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Evolution and phylogeography of the tubenose goby genus *Proterorhinus* (Gobiidae: Teleostei): evidence for new cryptic species

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Cryptic taxa present unique difficulties in the description of biological diversity, which DNA sequencing approaches often readily resolve. The tubenose goby *Proterorhinus*, along with other Ponto-Caspian fauna, has undergone recent Eurasian range expansion, as well as colonized the North American Great Lakes in 1990. We analysed mitochondrial (cytochrome *b* and cytochrome *c* oxidase subunit I) and nuclear (recombination activating gene 1; RAG1) DNA sequences and morphological characters from exotic Great Lakes as well as introduced and native Eurasian populations of *Proterorhinus marmoratus* (Pallas) *sensu lato* to assess their species identity and biogeographic patterns. The results obtained show marked genetic and morphological divergence that indicates species-level separation between fresh water and marine/brackish lineages, dating back approximately 3.82–4.30 million years. In addition, freshwater lineages within the Black and Caspian Sea basins show significant genetic and morphological differentiation, corresponding to an estimated 0.92–1.03 million years. We describe new evidence to support at least three separate species: the original *P. marmoratus* in marine and estuarine habitats within the Black Sea, a freshwater species in the Black Sea basin that was introduced to the North American Great Lakes, and another freshwater species inhabiting the Caspian Sea/Volga River basin. The freshwater tubenose goby in the Black Sea basin originally was described as *Proterorhinus semilunaris* (Heckel), and this is confirmed to be a valid taxon. The Caspian basin taxon may correspond with *Proterorhinus semipellucidus* (Kessler), a putative freshwater species in the Caspian basin that was originally described from a single specimen.

ADDITIONAL KEYWORDS: Black Sea – Caspian Sea – cytochrome *b* – COI – exotic species – phylogeography – Ponto-Caspian – RAG1 – systematics.

INTRODUCTION

Cryptic species are evolutionarily distinct yet morphologically indistinguishable from their relatives, which often precludes correct diagnosis and poses a fundamental problem in describing biological diversity. Morphology traditionally has been the primary means of identifying species; however, other types of information are essential to identify cryptic taxa in the absence of anatomically distinguishing characters (Bickford *et al.*, 2007; Kon *et al.*, 2007). Genetics, behaviour, and natural history all have been used to supplement morphology in defining species-level diversity among organisms, with DNA characters revolutionizing systematics in the past two decades (Hillis, 1988; Johnson, Dowling & Belk, 2004; Egge & Simons, 2006).

Cryptic divergence, in the absence of apparent morphological separation, has been discovered within a broad range of marine and freshwater taxa (Knowlton, 1993, 2000). Molecular data, such as DNA sequences, often reveal deep genetic divergences within taxa that once were regarded as synonymous, due to little morphological variation (Knowlton, 2000; Gómez *et al.*, 2002; Slapeta, López-García & Moreira, 2006). Several recent introductions of cryptic or

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Figure 1. A, freshwater tubenose goby (*Proterorhinus semilunaris*), UMMZ 231002, 32.1 mm standard length. B, enlargement of head showing tubular nostrils. Scale bar = 5 mm.

previously undescribed species have been detected through genetic analyses of invasive sister taxa; for example, the green crab *Carcinus aestuarii* in areas thought to contain its more widespread sister taxon *Carcinus maenas* (Geller *et al.*, 1997) and introduction to the Mediterranean Sea of genetically distinct lineages of hardyhead silverside fish *Atherinomorus lacunosus* from the Red Sea (Bucciarelli, Golani & Bernardi, 2002). In cases of invasive or introduced species, misidentification may mask our ability to predict their potential impacts in invaded areas, precluding correct ecological comparisons (Ruiz *et al.*, 1997).

The tubenose goby (*Proterorhinus*; family Gobiidae), along with members of the closely related genera *Ponticola* and *Neogobius*, is part of a growing number of Ponto-Caspian taxa introduced outside of their native range (Black/Caspian Sea basins) into western and central parts of Europe as well as to North America (Ricciardi & MacIssac, 2000). *Proterorhinus* was discovered in the North American Great Lakes in 1990 (Jude, Reider & Smith, 1992), attributed to ballast water exchange from transoceanic shipping vessels, and now is limited to the St Clair and Detroit Rivers, and the western margins of Lakes Erie, St Clair, and Superior. In central Europe, *Proterorhinus* expanded up the Danube River during the past two decades via shipping and the construction of canals (Ahnelt *et al.*, 1998; Prášek & Jurajda, 2005). Naseka *et al.* (2005) suggested that its recent expansion throughout the Volga River originated from the Don River (through the Volga-Don Canal) rather than from the lower Volga River and Caspian Sea. To assess the ecology of these introductions and identify source and non-native populations, it is essential to understand their correct species identity.

Proterorhinus was described by Pallas (1814) as *Gobius marmoratus* from Sevastopol, Ukraine, and is distinguished from other Ponto-Caspian gobiids by tubular nostrils that extend onto or beyond the upper lip (Fig. 1). Tubenose gobies are moderately large gobiids (up to 11 cm in total length) with infraorbital neuromast organs in seven transverse rows, and complete oculoscapular and preopercular lateral line canals; scaled nape and breast with cycloid scales, cheek naked; and overall body coloration yellow to light brown with five or six irregular dark brown blotches.

Several tubenose goby species were once recognized, including four marine and three freshwater taxa, of which the latter were: *Gobius semilunaris* Heckel 1837 from the Maritsa River, Bulgaria; *Gobius rubromaculatus* Kriesch 1873 from the Danube River near Budapest, Hungary; and *Gobius semipellucidus* Kessler 1877 from the mouth of the Karasu River, in Astrabad (Gorgon) Bay, Iran. Smitt (1899) provided the first usage of *Proterorhinus* as a subgenus of *Gobius*, which Berg (1916) elevated to generic status.

Berg (1949) later synonymized all *Proterorhinus* species as *Proterorhinus marmoratus* except for *Proterorhinus semipellucidus*, which is currently regarded as a synonym of *P. marmoratus* (Pinchuk, *et al*., 2003).

Recently, Stepien *et al.* (2005) and Stepien & Tumeo (2006) found marked genetic divergence between freshwater and marine specimens of *P. marmoratus* with mitochondrial (mt) cytochrome (cyt) *b* sequence data, concluding that they comprise separate species and suggesting *P. semilunaris* be resurrected for the freshwater clade in the Black Sea basin and North America. Freyhof & Naseka (2007) described a new freshwater species, *Proterorhinus tataricus*, from the Chornaya River in the Crimean Peninsula near Sevastopol, Ukraine using morphological characters; however, no genetic data or modern statistical, phylogeographic, or evolutionary analyses were presented. It is distinguished from *P. marmoratus* by greater number of mid-lateral scales and wider interorbital distance, and from the Black Sea freshwater *P. semilunaris* (*sensu* Stepien & Tumeo, 2006) in having a shorter and deeper head and more second dorsal fin rays. Freyhof & Naseka (2007) also concluded that 'marine' specimens from the Caspian Sea basin likely constituted another separate species, suggesting resurrection of *Proterorhinus nasalis* (originally described as *Gobius nasalis* by Filippi, 1863).

The present study expands on the work of Stepien *et al.* (2005) and Stepien & Tumeo (2006) by further investigating the divergence between freshwater and marine *Proterorhinus*, with the goals of describing their evolutionary and phylogeographic history and to evaluate whether there are additional cryptic taxa. Our investigation analyses DNA sequence data from two mt genes [cyt *b* and cytochrome oxidase *c* subunit I (COI)] and a nuclear gene [recombination activating gene 1 (RAG1)] to more fully reconstruct phylogenies, estimate divergence times, and describe spatial patterns in genetic variation within and among tubenose gobies from 18 locations throughout much of their known native and introduced ranges. We include locations within the Caspian Sea basin and the Kumo-Manych Depression (a geologic depression separating the Russian Plain and the northern foothills of the Caucasus Mountains) that were not sampled in prior work, as well as the type locality of *P. marmoratus* at Sevastopol, Crimea, Ukraine. In addition, we assess morphological characters of freshwater and marine specimens using multivariate ordination techniques to describe and quantify potential morphological separation. We thus provide the first analysis of the genus *Proterorhinus* using both multilocus DNA sequences and modern statistical survey of morphology.

MATERIAL AND METHODS

Specimen collection

We analysed specimens obtained by small beach seine or beam trawl from 18 freshwater and marine localities throughout much of the native and introduced range of *Proterorhinus* (Fig. 2; Table 1), with additional museum material used for morphology (see Supporting information; Appendix S1). Specimens were preserved immediately either in 95% ethanol for molecular analyses or 10% formalin for morphological analyses (following removal of right pectoral fin for genetic study).

Molecular analyses

Genomic DNA was isolated using a Qiagen DNEasy tissue extraction kit following the manufacturer's protocols. Two mt genes (cyt *b*, COI) and a nuclear gene (RAG1) were amplified via the polymerase chain reaction (PCR) using primers listed in Table 2. Use of independent loci from both the nuclear and mitochondrial genomes in a combined analysis can aid in the estimation of the true 'species tree' by searching for common relationships among lineages in individual gene trees (Avise, 2000), maximizing potential inference power. Different genes evolve at different rates, and thus are useful in resolving multiple hierarchical levels within a phylogeny or in estimating separation at different temporal scales (Quenouille, Bermingham & Planes, 2004).

