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Rapid Communication

Molecular analysis reveals the invasion of eastern tubenose goby *Proterorhinus nasalis* De Filippi, 1863 (Perciformes: Gobiidae) into the Baltic Sea

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Abstract

We report the first confirmed record of the invasive alien species, eastern tubenose goby *Proterorhinus nasalis* (De Filippi, 1863) in the Baltic Sea as a first verified record of this species outside Azov and Caspian Sea basins and the Volga River system. Several tubenose gobies with inconclusive morphometrical characteristics were caught from the Narva Bay, eastern part of Gulf of Finland in September 2020. Phylogenetic analysis of caught individuals confirmed their taxonomic classification as *Proterorhinus nasalis*. Relatively high abundance of tubenose gobies hints that *P. nasalis* may have established a naturalized population in the Narva Bay.

Key words: invasive species, Gulf of Finland, Ponto-Caspian gobies, *Proterorhinus* cf. *semipellucidus, Proterorhinus marmoratus,* European Northern Invasion Corridor

Introduction

Biological invasions can potentially have strong ecological or (and) economic impacts (e.g., Gollasch and Leppäkoski 1999; Bij de Vaate et al. 2002; Pauli and Briski 2018). It has been pointed out, that the most important factors controlling marine invasions are temperature and salinity (Gollasch and Leppäkoski 1999). A large fraction of invasive species in the Baltic Sea originates from freshwater or brackish water environments of Ponto-Caspian region (e.g., Gollasch and Leppäkoski 1999; Bij de Vaate et al. 2002; Pauli and Briski 2018). Previous studies have suggested that Ponto-Caspian freshwater-brackish taxa can adapt rapidly to highly variable salinity conditions in the Baltic Sea, and therefore may be more successful colonizers than species from other regions (reviewed in Pauli and Briski 2018).

The tubenose gobies of genus *Proterorhinus* Smitt, 1900 are native to fresh, brackish, and saline waters of the Azov–Black Sea Basin, the Caspian Sea, and the northeastern part of the Aegean Sea basin (reviewed in Manilo 2020). *Proterorhinus* species are difficult to distinguish by morphological data (Neilson and Stepien 2009a; Sorokin et al. 2011; Uspenskiy 2020) and

the phylogenetic relationships between different species within the genus *Proterorhinus* remain widely discussed and need further investigation (e.g., Neilson and Stepien 2009a, b; Sorokin et al. 2011; Manilo 2020). In this article, we follow the international database Eschmeyer's Catalog of Fishes (Fricke et al. 2021) in which only tubenose goby *Proterorhinus marmoratus* (Pallas, 1814), eastern tubenose goby *Proterorhinus nasalis* (De Filippi, 1863) and western tubenose goby *Proterorhinus semilunaris* (Heckel, 1837) are considered as valid species (*P. semipellucidus* and *P.* cf. *semipellucidus* are treated as synonymes to *P. nasalis*).

Several tubenose goby invasions have been recorded during the last decades. *Proterorhinus semilunaris* has been introduced to North America (e.g., Stepien and Tumeo 2006; Neilson and Stepien 2009a, b) and into several areas of central and western Europe (e.g., Adámek et al. 2010; Mombaerts et al. 2014; Grabowska et al. 2019). In the Baltic Sea basin, *P. semilunaris* is abundant in the Vistula River system (Grabowska et al. 2008, 2019).

