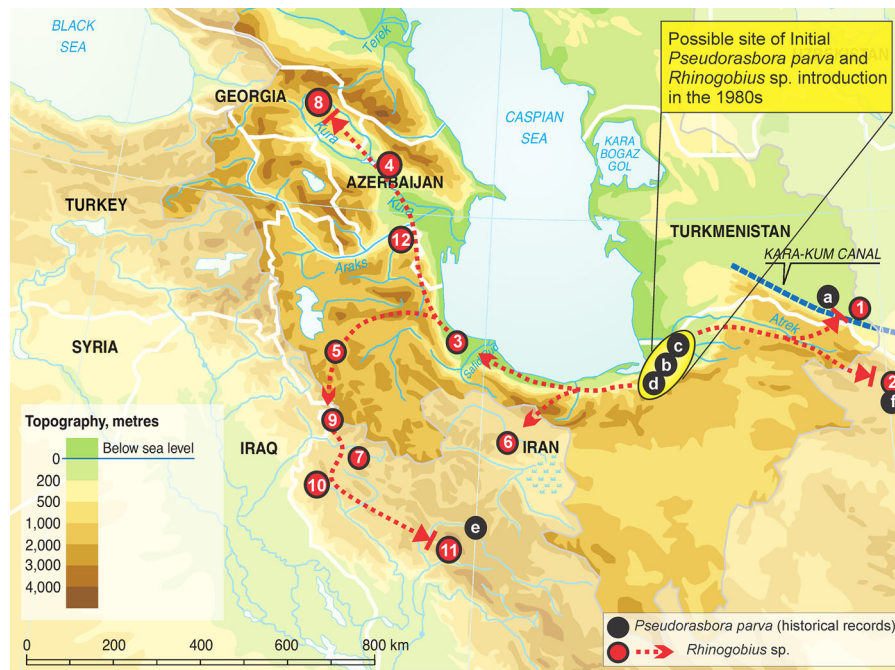


Fig. 11 Proposed invasion scenario for *Rhinogobius* sp. in the inland waters of Iran, Turkmenistan and the Caucasus based on genetic background and historical records of *Rhinogobius* sp. and *Pseudorasbora parva*. The yellow zone shows the area of initial introduction from Japan in the 1980s. Circled letters (a–g) represent the first recording sites of *P. parva* in the region in chronological order: a: Kara-Kum Canal (Welcomme, 1981, 1988); b: on 6 May 1991 from “Ab Bandans” of Avanness (37°03' N, 54°47' E); c: “Hesen Tebib” and “Shaheed Ziaee” (37°1'6" N, 54°47'11" E); d: “Tir Tash” and “Lemrask” (36°42'43.1382" N, 53°44'25.4508" E); e: Arak (34°05' N, 49°41' E); and f: Hari River (36°30'00.0" N, 61°10'00.0" E) (Coad & Abdoli, 1993). Circled numbers (1–10) represent the first recording sites of *Rhinogobius* sp. in the region in

background of the Iranian *Rhinogobius* species are similar to those of *P. parva*. In Iran, *P. parva* was first recorded on May 1991 from “Ab Bandans” of Avanness (vs. Feb 1996 for *Rhinogobius* in Hari River), and soon later from “Hesen Tebib” and “Shaheed Ziaee”, “Tir Tash”, all in the Caspian Sea basin with an approximate distance of 45–70 km to the Atrak River on the Iran–Turkmenistan border (Coad & Abdoli, 1993). Sal'nikov (1995, 1998) considers that fishes in the Tedzhen/Hari, Murgab, and Amu Darya Rivers of Turkmenistan may reach the Caspian Sea basin and conversely via the Atrak River basin. Following the initial discovery of *P. parva* in the Caspian Sea basin, specimens were found at fish ponds in Arak (central) and Mashhad (northeastern Iran;