PCR amplifications were performed in 25 μL volumes containing: 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ (2.5 mM for COI), 0.001% (w/v) gelatin, 200 μM each dNTP, 0.5 μM each primer, 1.5 units *Taq* polymerase, and approximately 100 ng (1–3 μL) template DNA. The PCR profile for cyt *b* and RAG1 included an initial denaturation at 94 °C for 2 min; 40 cycles of 94 °C for 45 s, a gene specific annealing temperature (Table 2) for 30 s, and 60 s extension at 72 °C; with a final extension at 72 °C for 3 min. The cycling profile for COI included an initial denaturation at 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 60 s; with a final extension at 72 °C for 2 min. PCR reactions were visualized on 1% agarose gels with ethidium bromide, with excess primers and unincorporated nucleotides removed with spin columns (QIAquick PCR Purification Kit, Qiagen; or QuickStep 2 PCR Purification Kit, Edge Biosystems). Amplicons were sequenced in both directions using dye-labelled terminators on an ABI 3730 (Applied Biosystems) genetic analyser at the Cornell University Life Sciences Core Laboratories Center. We aligned forward and reverse sequences for each gene per individual with BIOEDIT (Hall, 1999).

We employed both phylogenetic and population genetic approaches to evaluate variation in *Proter-*

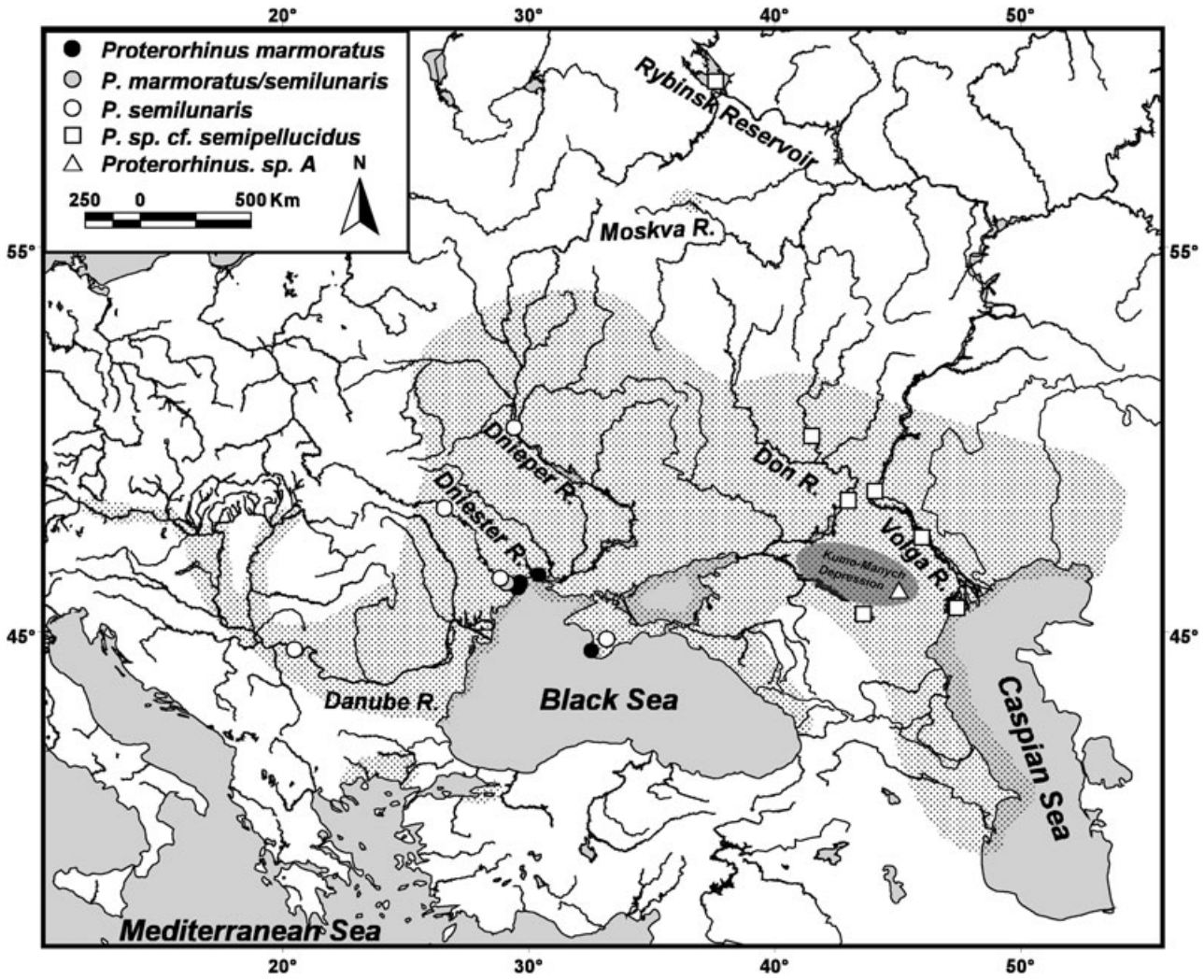

Figure 2. Collection locations of *Proterorhinus* specimens in native and invasive European populations. Shaded area indicates current range of *Proterorhinus* (*sensu* Pinchuk *et al.*, 2003).

orhinus. Phylogenetic analyses compared variation at three gene loci in *P. marmoratus* (*s.l.*) with representatives of the most closely related genera (Stepien *et al.*, 2005; Stepien & Tumeo, 2006), including the monkey goby *Neogobius fluviatilis* (Pallas 1814; formerly *Apollonia fluviatilis* per Stepien & Tumeo, 2006), round goby *Neogobius melanostomus* (Pallas 1814; formerly *Apollonia melanostoma* per Stepien & Tumeo, 2006), knout goby *Mesogobius batrachocephalus* (Pallas 1814), racer goby *Babka gymnotrachelus* (Kessler 1857), and bighead goby *Ponticola kessleri* (Günther 1861). The population genetic approach analysed variation at the cyt *b* locus to elucidate fine-scale biogeographic patterns and processes. Our phylogenetic approach included a representative from each *Proterorhinus* population, whereas the population genetic approach included all individuals in our collection. All sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov).

Our phylogenetic analyses centered on testing the validity of distinct marine and freshwater *Proterorhinus* lineages, and evaluated relative support from independent lines of evidence (mt and nuclear DNA) in separate analyses and a total DNA combined evidence approach. Several phylogenetic approaches, having different evolutionary assumptions, were used to evaluate our evolutionary hypothesis (Avise, 2004): maximum parsimony (MP) (PAUP*, version 4.0b10; Swofford, 2003), maximum likelihood (ML) (PhyML, version 2.4.4; Guindon & Gascuel, 2003), and Bayesian (MrBayes, version 3.1.2; Ronquist & Huelsenbeck, 2003).

Parsimony analyses were performed with branch and bound searches, and branch support was

Table 1. Collection location, salinity (parts per thousand), and number of tubenose goby individuals sampled for molecular analyses

Taxon	Body of water	Location	Salinity (ppt)	*N*	Latitude	Longitude	Code
Proterorhinus marmoratus	Dniester River	Delta near Bilyayivka, Ukraine	0–2	15	46.468333	30.216667	A
	Sukhyi Estuary	Illichivs'k, Ukraine	14–17	15	46.326700	30.667550	B
	Odessa Bay	Cape Langeron, Ukraine	14–17	13	46.483333	30.755000	C
	Odessa Bay	Cape Malyi Fontan, Ukraine	14–17	9	46.450000	30.766667	D
	Tyligul Estuary	Koza, Ukraine	4	15	46.690000	31.486783	E
	Black Sea	Sevastopol, Ukraine	17–18	23	44.604040	33.540840	F
Proterorhinus semilunaris	Lake Superior	St Louis R. estuary, MN, USA	0	9	46.666667	–92.200000	G
	Clinton River	1 km upstream of Lk St Clair, MI, USA	0	10	42.594282	–82.803323	H
	Danube River	Dobra, Iron Gate Gorge, Serbia	0	3	44.638100	21.909400	I
	Dniester River	Mohyliv-Podilskii, Ukraine	0	1	48.447860	27.782797	J
	Kurchurgan Reservoir	Hradenytsi, Ukraine	0	5	46.600000	29.986000	K
	Dnieper River	Kiev, Ukraine	0	3	50.490019	30.517685	L
	Simferopol Reservoir	Simferopol, Ukraine	0	8	44.928732	34.149352	M
Proterorhinus cf. *semipellucidus*	Rybinsk Reservoir	Rybinsk, Russia	0	6	58.362982	38.425064	N
	Buzuluk River	Alexivska, Russia	0	1	50.273600	42.182188	O
	Volga-Don Canal	Karpovska Reservoir, near Iliovka, Russia	0	3	48.643269	43.617069	P
	Chagraiskoye Reservoir	Zunda Tolga, Russia	0	3	45.617691	44.211077	Q
	Volga River	Volgograd Reservoir, Volgograd, Russia	0	1	48.870870	44.660139	R
	Volga River	Preshib, Russia	0	3	47.683923	46.509057	S
	Volga River delta	Damchik, Russia	0–6	3	45.788350	47.886953	T
Proterorhinus sp.	Chernozemel'skii Connector	120 km east of Elista, Russia	0	2	46.272008	45.615373	U