Proterorhinus marmoratus (Antsulevich et al. 2007; Kottelat and Freyhof 2007, reviewed in Zhokhov et al. 2017; Uspenskiy 2020), *P. nasalis* (Parin et al. 2014; reviewed in Zhokhov et al. 2017) but also *P. semilunaris* (reviewed in Zhokhov et al. 2017) have all been reported invasive in the Volga River basin and in the European Northern Invasion Corridor (*sensu* Bij de Vaate et al. 2002). According to Antsulevich et al. (2007), tubenose gobies reached the Neva Estuary in 2006 and the Baltic Sea in 2007. By now they have established a numerous population in the eastern end of Gulf of Finland (Uspenskiy 2020; Demchuk et al. 2021). Currently, those tubenose gobies have been determined as *P. marmoratus sensu lato* (see e.g. Neilson and Stepien 2009a) but have not been determined to the species level (*sensu* Fricke et al. 2021) or have been described as *P. semilunaris* (Demchuk et al. 2021). Thus, the taxonomic status of genus *Proterorhinus* in the European Northern Invasion Corridor and in the Baltic Sea is still unclear and needs genetic verification.

In this study, we present a phylogenetic analysis of tubenose gobies from Narva Bay, eastern part of Gulf of Finland and exhibit the first confirmed record of *P. nasalis* in the Baltic Sea basin.

Materials and methods

The first two specimens of tubenose gobies from the Estonian waters of the Baltic Sea were caught near Sillamäe harbour (59.4164N; 27.7066E), Narva Bay, Gulf of Finland (Figure 1) on 30 September 2020. Those fish were caught with standard Nordic coastal multi-mesh gillnets (HELCOM 2015), that were set at the sunset of 29 September 2020 to the depth of 3.5 m, parallel to the shoreline on a gravel bottom area. The first individual, identified as a tubenose goby (Figure 2) was photographed and preserved in ethanol (96.5% vol).