chronological order: 1: Kara-Kum Canal of Turkmenistan (Aliev et al., 1988); 2: Kashaf and Hari Rivers in the Hari River basin (Feb 1996: Abdoli et al., 2000; Coad & Abdoli, 2000); 3: Anzali Wetland in the Caspian Sea basin [July 2007: K. Abbasi unpub. data in Coad (2016)]; 4: Azerbaijan (Oct 2012; ZFMK, www.collections.zfmk.de); 5: Zarrineh River in Urmia Lake basin (July 2013: Eagderi & Moradi, 2017); 6: Jajrud river in the Namak Lake basin (2016: Eagderi et al., 2017); 7: Gaveh River (Sep 2016: Eagderi et al., 2018); 8: Georgia (Apr 2017; Japoshvili et al., 2020); 9: Zarivar Lake (May 2017: Sadeghi et al., 2019); 10: Eivashan River (Oct 2018: Eagderi et al., 2018); 11: Tange-Hamam River (May 2018; Mousavi-Sabet et al., 2019); and 12: Aras River (Jouladeh-Roudbar et al., 2020)

140 km distance to Hari River at Sarakhs). Coad and Abdoli (1993), Coad and Abdoli (2000), Coad (1996), and Abdoli et al. (2000) recorded *P. parva* along with *Rhinogobius* in the Hari River in Iran. Welcomme (1981, 1988 in: Courtenay & Stauffer, 1984) also reported *P. parva* from the Kara-Kum Canal in Turkmenistan. Nowadays, *P. parva* is widely distributed and established in Iranian inland waters, and it has been found in all localities where *Rhinogobius* has been recorded (Coad, 2016; Eagderi et al., 2017, 2018; Eagderi & Moradi, 2017; Mousavi-Sabet et al., 2019; Sadeghi et al., 2019; Jouladeh-Roudbar et al., 2020). Recently, based on mtDNA control region and COI sequence variation of 161 samples collected from 15 Iranian localities and additional sites in Europe and

Asia, Ganjali et al. (2020) characterized the pattern of genetic diversity and colonisation history of *P. parva* in Iran. Ganjali et al. (2020) show the presence of three *P. parva* haplotypes in Iran belonging to two distinct lineages: (ii) a Chinese lineage represented by a single haplotype found in the Shafarood River of the Caspian Sea basin; it penetrated recently to northern Iran through natural dispersal from Azerbaijan as an extension of the European wave of invasion; and (i) a widespread lineage with a common haplotype found throughout the country and one private haplotype confined to the Mashkid basin, originated from Japan through a single introduction of a small number of propagules and corresponded to the Iranian introduction of *P. parva* in the 1980s. After the Iran–Iraq war in 1988, the Iranian government began paying more attention to its fisheries and aquaculture industry (Karimpour et al., 2013). During this period, Japan was a good trading partner for Iran and the volume of Japanese exports to Iran was the second largest in the world, behind Germany (Nobuaki, 2012). Therefore, similarities in the genetic background, invasion history, and historical records in Iranian waters might indicate that the initial and simultaneous introduction of *P. parva* and *Rhinogobius* sp. into the Iranian inland waters originated from Japan as a by-product of Asian carp imports for aquaculture, possibly associated with the strong commercial link between Japan and Iran in the 1980s (Fig. 11). Following this initial introduction to the northeastern part of the southern Caspian Sea basin, both species penetrated eastward through the Atrak River basin into the Tedzhen/Hari River and Kara-Kum Canal in Turkmenistan. In addition, these species spread toward west and southwest in Iranian inland waters and historical records for both species support this conclusion. The Iranian *Rhinogobius* species has penetrated into Azerbaijan and recently, into Georgia (Epitashvili et al., 2020; Japoshvili et al., 2020), and is expected to be found in Armenian, and to enter the Black Sea basin very soon (Kuljanishvili et al., 2020). Similar to *P. parva*, the presence of only one *Rhinogobius* sp. mitochondrial haplotype in the inland waters of Iran and the Caucasus as well most likely refers to a single introduction of a small number of propagules.