Table 2. Primers pairs used for polymerase chain reaction amplification (including reaction annealing temperatures T_A) and DNA sequencing of the tubenose goby

Gene	Primer name	Sequence (5′ to 3′)	T_A (°C)	Reference
Cytochrome *b*	AJG15	CAAAAACCATCGTTGTAATTCAACT	52	Akihito *et al.* (2000)
	H15343goby	GGGTTATTAGATCCTGTTTCGTGTAGG		This study
	L15162goby	GCTATGTCCTACCATGGGGGCAAATATC	52	This study
	H5	GAATTYTRGCTTTGGGAG		Akihito *et al.* (2000)
Cytochrome oxidase *c* subunit I	L6468	GCTCAGCCATTTTACCTGTG	53	Thacker (2003)
	H7127	ACYTCTGGGTGACCAAAGAATC		Thacker (2003)
	L7059	CCCTGCMGGTGGAGGAGACCC	53	Thacker (2003)
	H7696	AGGCCTAGGAAGTGTTGAGGGAAG		Thacker (2003)
Recombination activating gene 1	RAG1F1	CTGAGCTGCAGTCAGTACCATAAGATGT	50	Lopez, Chen & Orti (2004)
	R811goby	TCATAGCGCTCTAGGTTCTCC		Present study
	F709goby	CTTATGTCTGCACGCTCTGC	50	Present study
	RAG1R1	GTGAGTCCTTGTGAGCTTCCATRAAYTT		Lopez *et al.* (2004)

evaluated via nonparametric bootstrapping (1000 replications) and decay indices (Bremer, 1994). For likelihood and Bayesian analyses, MODELTEST, version 3.7 (Posada & Crandall, 1998) determined the simplest best-fit model for the dataset using the Bayesian information criterion (Posada & Buckley, 2004). For the cyt *b* data, the best-fit model was HKY+I+G with a shape parameter (α) of 1.1964 and a proportion of invariant sites (i) of 0.4805; for COI, the best-fit model also was HKY+I+G ($\alpha = 1.1987$; $i = 0.57$); and for RAG1, HKY+G ($\alpha = 0.0176$). Bayesian analyses using Metropolis coupled Markov chain Monte Carlo sampling were run for five million generations, with sampling every 1000 generations, to assure convergence of likelihood values. Four separate chains were run in each of two simultaneous analyses, and burn-in period was determined by plotting log likelihood values at each generation to identify the point where values reached stationarity, which occurred after 200 000 generations. We chose a conservative burn-in period of one million generations, and discarded prior trees and parameter values. Branch support was calculated from 1000 bootstrap replications for likelihood analyses and via the posterior probability distribution of clades for Bayesian analyses.

We performed a partition homogeneity test to determine combinability of the three molecular datasets, using reduced numbers of marine and freshwater *Proterorhinus* (four sequences from each lineage) to minimize phylogenetic noise associated with highly similar sequences (Dolphin *et al.*, 2000) and removing uninformative characters (Cunningham, 1997). No significant incongruence was found among the three genes (1000 replicates; $P = 0.158$): all were combined for simultaneous analysis using search strategies identical to the separate analyses, with GTR+I+G ($\alpha = 0.819$; $i = 0.60$) selected as the best-fit model. A single model approach was used for the ML analysis, whereas a partitioned mixed-model approach was used for Bayesian analysis. The models of sequence evolution identified for each individual gene region were assigned using the APPLYTO command, and the appropriate model parameters were estimated for each gene using the UNLINK command.

Population genetic analyses focused on describing fine scale genetic variation across the current range of *Proterorhinus*, using cyt *b* sequences from all individuals. A second ML search was performed including all discovered haplotypes, the outgroups from the original phylogenetic analysis, and additional gobioid outgroup taxa (Gobiidae: Amblyopinae – *Taenioides limicola* [AB021253]; Gobiinae – *Gobiosoma bosc* [AY848456]; Gobionellinae – *Gymnogobius petschiliensis* [AY525784]; Oxudercinae – *Periophthalmus argentilineatus* [AB021251]; Eleotridae: Butinae – *Butis amboinensis* [AB021232]; Eleotrinae – *Eleotris fusca* [AB021236]; Rhyacichthyidae – *Rhyacichthys aspro* [AP004454]). The search strategy (including bootstrap analysis) was identical to that in the initial phylogenetic analyses, except that a slightly more complex model was chosen for this extended cyt *b* dataset (TrN+I+G; $\alpha = 0.9223$; $i = 0.4706$).

To identify boundaries among evolutionary lineages, we used Wiens & Penkrot's (2002) tree-based method, which evaluates concordance between geography and a haplotype phylogeny to identify gene flow or isolation among populations of a focal species. Deep divergences among geographically discordant lineages can indicate putatively independent ones hidden within a taxon (Wiens & Penkrot, 2002). The null hypothesis of lineage monophyly as a chance outcome of random branching processes was tested

sensu Rosenberg (2007). Tajima's (1989) *D* test, implemented in ARLEQUIN, version 3.11 (Excoffier, Laval & Schneider, 2005), examined whether patterns of variation within *Proterorhinus* fit a hypothesis of neutrality.

To estimate divergence times, we calculated pairwise sequence divergence (using the TrN model above) within and among major *Proterorhinus* lineages in MEGA, version 4 (Tamura *et al.*, 2007), and using a penalized likelihood approach in R8S, version 1.71 (Sanderson, 2003). We utilized an average pairwise sequence divergence rate for cyt *b* of 2.05% per million years estimated from sister species of *Evorthodus* gobies by Rocha *et al.* (2005). Divergence times were further examined with a penalized likelihood (Sanderson, 2002) approach in R8S (Sanderson, 2003) using an initial age estimate generated under a molecular clock assumption, from which our sequences significantly departed. We conducted a second analysis using penalized likelihood: a semiparametric approach incorporating a roughness penalty to constrain autocorrelation in rate variation between ancestor and descendent branches, with the optimal smoothing parameter determined by cross-validation in R8S. Divergence time estimates under penalized likelihood require a fixed age for at least one node within the phylogeny. Additional outgroups for our ML and age estimation analyses included a node representing the family Gobiidae, set to 53 Myr for the penalized likelihood analyses as its oldest known fossils date to 51–56 Mya (Bajpai & Kapur, 2004). In addition, Rückert-Ülkümen (2006) described fossil otoliths of *Neogobius* as dating to the late Miocene-early Pliocene (approximately 10 Mya). As the otoliths of *Ponticola*, *Neogobius*, and *Proterorhinus* are similar, we thus used a conservative approach and set the age of their most recent common ancestor to 10 Mya.

Nested clade analysis further explored phylogeographic patterns among lineages, testing the association of haplotypes with geographic location (Templeton, 1998). A statistical parsimony network was created using TCS, version 1.21 (Clement, Posada & Crandall, 2000); haplotypes were nested *sensu* Templeton (1998), and ambiguities or reticulations in the parsimony network were resolved according to Pfenninger & Posada (2002). For networks that could not be connected with statistical parsimony, the ML tree was employed as a guide and grouped individual networks as sister clades at equal nesting levels, with outgroup rooting (using nearest sister group from phylogenetic analyses) determining the tip/interior status of a clade. GEODIS, version 2.5 (Posada, Crandall & Templeton, 2000) tested for a significant association of haplotypes and geography, and the inference key of Templeton (2004; updated by D. Posada, 2005) was used to identify the likely cause(s) of association for significant clades.

Morphological analyses

We quantified morphological variability within and among freshwater and marine tubenose gobies using meristic and mensural characters. All counts and measurements used a Leica MZ-12.5 dissecting microscope, and mensural data (to 0.01 mm) employed vernier calipers. Meristic data included numbers of first dorsal spines, second dorsal and anal fin elements, pectoral fin segmented rays, and lateral scale rows (post erodorsal tip of opercle to base of caudal fin). Measurements were standard length (tip of snout, not including lower jaw, to midpoint of caudal fin base), head width (maximum width at preopercular margin), head depth (maximum depth at posterior dorsal head margin), head length (tip of snout not including jaw to post erodorsal tip of opercle), eye diameter (horizontal diameter), snout length (tip not including jaw to anterior eye margin), interorbital width (least distance between left and right orbits), caudal peduncle length (posterior end of anal fin base to midpoint of caudal fin base), minimum caudal peduncle depth, caudal peduncle width (at minimum depth), pectoral fin length (insertion of longest fin ray to tip), maximum body depth (at anterior margin of first dorsal fin), maximum body width (behind pectoral fin base), pelvic disc length (insertion to post eriormost point), preorbital distance (distance between lip and orbit), and abdomen length (insertion of pelvic fin to vent).