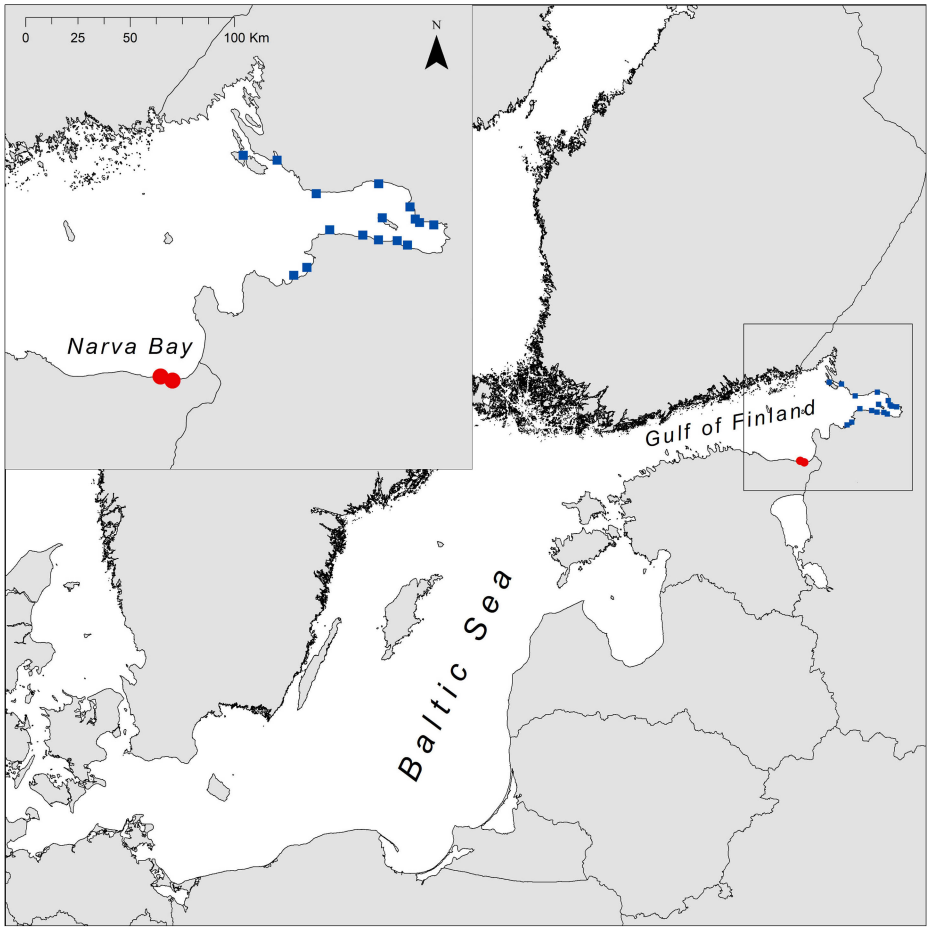

Figure 1. Map describing capture cites of tubenose gobies from the Baltic Sea. Red circles denote *Proterorhinus nasalis* (this study). Blue squares denote *P. marmoratus sensu lato* (Uspenskiy 2020).

Figure 2. First specimen of *Proterorhinus nasalis* from the Baltic Sea, caught near Sillamäe harbour (59.4164N; 27.7066E), Narva Bay, Gulf of Finland on 30 September 2020. Photo by Lauri Saks.

Additional 21 individuals were caught during 5 October 2020 near Sillamäe harbour, Narva Bay using beach seine (code-end mesh size 5 mm). The tubenose gobies were placed into a 40 litre plastic container which was filled with local brackish sea water, aerated with a battery operated aquarium aerator and transported to the laboratory. Until DNA sampling, the fish were housed in a 40 litre aquarium equipped with a filter and aerator

Table 1. Morphometrical (TL = Total length, SL = Standard length, HL = Head length, ED = Eye diameter,) and meristic (A = Number of anal fin rays) characteristics of *Proterorhinus nasalis* individuals in the dataset of phylogenetic analysis.

Fish nr	TL	SL	HL	ED	A	HL/SL	ED/HL
1	80	67	15.7	3.7	13	23.4	23.6
2	94	79	22.1	4.4	14	28.0	19.9
3	90	74	22.9	4.9	14	30.9	21.4
4	65	54	14.7	3.8	13	27.2	25.9
5	80	67	20.2	4.0	14	30.1	19.8
6	60	50	14.2	3.5	13	28.4	24.6

and filled with the water collected at the capture site. Captured individuals were provided with commercially available fish diet for demersal and benthic fish. By the time of DNA sampling most of the smaller individuals were, however, absent from the aquarium, most likely due to predation by larger individuals.

As the morphometrical and meristic characteristics of the individuals (Table 1) were not suitable for exact determination of the species, DNA samples for molecular species determination were collected on 22 December 2020. Each individual was removed from the aquarium with a small net, measurements were taken with a ruler (total length – TL, and standard length – SL) and a calliper (head length – HL, eye diameter – ED) and anal fin rays were counted. A small piece (up to 5 mm long) was cut with scissors from the anterior dorsal edge of the posterior dorsal fin from all the fish (and also from the previously fixed individual, caught on 29 September 2020). All the fish were released back to the aquarium immediately after the sampling where they fully recovered. No negative effects of fin clipping were recorded and complete regeneration of the affected fins was observed in case of all the individuals within two weeks after the procedure. Fin clip samples were fixed in a 1.5 ml of ethanol (96.5% vol) and stored in individually marked screw cap plastic tubes. As an internal blind control a fin clip of one round goby (*Neogobius melanostomus*) individual was also collected and a fin clip sample of one tubenose goby individual (fish nr. 1 in the Table 1) was stored separately in two tubes. Altogether the analyzed dataset consisted of six fin clipped *Proterorhinus* individuals (Table 1), one previously fixed *Proterorhinus* individual and one round goby individual (DNA ID goby1–goby6 and goby8–goby9 in Table 2).

Total genomic DNA was extracted from tissue samples using Qiagen DNeasy Blood & Tissue Kit (Hilden, Germany) following the manufacturer's protocols. Mitochondrial cytochrome oxidase subunit I (COI) gene was amplified via the polymerase chain reaction (PCR) using goby-specific primers as listed in Thacker (2003). 5' end of the COI gene was amplified using primers L6468 and H7127, and 3'end was amplified using primers L7059 and H7696.