Phylogeography of the Caucasian dwarf goby

The Caucasian dwarf goby, *K. caucasica*, is a widespread Ponto-Caspian species (Berg, 1949; Svetovidov, 1964), in the Caspian Sea, Sea of Azov, and Black Sea, and also introduced to the Aral Sea but in addition found outside this region, in the Aegean and eastern Ionian catchments (Miller, 2004b). The specific name of *causicus*, proposed by Kawrajsky for museum use, was originally published as a *nomen nudum* by Radde (1899) and not until 1916 was the taxon described by Berg (with the type locality being a swamp near Batum and Lake Inkit near Pitzunda, Georgia, Black Sea), the name being attributed to Kawrajsky. The phylogeographic outcome retrieved here for *K. caucasica* is a shallow genealogy with major lineages mainly allopatric. Four main clades were identified among the Caucasian dwarf goby COI haplotypes, corresponding to three major hydrogeographic basins, the Aegean (Hg1–Hg2), Black (Hg3), and Caspian Sea (Hg4) basins. The implication is that contemporary gene flow has been low enough in relation to population size to have permitted lineage sorting and random drift (or, perhaps, diversifying selection) to promote genetic divergence among basins/populations that nonetheless were in historical contact recently. The phylogeographic break between the Caspian and Black Sea lineages (either population, subspecies, or species-level separations) was also documented for some mysid crustaceans (Audzijonyte et al., 2006), *Pontogammarus* amphipods and onychopod cladocerans (Cristescu et al., 2003), the round goby *N. melanostomus* (this study; Brown & Stepien, 2008), the cyprinid fish *Rutilus frisii* (Kotlik et al., 2008), the tubenose gobies of genus *Proterorhinus* (this study; Neilson & Stepien, 2009b), the chub *Leuciscus cephalus* (Durand et al., 1999), and the Eurasian monkey gobies *N. fluviatilis* and *N. pallasi* (Neilson & Stepien, 2011). These cases characterize what is implied by the principles of genealogical concordance; concordance in the geography of gene-tree partitions across multiple co-distributed taxa implicates shared historical biogeographic factors in shaping genealogies (Avise & Ball, 1990). This fact corresponds to the geological history of the Ponto-Caspian region, as the historic Black and Caspian Sea basins have been intermittently separated and connected over the past 5 Ma associated with Pliocene and Pleistocene glaciations (Reid & Orlova, 2002),

promoting isolation, adaptation, and divergence into localized, distinct lineages in many taxonomic groups (Dumont, 1998, 2000; Naseka & Bogutskaya, 2009).

Management units (MUs) can be distinguished by considerable divergence in allele frequencies, regardless of depth in a genealogy (Moritz, 1994). Mitochondrial haplotypes are particularly important for distinguishing MUs because of their typical fourfold smaller effective population size (compared to haplotypes at autosomal loci), and because of their special relevance to demographic and reproductive connections among populations (Avice, 2000). Even shallow matrilineal subdivisions can be relevant to conservation efforts. Accordingly, we may consider populations of *K. caucasica* in the Caspian, Black, and Aegean Sea basins and Axios as separate MUs. However, since this phylogeographic outcome is established based on a limited number of *K. caucasica* specimens, one cautionary point should be made. As larger numbers of individuals are assayed, the power to detect a different phylogeographic pattern increases (Avice, 2000). For this reason, the shallow but allopatric phylogeographic pattern retrieved here for the Caucasian dwarf goby should be considered as preliminary.

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Data availability The COI sequence dataset generated and analyzed during this study is available in the GenBank repository. Specimens used in the present study are deposited as voucher specimens in the Zoological Museum of Shiraz University, Collection of Biology Department (ZM-CBSU), and the SNSB-Bavarian State Collection of Zoology, Munich (SNSB-ZSM).

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors. All necessary permits for sampling have been obtained by the authors from the Iranian authorities. The research work was approved by Ethics Committee of Biology Department, Shiraz University (SU-9630190).

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