We performed multivariate analyses in R (R Development Core Team, 2008) to determine whether freshwater and marine lineages of tubenose gobies are morphologically distinguishable. A principal components analysis on natural log-transformed measurements separated morphological variation into linear combinations of variables that describe overall body size and shape variation among lineages. The first principal component (PC1) primarily describes body size variation, whereas the remaining components encompass body shape variation. We utilized the components that explained 95% of the morphological variance (PC1–4) in further analyses. We tested the hypotheses that freshwater and marine tubenose gobies differ in a) body size and shape, and b) body shape alone, using multivariate analyses of variance (MANOVA), with PC1–4 as dependent variables in the former, and the latter with the shape components alone (PC2–4). Differences in lineage mean score for each principal component were assessed with analysis of covariance (ANCOVA) (allometric components) or analysis of variance (ANOVA) (non-allometric components).

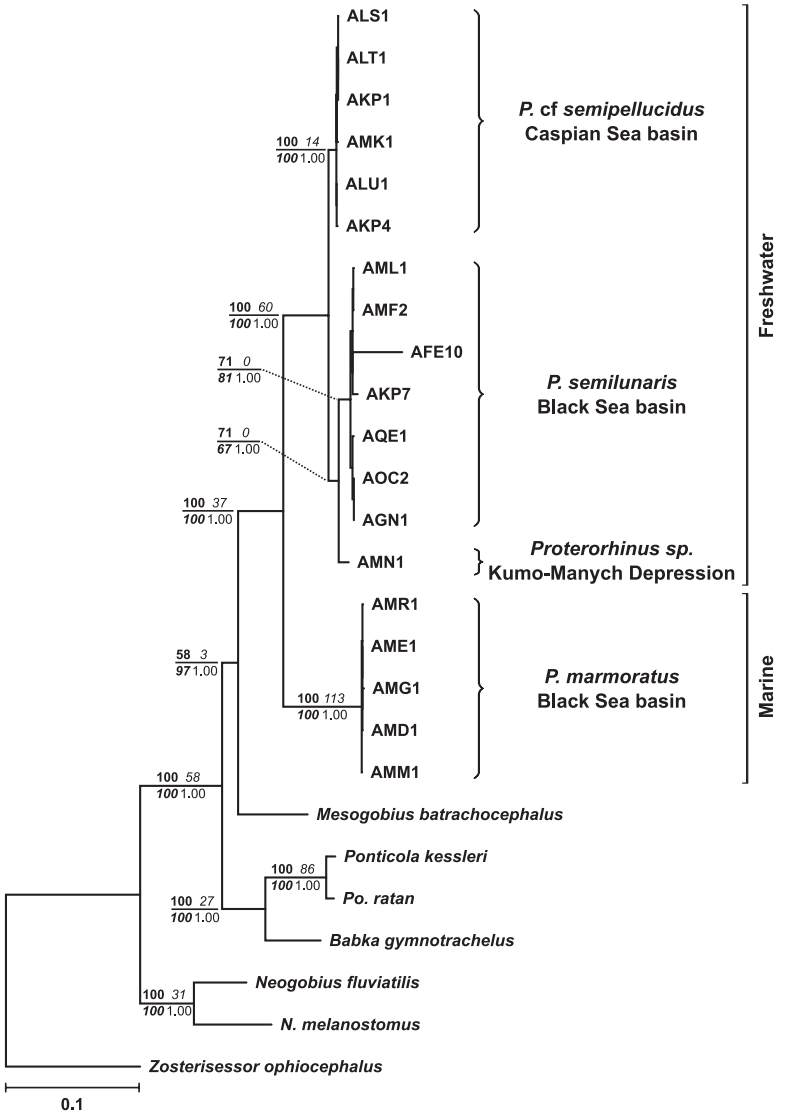

Figure 3. Maximum likelihood phylogeny of *Proterorhinus* and other 'neogobiin' outgroups based on total evidence analysis of cytochrome *b*, cytochrome oxidase *c* subunit I, and recombination activating gene 1 sequence data. Numbers above branches are bootstrap support values from 1000 pseudoreplications (bold) and decay indices (italic) for maximum parsimony, below branches are likelihood bootstrap support (bold italic) and posterior probability values (plain) for Bayesian analysis.

RESULTS

PHYLOGENETIC AND PHYLOGEOGRAPHIC PATTERNS

The aligned dataset for the combined three gene regions for 18 marine and freshwater specimens of *Proterorhinus*, spanning its range, as well as five 'neogobiin' gobies and a gobiin outgroup contained 3968 bp (cyt *b* – 1141 bp; COI – 1271 bp; RAG1 – 1556 bp). Cyt *b* sequences are available as GenBank accession nos. EU331208, EU444604, EU444607, EU444610–612, EU444618, EU444620–21, EU444624, EU444626, EU444630, EU444632, EU444636, EU444649, and EU444667–72; COI sequences as EU444673–98; RAG1 sequences as EU444699–724. Nucleotide compositions for each gene are stationary across taxa ($\chi^2 > 5.62$; d.f. = 75; $P > 0.99$).

Phylogenetic analyses of the three-gene dataset are highly congruent among tree-building methods and well-supported (Fig. 3). MP of the three-gene dataset found 48 most parsimonious trees of 2089 steps (CI = 0.648, RI = 0.790, RC = 0.512, HI = 0.352). The majority rule consensus is well-resolved, with high bootstrap support for each species (many at or near

100%) and generally high decay indices. Marine and freshwater clades of *Proterorhinus* have 100% bootstrap support and large decay indices (freshwater clade = 60; marine clade = 113). ML and Bayesian analyses of the combined dataset likewise show a similar topology to the consensus parsimony tree, in addition to high support for relationships (100% likelihood bootstrap support; 1.00 posterior probability) among all species, as well as the marine and freshwater clades of *Proterorhinus* (Fig. 3). Primary differences among the analyses occur in the terminal branching order of individuals within each of the major lineages; branching order among species as well as among major lineages of *Proterorhinus* are identical across analyses.

The freshwater *Proterorhinus* clade comprises two primary lineages: one from the Caspian Sea basin; the second from freshwater Black Sea basin locations, along with a single specimen from the Kumo-Manych Depression [AMN1 (Fig. 3); population U (Table 1)]. The freshwater Caspian Sea clade has very high support in all analyses [parsimony bootstrap (MPBS) = 100, decay index (DI) = 14, likelihood bootstrap (MLBS) = 100, posterior probability (BPP) = 1.00]. The clade from freshwater Black Sea locations + Kumo-Manych Depression also is highly supported (MPBS = 71, DI = 0, MLBS = 67, BPP = 1.00), as well as a smaller subclade limited to freshwater Black Sea locations (MPBS = 71, DI = 0, MLBS = 81, BPP = 1.00; Fig. 3). Individual analyses of the separate genes (not shown) largely are congruent with the total evidence analyses, again differing primarily in the branching order of the shallowest nodes. In each mitochondrial gene (cyt *b* and COI), marine and freshwater specimens of *Proterorhinus* separate into two distinct, well-supported clades (MPBS > 83, DI > 8, MLBS > 97, BPP = 1.00). Within the freshwater clade, distinct Black and Caspian Sea lineages are resolved with cyt *b* and are strongly supported (Black Sea lineage – MPBS > 98, DI > 7, MLBS > 98, BPP = 1.00; Caspian Sea lineage – MPBS > 98, DI > 8, MLBS > 98, BPP = 1.00), whereas only the Caspian Sea lineage is resolved with the COI gene data (MPBS > 99, DI > 11, MLBS > 96, BPP = 0.94). The relationships among *Proterorhinus* and other Ponto-Caspian 'neogobiins' thus are generally congruent with prior studies of 'neogobiin' systematics (Stepien *et al.*, 2005; Stepien & Tumeo, 2006): *Proterorhinus* is closely related to *Mesogobius*, with a *Proterorhinus* + *Mesogobius* clade comprising the sister group to *Babka* + *Ponticola* [recently redefined by Neilson & Stepien (2009)], and *Neogobius* [recently redefined, containing *N. fluviatilis* and *N. melanostomus*; Neilson & Stepien (2009)] as a sister clade to all other 'neogobiins'.

ML analysis of cyt *b* sequences from 151 individuals in 18 population sites (EU444604–EU444666; Table 3; Fig. 4) show a pattern similar to that of the larger phylogenetic analysis (Fig. 3). Tree-based lineage delimitation identifies three phylogenetically and geographically distinct haplotype clades, representing independent evolutionary lineages.