PCR was performed in a total volume of 20 µl, with the reaction mixture containing 10X Advantage® 2 PCR Buffer, 1X Advantage® 2 Polymerase Mix

Table 2. Species, voucher data and GenBank accession numbers used in this study. *Proterorhinus* cf. *semipellucidus* is considered as junior synonym to *Proterorhinus nasalis* (Fricke et al. 2021).

Species	Specimen ID	DNA ID	GenBank Accession Number
Proterorhinus nasalis	TUZ700166	Goby1	MW984354
Proterorhinus nasalis	TUZ700167	Goby2	MW984353
Proterorhinus nasalis	TUZ700168	Goby3	MW984352
Proterorhinus nasalis	TUZ700169	Goby4	MW984351
Proterorhinus nasalis	TUZ700170	Goby5	MW984350
Proterorhinus nasalis	TUZ700171	Goby6	MW984349
Neogobius melanostomus	TUZ700173	Goby8	MW984348
Proterorhinus nasalis	TUZ700174	Goby9	MW984347
Proterorhinus cf. *semipellucidus*	PseAKP1	PseAKP1	EU444675
Proterorhinus cf. *semipellucidus*	PseAKP4	PseAKP4	EU444676
Proterorhinus cf. *semipellucidus*	PseALS1	PseALS1	EU444678
Proterorhinus cf. *semipellucidus*	PseALT1	PseALT1	EU444679
Proterorhinus cf. *semipellucidus*	PseALU1	PseALU1	EU444680
Proterorhinus cf. *semipellucidus*	PseAMK1	PseAMK1	EU444685
Proterorhinus semilunaris	PseAGN1	PseAGN1	EU444674
Proterorhinus semilunaris	PseAKP7	PseAKP7	EU444677
Proterorhinus semilunaris	PseAMF2	PseAMF2	EU444683
Proterorhinus semilunaris	PseAML1	PseAML1	EU444686
Proterorhinus semilunaris	PseAOC2	PseAOC2	EU444690
Proterorhinus semilunaris	PseAQE1	PseAQE1	EU444691
Proterorhinus marmoratus	PmaAMD1	PmaAMD1	EU444681
Proterorhinus marmoratus	PmaAME1	PmaAME1	EU444682
Proterorhinus marmoratus	PmaAMG1	PmaAMG1	EU444684
Proterorhinus marmoratus	PmaAMM1	PmaAMM1	EU444687
Proterorhinus marmoratus	PmaAMR1	PmaAMR1	EU444689

(TAKARA BIO INC, Kusatsu, Shiga, Japan), 0.2 mM dNTP (Thermo Fisher Scientific, Waltham, MA, USA), 5 pmol of primers and 20–80 ng of purified genomic DNA. PCR was performed on a Biometra T1 Thermocycler (Analytik Jena, Jena, Germany). The cycling profile 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 (Neilson and Stepien 2009a). PCR products were visualized on a 1.6% agarose gel with ethidium bromide and 10 µl of the PCR solution was treated with FastAP thermosensitive alkaline phosphatase and exonuclease I (Thermo Fisher Scientific). One unit of both enzymes was added to PCR solution, which was incubated for 16 min at 37 °C, followed by 15 min inactivation at 80 °C. Annealing temperature used for cycle sequencing was 50 °C. All four DNA strands were sequenced with 3 pmol of primers and sequences were resolved by a 3730xl DNA Analyzer automated sequencer (Applied Biosystems, Foster City, CA, USA) in the Estonian Biocentre (Tartu, Estonia). The studied material with the extracted genomic DNA is housed in the Museum of Natural History of the University of Tartu (Table 2). Consensus sequences were created in GENEIOUS R7.1.7 (Kearse et al. 2012) using sequence data from four DNA strands. Sequences were double-checked by eye, and edited and aligned in BIOEDIT 7.2.5 (Hall 1999). All specimens were cross-checked with their DNA barcodes in the Barcode of Life Data Systems (Ratnasingham and Hebert 2007).