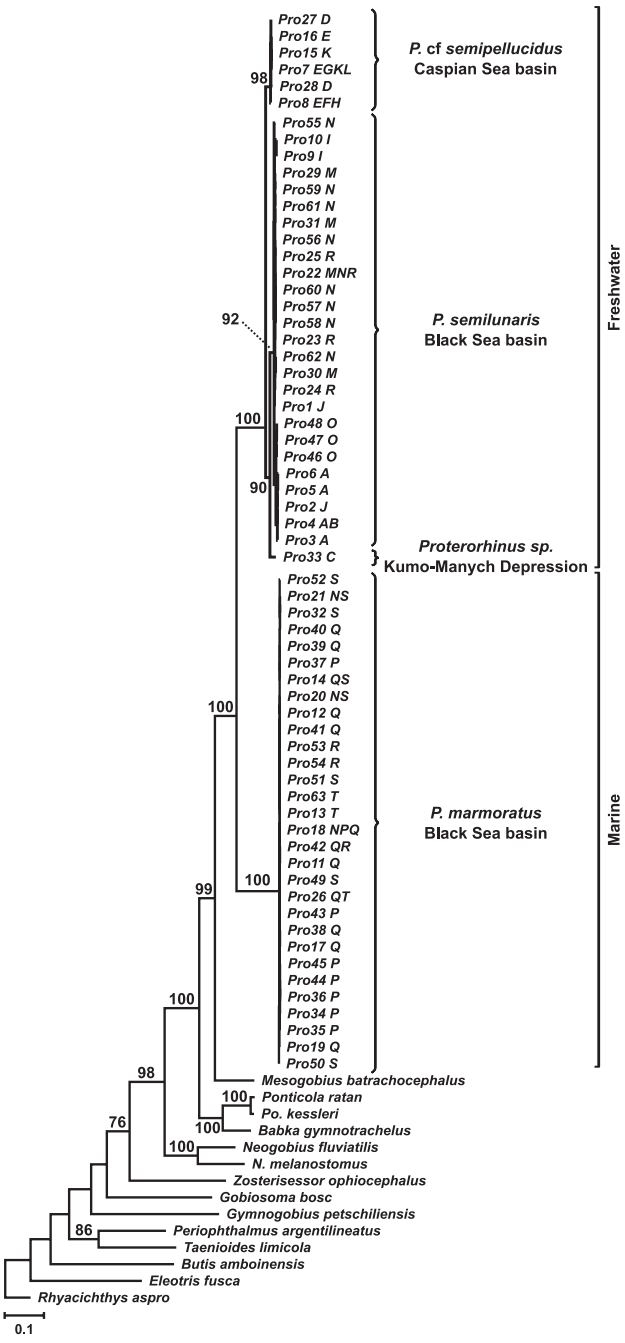

Figure 4. Maximum likelihood analysis of *Proterorhinus* cytochrome *b* haplotypes. Letters represent population codes from Table 1. Numbers around branches indicate maximum likelihood bootstrap support (1000 pseudoreplications).

Table 3. Mitochondrial cytochrome *b* haplotype distribution for populations of the tubenose goby *Proterorhinus*

Haplotype	Genbank accession	Population																			
		Proterorhinus marmoratus						*Proterorhinus semilunaris*							*Proterorhinus* cf *semipellucidus*							
		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U
1	EU444604										**1**											
2	EU444605												**3**									
3	EU444606								**1**													
4	EU444607							**9**	**7**													
5	EU444608								**1**													
6	EU444609								**1**													
7	EU444610														5		3			1		
8	EU444611															1			1	1	3	
9	EU444612									**2**												
10	EU444613									**1**												
11	EU444614		*1*																			
12	EU444615		*1*																			
13	EU444616					*1*																
14	EU444617		*1*	*1*																		
15	EU444618														1							
16	EU444619																			1		
17	EU444620		*1*																			
18	EU444621	*3*	*4*	*5*	*1*	*12*	*5*															
19	EU444622		*1*																			
20	EU444623	*1*		*1*																		
21	EU444624	*1*		*1*																		
22	EU444625	**2**			**2**							**1**										
23	EU444626				**1**																	
24	EU444627				**1**																	
25	EU444628				**1**																	
26	EU444629		*1*			*1*																
27	EU444630																	2				
28	EU444631																	1				
29	EU444632											**1**										
30	EU444633											**1**										
31	EU444634											**2**										
32	EU444635			*1*																		
33	EU444636																					***2***

Table 3. *Continued*

Haplotype	Genbank accession	Population																			
		Proterorhinus marmoratus						*Proterorhinus semilunaris*							*Proterorhinus* cf *semipellucidus*							
		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U
34	EU444637						*6*															
35	EU444638						*7*															
36	EU444639						*1*															
37	EU444640						*1*															
38	EU444641		*1*																			
39	EU444642		*1*																			
40	EU444643		*1*																			
41	EU444644		*1*																			
42	EU444645		*1*		*1*																	
43	EU444646						*1*															
44	EU444647						*1*															
45	EU444648						*1*															
46	EU444649													**6**								
47	EU444650													**1**								
48	EU444651													**1**								
49	EU444652			*1*																		
50	EU444653			*1*																		
51	EU444654			*1*																		
52	EU444655			*1*																		
53	EU444656				*1*																	
54	EU444657				*1*																	
55	EU444658	**1**																				
56	EU444659	**1**																				
57	EU444660	**1**																				
58	EU444661	**1**																				
59	EU444662	**1**																				
60	EU444663	**1**																				
61	EU444664	**1**																				
62	EU444665	**1**																				
63	EU444666					*1*																

Marine haplotypes are italic; Black Sea freshwater haplotypes are indicated in bold; Caspian Sea freshwater haplotypes in plain text; Kumo-Manych Depression haplotypes in bold italic. Population letter codes follow Table 1.

Table 4. Cytochrome *b* sequence divergence among major lineages of *Proterorhinus*, including Tamura–Nei distances within (on diagonal) and among (below diagonal) lineages, and lineage pairwise θ_{ST} values (above diagonal)

	Proterorhinus cf *semipellucidus*	*Proterorhinus semilunaris*	*Proterorhinus marmoratus*
Proterorhinus cf *semipellucidus*	**0.002**	0.873*	0.991*
Proterorhinus semilunaris	0.040	**0.006**	0.980*
Proterorhinus marmoratus	0.160	0.173	**0.001**

*Significant difference following sequential Bonferroni correction (Rice, 1989).

Freshwater and marine clades of *Proterorhinus* show marked phylogenetic divergence with 100% bootstrap support. Within the freshwater clade, among-basin differences (Black Sea, Caspian Sea, Kumo-Manych Depression) also have strong support (> 90%). Although there is strong support for the separation of the Black Sea freshwater clade and the Kumo-Manych haplotype, the paucity of samples (two individuals, one haplotype) from the latter area excludes it from further analyses. Primary lineages have large ratios of between- to within-lineage molecular divergences, indicating pronounced evolutionary separation (Table 4). Tamura–Nei distances among lineages range from 0.039–0.173, whereas those within lineages are 0.002–0.007 (Table 4). Average divergence between marine and freshwater lineages is 0.167.

Estimated divergence times within and among the marine and freshwater lineages of *Proterorhinus* (Table 5) range from 0.05 Mya (marine lineage) to 6.18 Mya (divergence of *Proterorhinus* and *Mesogobius*). Pairwise divergence estimates among lineages appear generally lower than those using penalized likelihood; however, age estimates from both methods are largely congruent (Table 5). Statistical tests of monophyly (Rosenberg, 2007) between marine and freshwater lineages, as well as between Black and Caspian basin freshwater lineages, are highly significant ($P << 0.001$ for both tests) indicating that the lineages are distinct taxa.

Nested clade analysis is consistent with tree-based lineage delimitation (Fig. 5, Table 6). *Proterorhinus* lineages are strongly associated with geographic location, reflecting allopatric fragmentation (Fig. 5, Table 6). Within *P. semilunaris* (clade 4-3; Fig. 5, Table 6), a significantly large interior-tip distance suggests either isolation by distance or long distance dispersal between the Danube and Dneiper River populations. Range expansion is inferred for *P. marmoratus* (clade 3-1; Fig. 5, Table 6), congruent with Tajima's (1989) *D* test (Table 7).

Table 5. Age estimates (million years before present) based on pairwise divergence and penalized likelihood (Sanderson, 2002) for major lineages of *Proterorhinus* (from Fig. 4)

Clade	Pairwise age	Penalized likelihood age
Proterorhinus marmoratus (Black Sea basin marine)	0.05	0.09
Proterorhinus cf *semipellucidus* (Caspian Sea basin)	0.07	0.13
Proterorhinus semilunaris (Black Sea basin freshwater)	0.17	0.26
Kumo-Manych Depression freshwater *Proterorhinus* and *P. semilunaris*	0.68	0.72
Proterorhinus semilunaris and *P.* cf *semipellucidus*	0.94	1.15
Freshwater *Proterorhinus* and *P. marmoratus*	4.22	4.40
Proterorhinus and *Mesogobius*	4.85	6.18

Morphological analyses

The three major clades of *Proterorhinus* broadly overlap in morphometric and meristic characters (Table 8). Species means differ slightly, however, for several morphometric characters, including: head length, maximum head depth, maximum body depth, and snout length (Table 8). These reveal subtle difference in body shape, with the marine lineage being more robust and deep-bodied than the more elongate freshwater lineages. PC analysis further examines difference in body shape: the first four PC explain 95.8% of the variance (PC1 – 90.1%; PC2 – 3.0%; PC3 – 1.4%; PC4 – 1.3%) and, thus, we restrict further analyses to these. PC1 primarily describes overall size variation, showing high correlation with standard length ($r = –0.971$; $P < 0.01$) and approximately equal loadings on all 17 morphometric variables except interorbital distance and preorbital width (Table 9). PC2–4 have low correlation with standard length ($|r| \leq 0.175$; $P > 0.05$ for all components), and represents size-independent shape variation. The strongest influences on PC2–4 are measures of overall head shape (interorbital distance, preorbital width, and

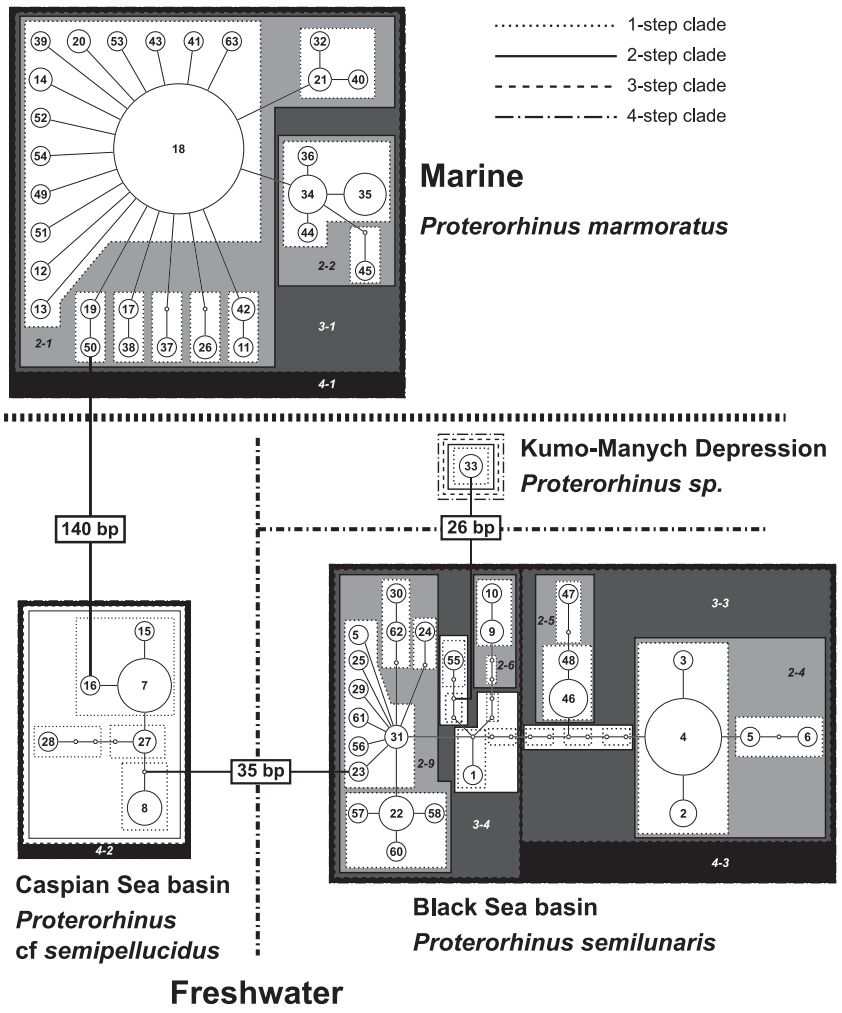

Figure 5. Statistical parsimony network among *Proterorhinus* cytochrome *b* haplotypes showing nested clades, highlighting major divisions between habitat type (marine versus freshwater; clades 4-1 versus 4-2/4-3), and among major freshwater basins (Black and Caspian Sea basins, Kumo-Manych Depression; clades 4-2, 4-3 and 4-4). Significant clades from Table 6 are shaded and labelled.

snout length) and body shape (body depth, caudal peduncle width and depth). Visual inspection of the principal components shows that, although the three clades overlap, there are differences among their mean scores (i.e. different centroids; Fig. 6). MANOVA using both body size and shape information (PC1–4) detects significant difference among the three lineages (Wilks' $\lambda = 0.416$, $F_{8,234} = 16.128$, $P << 0.001$); body shape information (PC2–4) alone recovers a slightly larger difference (Wilks' $\lambda = 0.447$, $F_{6,236} = 19.498$, $P << 0.001$). Univariate ANCOVA for PC1 with SL as the covariate indicates significant differences in lineage mean score ($P < 0.001$), and Tukey's HSD post-hoc comparisons identifies differences between *P.* cf *semipellucidus* and both *P. marmoratus* and *P. semilunaris* ($P < 0.001$ for both comparisons), with no significant difference in mean PC1 score between *P. marmoratus* and *P. semilunaris* ($P = 0.85$). Univariate ANOVAs for the remaining components discern significant difference among lineages for mean scores on PC2 ($P < 0.001$) and PC4 ($P = 0.015$), with no significant difference for PC3 ($P = 0.401$). Post-hoc comparison tests for PC2 indicate significant differences between *P. semilunaris* and both *P. marmoratus* and *P.* cf *semipellucidus* ($P < 0.001$ for both comparisons), with no difference between *P. marmoratus* and *P.* cf *semipellucidus* ($P = 0.467$). Post-hoc comparisons for PC4 depict a significant difference between *P. marmoratus* and *P. semilunaris* ($P = 0.019$) alone. In general, *P. semilunaris* has a wider interorbital diameter, wider caudal peduncle, and a longer snout than *P. marmoratus* and *P.* cf *semipellucidus*, whereas *P.* cf *semipellucidus* has a shallower body than *P. marmoratus* and *P. semilunaris*.

Table 6. Nested clade analysis of *Proterorhinus* cytochrome *b* haplotypes showing significant nesting clades and subclades, clade dispersion (D_C) and displacement (D_N) values, inference chain, and the resulting inferred pattern

Nesting clade	Significant subclade	D_C	D_N	Inference chain	Inferred pattern
Total	4-1	(S) $P < 0.0001$	(S) $P < 0.0001$	1-2-3-4-9-No	Allopatric fragmentation among marine and freshwater habitats in the Black and Caspian Sea basins
	4-2		(L) $P < 0.0001$		
	4-3	(S) $P < 0.0001$			
	I-T	(L) $P < 0.0001$	(L) $P < 0.0001$		
4-3	3-3		(L) $P = 0.0191$	1-19-20-No	Inadequate geographic sampling between Crimean Peninsula and Danube/Dniester Rivers
	3-4		(S) $P = 0.0210$		
	I-T		(S) $P = 0.0182$		
3-4	2-6		(L) $P = 0.0004$	1-2-3-5-6-7-8-No	Either isolation by distance or long distance dispersal between Danube and Dnieper River basins
	2-9	(S) $P < 0.0001$	(S) $P < 0.0001$		
	I-T		(L) $P < 0.0001$		
3-3	2-4	(S) $P = 0.0064$		1-19-20-No	Inadequate geographic sampling between Dniester River and Crimean Peninsula
	2-5	(S) $P = 0.0064$	(L) $P = 0.0064$		
3-1	2-1	(S) $P < 0.0001$	(S) $P < 0.0001$	1-2-11-Yes	Range expansion within northwestern Black Sea
	2-2	(S) $P < 0.0001$	(L) $P < 0.0001$		
	I-T	(S) $P < 0.0001$			

For clade dispersion and displacement values, (S) and (L) indicate a significantly small or large value, respectively.

Table 7. Tajima's (1989) *D* test for selective neutrality for major lineages of *Proterorhinus*

Lineage	*N*	θ_π	θ_S	Tajima's *D*	*P*
Proterorhinus cf *semipellucidus*	20	1.89	2.54	–0.87	0.202
Proterorhinus semilunaris	54	7.13	8.78	–0.63	0.280
Proterorhinus marmoratus	75	1.58	6.14	–2.34	**0.001**

Significantly negative *D* values (bold) indicate an excess of recent mutations and suggest recent population size expansion.

DISCUSSION

The conceptual foundation of what constitutes separate taxa is of fundamental importance in evolutionary studies. Numerous species concepts are described in the literature (Coyne & Orr, 2004; de Queiroz, 2005), differing primarily in the theoretical definition used to identify biological species and the operational criteria used to delimit them in nature. Whereas most researchers have an intuitive notion of their own personal species concept, few studies explicitly state the criteria or concepts used to recognize distinct taxa. Two species concepts predominate systematic studies: the evolutionary species concept (ESC: *sensu* Wiley & Mayden, 2000) and the phylogenetic species concept (PSC: *sensu* Mishler & Theriot, 2000). The ESC defines a species as a group of organisms with its own independent evolutionary trajectory separate from others in space and time. The PSC defines a species as the least inclusive unit within a phylogenetic classification as evidenced by monophyly. Whereas the ESC provides a sound theoretical definition for what constitutes a species (independent evolutionary lineages), the PSC provides robust operational criteria for delimiting among them (monophyly following Mishler & Theriot, 2000; i.e. diagnosable combinations of characters/synapomorphies, or recent genetic coalescence in other versions of the PSC). We thus use this approach to delimit species of *Proterorhinus*, coupled with the additional operational threshold to identify species of interspecific variation of ten or more times the mean intraspecific variation (Hebert *et al.*, 2004).