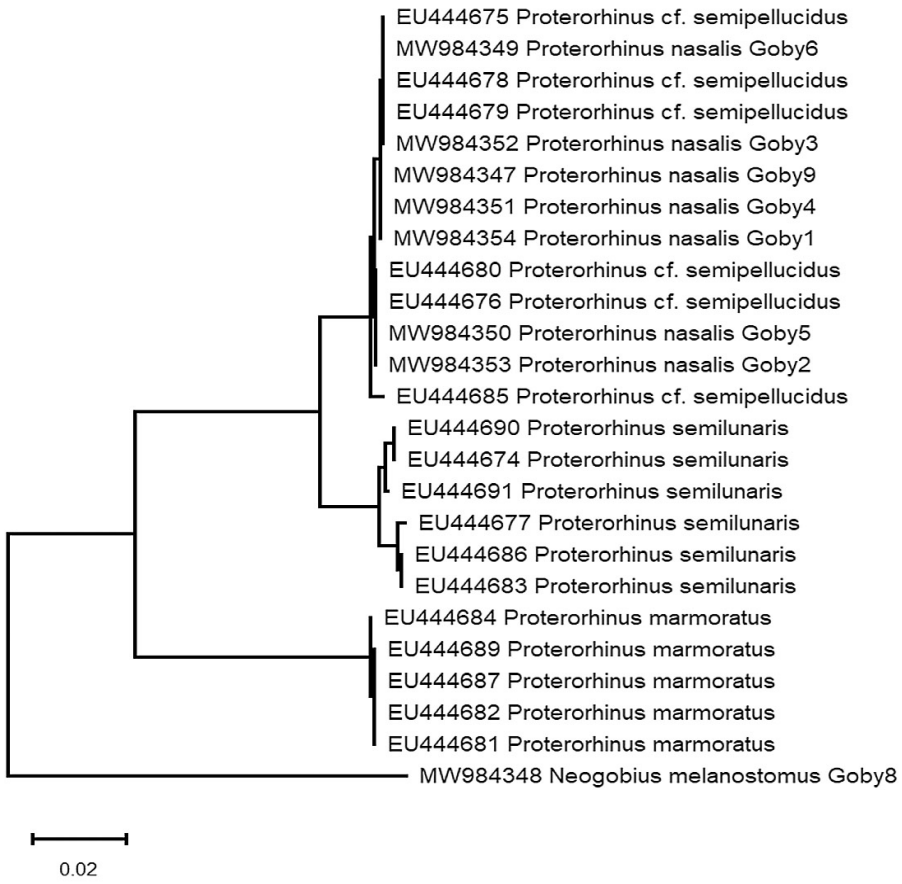

Figure 3. Molecular phylogenetic analysis by Neighbor Joining method. *Proterorhinus* cf. *semipellucidus* is considered as junior synonym to *Proterorhinus nasalis* (Fricke et al. 2021).

Full (~ 1300 bp) COI sequence data for *P. semilunaris*, *P. marmoratus* and *P.* cf. *semipellucidus* provided by Neilson and Stepien (2009a) were downloaded from GenBank (Sayers et al. 2021), and used in the phylogenetic analyses together with the original sequences. *Neogobius melanostomus* (Pallas, 1814) was used as an outgroup. All original sequences were deposited in GenBank, their accession numbers are listed in Table 2. The evolutionary history of analysed *Proterorhinus* sp. was inferred (no bootstraping was applied) by using the Neighbor-Joining (NJ) method (Saitou and Nei 1987) and evolutionary distances were computed using the p-distance method (Nei and Kumar 2000). Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018) and the optimal tree is shown on Figure 3.