The results obtained in the present study describe three distinct evolutionary lineages of tubenose gobies: a marine lineage comprising *P. marmoratus* from the Black Sea proper, and two freshwater lineages in the Black (*P. semilunaris*) and Caspian (*P.* cf *semipellucidus*) Sea basins. A potential fourth lineage within the Kumo-Manych Depression also is suggested, which merits further sampling and investigation. Appreciable cyt *b* genetic divergence occurs among the three lineages (Tamura–Nei

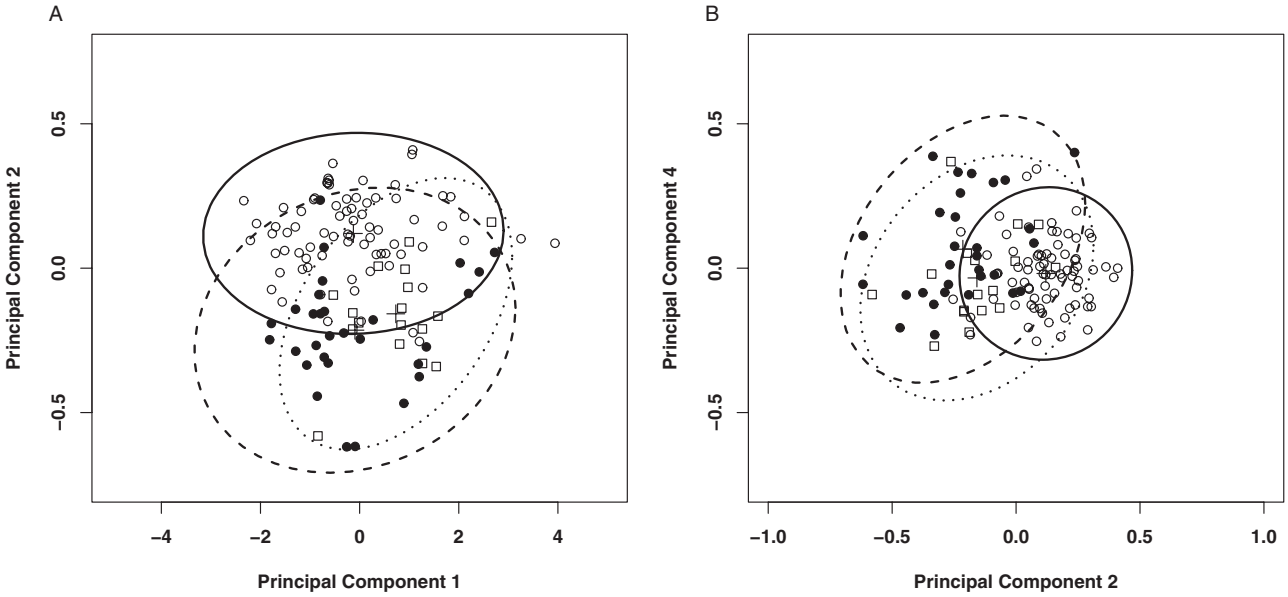

Figure 6. Plots of the (A) second versus first and (B) fourth versus second principal components (PC) from a principal components analysis of 123 tubenose gobies. ● = *Proterorhinus marmoratus*; ○ = *P. semilunaris*, □ = *P.* cf *semipellucidus*. 95% confidence ellipses and centroids (+) are drawn for each species. A multivariate analysis of variance (MANOVA) using both body size and shape information (PC1–4), as well as body shape alone (PC2-4) detected highly significant differences among taxa (PC1–4, Wilks' $\lambda = 0.416$, $F_{8,234} = 16.128$, $P << 0.001$; PC2–4, Wilks' $\lambda = 0.447$, $F_{6,236} = 19.498$, $P << 0.001$).

distance = 0.04–0.17), within the range seen for many intrageneric comparisons of fishes (0.01–0.40; Johns & Avise, 1998) as well as in the 'neogobiin' genus *Neogobius* (Tamura–Nei distance between *N. fluviatilis* and *N. melanostomus* = 0.174; Brown & Stepien, 2008; M. Neilson & C. A. Stepien, unpubl. data), with coincident low divergence within lineages (0.001–0.006) similar to intraspecific variation in other gobiids (Harada *et al.*, 2002; Mukai, Suzuki & Nishida, 2004; Brown & Stepien, 2008). Monophyly of these lineages is highly significant ($P << 0.0001$), confirming that they are distinct taxa.

Divergence times among the major lineages of *Proterorhinus* estimated both with (pairwise divergence) and without (penalized likelihood) a molecular clock are largely congruent. As the ages specified for the oldest fossils of the Gobiidae and *Neogobius* likely underestimate their true ages, our approximations for *Proterorhinus* are minimum estimates. Utilizing estimated divergence times, we reconstruct the evolutionary history of *Proterorhinus* within the Ponto-Caspian basin in relation to the region's geologic history. Names for the historic stages of the Black and Caspian Sea basins are used *sensu* Reid & Orlova (2002). A marine ancestor of *Proterorhinus* and *Mesogobius* likely inhabited the Sarmatian Sea, which encompassed both the Black and Caspian Sea basins approximately 8–15 Mya during the mid- to late Miocene epoch. At this time, the Pontian Lake-Sea in the Black Sea basin was connected to a large water body located in the Pannonian Depression (geologic depression west of the Ponto-Caspian basin and Carpathian Mountains in present day Serbia and Romania), merging their two faunas (Reid & Orlova, 2002). *Proterorhinus* and *Mesogobius* then diverged approximately 4.8–6.2 Mya in the Pontian Lake-Sea in the Black Sea basin, perhaps due to specialization and competition with Pannonian taxa.

The first major division within *Proterorhinus* occurred approximately 4.2–4.4 Mya, separating the marine and freshwater taxa. Their early Pliocene divergence likely resulted from decrease in salinity of the Kimmerian Lake-Sea (proto-Black Sea basin), leading to an overall shift from marine to brackish water fauna (Zaitsev & Mamaev, 1997; Reid & Orlova, 2002). The separation between the Black and Caspian Sea freshwater lineages occurred during the mid-early Pleistocene epoch (approximately 0.94–1.15 Mya) when freshwater *Proterorhinus* moved along with other Black Sea basin fauna from the Gurian Lake-Sea in the Black Sea basin into the Apsheron Lake-Sea in the Caspian Sea basin. This migration occurred across the Kumo-Manych Depression during the Aspheronian transgression: the second major Pleistocene connection between the Black and Caspian Sea basins (Reid & Orlova, 2002; Cristescu, Hebert & Onciu, 2003). This transbasin connection persisted intermittently for the last approximately 0.9 Myr due to fluctuating water levels associated with major glacial and interglacial periods (Reid & Orlova, 2002),

Table 8. Morphometrics and meristics of *Proterorhinus* taxa

	Proterorhinus marmoratus ($N = 30$)		*Proterorhinus semilunaris* ($N = 76$)		*Proterorhinus* cf *semipellucidus* ($N = 17$)	
Measurement	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
Standard length	47.0 ± 11.7	24.2–66.4	43.7 ± 10.4	17.5–68.0	38.8 ± 7.8	23.0–55.0
% Standard length						
Caudal peduncle length	14.6 ± 1.7	12.2–19.5	15.6 ± 1.3	11.6–18.5	15.5 ± 2.0	11.8–20.6
Caudal peduncle depth	11.2 ± 1.0	9.1–13.8	10.7 ± 0.9	8.0–12.8	11.3 ± 1.6	7.3–14.2
Caudal peduncle width	4.2 ± 0.8	2.5–5.9	4.5 ± 0.6	3.1–7.1	4.2 ± 0.7	3.0–5.4
Pelvic disc length	21.3 ± 1.8	17.7–24.8	22.5 ± 1.5	18.4–26.5	22.3 ± 1.8	17.4–25.1
Abdomen length	23.7 ± 2.9	18.5–29.8	23.6 ± 2.0	19.0–31.7	23.4 ± 1.8	18.4–25.8
Pectoral fin length	23.6 ± 2.7	18.4–28.0	25.5 ± 2.3	20.3–32.3	23.5 ± 2.0	19.4–26.5
Body depth	21.3 ± 2.3	17.3–25.5	21.1 ± 2.0	15.5–24.7	18.3 ± 2.4	14.5–22.5
Body width	13.9 ± 1.7	9.9–17.1	14.2 ± 1.8	10.3–19.6	12.9 ± 1.8	9.6–15.8
Head length	26.7 ± 1.9	21.0–28.8	29.2 ± 1.2	27.0–31.9	28.5 ± 2.1	23.7–32.6
% Head length						
Head width	68.5 ± 7.1	58.8–92.1	64.5 ± 6.4	52.1–80.1	58.8 ± 4.6	49.3–68.0
Head depth	72.7 ± 7.0	61.3–97.6	65.1 ± 4.9	54.8–76.3	62.0 ± 5.0	52.0–71.2
Eye diameter	22.2 ± 3.1	17.7–29.2	21.7 ± 2.7	17.6–31.4	23.0 ± 2.5	18.8–26.7
Interorbital distance	10.0 ± 2.7	5.9–14.9	12.6 ± 3.0	5.9–21.1	8.1 ± 1.7	5.3–10.9
Snout length	27.5 ± 3.8	21.7–34.3	31.2 ± 4.1	20.6–38.8	27.0 ± 1.7	24.2–30.4
Preorbital width	16.8 ± 2.0	13.1–19.8	16.6 ± 2.4	9.8–24.6	13.9 ± 2.5	9.3–18.1
Meristic	Mode	Range	Mode	Range	Mode	Range
Scales in lateral series	44	39–49	42	39–53	43	39–47
First dorsal fin spines	6	5–7	6	5–6	6	5–6
Second dorsal fin elements	16	15–18	16	14–17	17	15–18
Anal fin elements	15	12–16	13	12–15	15	13–16
Pectoral-fin segmented rays	15	15–17	15	14–16	15	14–16

Table 9. Summary of principal components analysis (eigenvalues and loadings) calculated from 17 linear measurements from three *Proterorhinus* lineages

	PC1	PC2	PC3	PC4
Eigenvalue	1.206	0.219	0.152	0.145
% Eigenvalue	90.1	3.0	1.4	1.3
Standard length	–0.212	–0.184	–0.020	0.084
Caudal peduncle length	–0.197	–0.104	0.198	0.097
Caudal peduncle depth	–0.240	–0.234	**0.309**	0.086
Caudal peduncle width	–0.190	–0.014	**0.690**	**–0.610**
Pelvic disc length	–0.202	–0.105	–0.192	–0.110
Abdomen length	–0.215	–0.255	–0.199	–0.134
Pectoral fin length	–0.239	–0.063	–0.261	–0.069
Body depth	–0.275	–0.133	0.031	**0.338**
Body width	–0.271	–0.208	0.138	0.178
Head length	–0.212	–0.031	–0.135	–0.039
Head width	–0.261	–0.081	0.034	0.188
Head depth	–0.254	–0.178	–0.008	0.176
Eye diameter	–0.139	–0.062	–0.080	–0.035
Interorbital distance	**–0.399**	**0.819**	0.141	0.264
Snout length	–0.283	0.214	**–0.375**	**–0.495**
Preorbital width	**–0.305**	–0.008	–0.196	–0.202

Loadings with absolute magnitude greater than 0.3 shown in bold.

with the most recent connection closing approximately 9000 years ago. Separation of the waterbodies remaining within the depression after these repeated openings and closures likely led to the divergence of the fourth putative freshwater lineage of *Proterorhinus* from *P. semilunaris* approximately 0.68–0.72 Mya. Ages estimated for the individual marine, Black Sea freshwater, and Caspian Sea freshwater clades (Tables 4, 5) likely reflect population size changes associated with late Pleistocene glaciations and fluctuations in water levels, indicated by the significant Tajima's (1989) *D* value for *P. marmoratus* (Table 7).

The separation among Black and Caspian Sea lineages of *Proterorhinus* echoes the pattern seen in numerous Ponto-Caspian organisms. Brown & Stepien (2008) describe an approximately 1 Myr separation between Black and Caspian Sea lineages of *Neogobius melanostomus*, whereas an older separation (approximately 4 Mya) characterizes its sister species *N. fluviatilis* between those basins (M. Neilson & C. A. Stepien, unpubl. data). Durand, Persat & Bouvet (1999) estimated a Pliocene divergence between Danube River and Caspian Sea lineages of the chub *Leuciscus cephalus*. Cristescu *et al.* (2003, 2004) found a similar pattern of genetic structure in several Ponto-Caspian crustaceans, with differentiation times between Black and Caspian Sea lineages ranging from approximately 1 Myr for benthic species and 6–8 Myr for planktonic species. Stepien, Taylor & Dabrowska (2002) and Gelembiuk, May & Lee (2006) estimated more recent separation time (approximately 532 000 and 166 000 years, respectively) between Black and Caspian Sea populations of *Dreissena polymorpha*. These repeated patterns of genetic divergence between Black and Caspian Sea basin lineages highlight the role of intermittent basin connectivity/separation in shaping the evolutionary history of Ponto-Caspian taxa.

Morphological separation of the three major clades of *Proterorhinus* is not as marked as their genetic divergence: all overlap for individual morphometric variables, yet differ slightly in mean values for several characters (Table 8). Some of these characters likely contribute to the significant overall differences in body shape observable among the taxa (Fig. 6, Table 9). Although overlapping principal component scores indicate that morphometrics alone cannot classify specimens to individual taxa, a significant MANOVA (and subsequent univariate ANOVAs) for PC1–4 shows that the three major lineages represent three distinct statistical populations, occupying different areas in morphospace. Habitat specific (marine versus freshwater) morphological differences have been observed in other fishes, including sticklebacks (*Gasterosteus aculeatus* complex; Walker & Bell, 2000) and sea bass (*Dicentrarchus labrax*; Corti, Loy & Cataudella, 1996). In addition, significant genetic divergence among morphologically cryptic species is widespread in fishes. For example, Egge & Simons (2006) described the morphologically cryptic North American madtom catfish *Noturus maydeni* using a

combination of karyology, allozymes, and fixed DNA sequence differences. Bonefishes, a circumtropical species complex originally described as a single species (*Albula vulpes*), contain eight morphologically cryptic species with high degree of genetic divergence (genetic distance = 5.56–30.6%; Colborn *et al.*, 2001); Quattro *et al.* (2006) described large phylogenetic separation, as well as differences in vertebral counts, between the widespread scalloped hammerhead (*Sphyrna lewini*) and a cryptic species in the western North Atlantic Ocean. Despite large differences in ecology and taxonomy, these three examples all exhibit a common pattern: high degree of genetic divergence in the absence of significant morphological divergence among cryptic taxa.

The combined morphological and molecular separation of geographic lineages of *Proterorhinus* indicates that there are at least three separate species of *Proterorhinus*: (1) *P. marmoratus* from marine habitats within the Black Sea; (2) *P. semilunaris* from freshwater drainages in the Black Sea basin and introduced populations in the North American Great Lakes; and (3) *P.* cf *semipellucidus* from the upper Don River and Volga River basin. Freyhof & Naseka (2007), in a description of a new freshwater species of *Proterorhinus* (*tataricus*) from the Crimean Peninsula, additionally found that Caspian Sea basin tubenose gobies appear distinct from all other species (*P. marmoratus*, *P. semilunaris*, and *P. tataricus*) and ascribed those populations to *P. nasalis*. However, the specimens examined by Freyhof & Naseka (2007) were derived primarily from marine regions of the Caspian Sea proper and included only two specimens from the Volga River at Zam'yany (which also was sampled in the present study). Moreover, Freyhof & Naseka (2007) did not incorporate genetic data or employ modern statistical analysis of their morphological data.

Given the morphological and genetic differences observed in our study between *P. marmoratus* and *P. semilunaris*, as well as strong salinity differences between the northern and southern Caspian Sea basins (0–2‰ in the northern reaches near the Volga River delta versus 12–14‰ in the south Sea), it is likely that the Caspian Sea basins house separate freshwater and 'marine' *Proterorhinus* taxa. Thus, Caspian Sea watersheds presumably contain a freshwater taxon in the Volga River and other northern drainage basins and a second 'marine' taxon in the more saline southern basin (south of the Baku Peninsula, Azerbaijan). The earliest assigned name for the tubenose goby in the Caspian Sea basin was *Gobius nasalis*, described by Filippi (1863) from specimens from the Caspian Sea near Baku, Azerbaijan. The only previously described freshwater species of tubenose goby in the Caspian Sea basin was *G. semipellucidus*, described by Kessler (1877) from a single specimen from the lower Karasu River at Astrabad (Gorgon) Bay, Iran. Although we do not dispute the resurrection of *P. nasalis* by Freyhof & Naseka (2007) for truly 'marine' specimens of Caspian Sea basin tubenose goby, it is likely that the freshwater specimens included in the present study constitute yet another separate taxon. This problem thus merits further sampling and investigation using both morphological and molecular techniques, as well as state-of-the-art evolutionary analyses. We thus tentatively identify the freshwater Caspian Sea basin lineage as *P.* cf *semipellucidus*.

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SUPPORTING INFORMATION

Some taxonomic nomenclatural changes are being made, in consultation with the American Fisheries Society Fish Names Committee and the International Commission for Zoological Nomenclature. Notably, *Apollonia* is now being synonymized with the genus *Neogobius*; *Neogobius ratan* and *N. kessleri* are being removed from the genus *Neogobius* and placed in the new genus *Ponticola*; and *N. gymnotrachelus* is being placed in the new genus *Babka*. This is due to paraphyly. These changes are detailed in a new paper now accepted for publication by Neilson and Stepien in the journal Molecular Phylogenetics and Evolution, Escape from the Ponto-Caspian: Evolution and biogeography of an endemic goby species flock (Benthophilinae: Gobiidae: Teleostei), 2009.

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Material examined. Institutional abbreviations follow Leviton *et al.* (1985) with the following addition: GLGL, Great Lakes Genetics Laboratory.

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The systematics, taxonomy, and nomenclature of the Ponto-Caspian 'neogobiin' gobies (including *Apollonia, Neogobius, Mesogobius*, and *Proterorhinus*) are more fully resolved and clarified in a work by Neilson & Stepien published in *Molecular Phylogenetics and Evolution*. Please refer to this publication for current information about the names of taxa examined in the present work.