Results

The full COI gene was successfully sequenced for all specimens in this study, except for *Neogobius melanostomus* for which only the 5′ half was obtained. The aligned dataset of the COI gene region for selected *Proterorhinus* species contained 1271 bp and for an outgroup (*Neogobius*) 708 bp. Consensus sequences obtained from eight *Proterorhinus* sp. and

one *Neogobius melanostomus* samples were cross-checked in the BOLD Identification Engine (Ratnasingham and Hebert 2007) to search for the nearest matches at species level in public libraries of sequences. The search for *Proterorhinus* sp. barcodes resulted in positive match for *Proterorhinus* cf. *semipellucidus*, considered as junior synonym to *Proterorhinus nasalis* (Fricke et al. 2021). For further insight, available data for additional *Proterorhinus* species was searched and downloaded from GenBank and added to the original dataset for phylogenetic analyses. As a result of phylogenetic analysis, a phylogenetic tree was constructed (Figure 3). Topology of the NJ tree corresponds with findings of Neilson and Stepien (2009a), as *P. marmoratus*, *P. semilunaris* and *P. nasalis* grouped into three monophyletic clades.

Discussion

The tubenose goby specimens caught from the Narva Bay, Gulf of Finland, were clearly demonstrated to be *P. nasalis* (Figure 3) and represent the first record of this species from the Baltic Sea. To our knowledge, this is the first verified record of *P. nasalis* outside Azov and Caspian Sea basins and the Volga River basin.

Relatively high abundance of tubenose gobies (21 individuals), caught on October 5th, indicates that *P. nasalis* may have established a naturalized population in the Narva Bay. Possible effects of the tubenose gobies interacting with native fish assemblages include predation and competition for shelter and food (e.g. French and Jude 2001; Van Kessel et al. 2011; Uspenskiy 2020). There are several small sized native benthic species in the littoral zone of the brackish Baltic Sea (e.g., Taal et al. 2017) which may suffer deleterious consequences from this invasion. At the same time, it is likely that *P. nasalis* would deliver a novel link in the energy pathway from benthic invertebrates (Froese and Pauly 2021) to piscivorous fish (e.g., European perch *Perca fluviatilis* (Linnaeus, 1758) and European smelt *Osmerus eperlanus* (Linnaeus, 1758)) at higher trophic levels. However, additional studies are needed to confirm that *P. nasalis* has established a self-sustaining population in the Narva Bay and to evaluate the further potential ecological impact of this invasion.

We cannot completely rule out the possibility that the tubenose goby populations in eastern Gulf of Finland may represent more than one distinct species. Although, as the previous reports (Antsulevich et al. 2007; Uspenskiy 2020, but see also Demchuk et al. 2021) were not determined to the species level (*sensu* Fricke et al. 2021), the current study confirms that there is only one recognized species (*P. nasalis*) from the genus *Proterorhinus* in the Baltic Sea. However, considering the recent invasion history of *P. semilunaris* (e.g. Stepien and Tumeo 2006; Neilson and Stepien 2009a, b; Adámek et al. 2010; Mombaerts et al. 2014; Grabowska et al. 2019),

favourable local conditions for Ponto-Caspian species (e.g. Gollasch and Leppäkoski 1999; Bij de Vaate et al. 2002; Pauli and Briski 2018) and overlapping external morphology of genus *Proterorhinus* (e.g. Neilson and Stepien 2009a; Sorokin et al. 2011; Parin et al. 2014; Zhokhov et al. 2017; Uspenskiy 2020), it cannot be totally excluded that the *P. semilunaris* and *P. marmoratus* are also already present in the eastern Gulf of Finland *sensu stricto*, as well as in the European Northern Invasion Corridor *sensu lato* (*sensu* Bij de Vaate et al. 2002). Thus, further studies with wider geographical scale and relying on molecular methods that allow phylogenetic analysis are needed to evaluate this hypothesis.

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