




The uselessness of cytochrome oxidase gene analysis for genetic identification of selected Gobiidae species from some European countries

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RECEIVED 13.05.2022

ACCEPTED 15.09.2022

AVAILABLE ONLINE 31.12.2022

Abstract: The family Gobiidae is the largest teleost family, consisting of more than 2,000 species in more than 200 genera. They have adapted to a wide range of habitats, inhabiting mostly marine and brackish waters. Recently invasive Ponto-Caspian gobies have caused significant changes to the structure of fish assemblage throughout the European waters. The aim of the study was to verify the possibility of identifying three species of the genus Gobiidae based on the analysis of the mitochondrial region of the cytochrome oxidase subunit 1 (COI) gene. 339 samples originated from seven countries and 17 different sites were investigated. Qualitative and quantitative assessment of the extracted DNA was conducted by measuring light absorbance and electrophoretic separation. Approximately 650-bp (base pairs) fragments were amplified from the 5' region of the mitochondrial COI with the PCR (polymerase chain reaction) reaction. Sequences of 652 bp were obtained and compared with reference sequences in the National Center for Biotechnology Information (NCBI). Based on the sequence analysis, we determined that in the analysed geographical regions, the goby preliminary identified morphologically as the round goby was represented by three species: round goby (*Neogobius melanostomus*), tubenose goby (*Proterorhinus marmoratus*), and monkey goby (*Neogobius fluviatilis*). The latter two are newly reported for their new habitats. Genetic characterisation of such populations offers the opportunity to assess their genetic stability, which provides agencies managing the aquatic environment in EU countries with a tool for revising the legislation, including regulations relevant for updating the status of native and invasive species in European waters.

Keywords: cytochrome oxidase subunit 1 (COI) gene, Gobiidae, invasive and native species, mt DNA

INTRODUCTION

Round goby (*Neogobius melanostomus*) occurs naturally in the Ponto-Caspian region where it inhabits the catchment areas of the Sea of Azov and the Black and Caspian Seas [KOTTELAT, FREYHOF 2007], demonstrating a rapid expansion in Europe [OJAVEER 2006, VAN BEEK 2006] and North America [CORCUM *et al.* 2004] in the recent decades. It has been suggested that the spread of this species is vectored by ballast water of cargo ships and intensive ship freight in recent decades [POLAČEK *et al.* 2008]. Colonisation by this species is facilitated by its tolerance to a wide

range of salinity (5–25 ppm) [KARSIOTIS *et al.* 2012], high temperature (critical thermal maximum 33.4°C) [CROSS, RAWDING 2009] and low level of oxygen in water (critical lethal threshold ranging from 0.4 to 1.3 mg·dm⁻³) [CHARLEBOIS *et al.* 2001].

In European waters, beyond the area of its natural occurrence, the species was first found in 1990 in the Baltic Sea confluence in Gdańsk Bay (Poland) [SKÓRA, STOLARSKI 1993]. In the subsequent years, its presence was also noticed in nearby reservoirs [KUCZYŃSKI 1995; SAPOTA 2005], and probably since 1996, it has been present in the western Baltic Sea and the Oder River estuary [CZUGAŁA, WOŹNICZKA 2010]. In the German

region of the Baltic Sea, round goby was first found in 1998 in fish catches in the vicinity of the Zicker Peninsula (Rügen Island) [WINKLER 2006], while in 2002, it was found further to the west, near the Darß Peninsula (Darßer Ort) [CORKUM *et al.* 2004; WINKLER 2006]. In the following years, the species colonised the Rhine Delta and the Lek River near the village of Schoonhoven (2004), the Elbe River (2008), the Scheldt and Albert Canals in Belgium (2010), and the Weser River (2012) [BRUNKEN *et al.* 2012; HEMPEL, THIEL 2013; VAN BEEK 2006; VERREYCKEN *et al.* 2011]. At the beginning of the 21st century, the species was also seen in the northern and eastern parts of the Baltic Sea [OJAVEER 2006].

It is believed that the second route of colonisation of European waters by round goby was the catchment area of the Danube River, as evidenced by the successive occurrence of this fish at sites in the middle and upper course of the river [ONDRÁČKOVÁ *et al.* 2012; POLAČIK *et al.* 2008]. For example, in the Austrian section of the Danube near Vienna, the species was found in 2000 [WIESNER *et al.* 2010], while in the German section of the river between Passau and Straubing – in 2004 [PAINTER, SEIFERT 2006; ZAUNER, RATSCHAN 2004]. Results of studies by CERWENKA *et al.* [2014] and JANAČ *et al.* [2017] suggest that round goby has also used this route to colonise the catchment area of the Rhine River.

Although a number of studies on the colonisation of European waters by round goby have been published, analysis of their results usually does not allow a thorough understanding of the expansion of this species [JANAČ *et al.* 2017]. Much more reliable results regarding routes of invasion of foreign species can be obtained using indirect methods, mainly based on genetic assays [HANDLEY *et al.* 2011]. Among the methods used to determine interspecies variability in fish is analysis of selected fragments of genome by sequencing [MALEWSKI *et al.* 2021], and much less frequently analysis of fragments after digestion with restriction enzymes (RFLP). In turn, analysis of single-nucleotide polymorphisms (SNPs) allows identification of gene fragments encompassing point mutations which may affect the structure of the gene product, thus modifying features of fish phenotype [MALEWSKI *et al.* 2021]. Cytochrome B gene analysis was used by DOUGHERTY *et al.* [1996] to indicate the phylogenetic relationships of *N. melanostomus* in the Great Lakes of the USA.

DILLON and STEPIEN [2001] analysed genetic and biogeographic relationships in the same geographical area, but they did not detect any population variation among the analysed *P. marmoratus* individuals, while they identified as many as 17 haplotypes in *N. melanostomus*. Very interesting are the conclusions of BROWN and STEPIEN [2008], drawn on the basis of the analysis of seven new microsatellite DNA loci in the cytochrome B gene from 432 individuals representing 22 locations. They found that the native populations of *N. melanostomus* in the Black Sea and Caspian Sea basins diverged by 1.4%, which corresponds to the geographic isolation and taxonomic separation of the Black Sea.

The aim of this study was to test and verify the possibility of using the cytochrome oxidase gene analysis for genetic identification of three Gobiidae species: round goby (*Neogobius melanostomus*), tubenose goby (*Proterorhinus marmoratus*), and monkey goby (*Neogobius fluviatilis*).

Moreover, we determined the amounts of other members of the Gobiidae family erroneously identified as round goby during

catches, but actually representing other, non-invasive species. This study constitutes a reference for agencies in EU countries responsible for legislation relevant to the selection of stocking material.

MATERIALS AND METHODS

Material for the study were fish frozen after catching, phenotypically classified during catches as round goby *Neogobius melanostomus*. The samples originated from seven countries and 17 different research sites: Poland (Oder River estuary, Bay of Puck, Vistula River estuary, Szczecin Lagoon), Estonia (Eru Bay), Bulgaria (Yantra River, Iskar River, Danube River, Black Sea), Belgium (Albert Canal), Germany (Salzhaff, Peenestrom, Bay of Greifswald, Gummlin, Baltic Sea – Rügen) (Fig. 1). The material consisting of 339 individuals was collected in the years 2017–2018. Fragments of fins maintained in isopropyl alcohol until analysis were also used in some cases for molecular assays (Tab. 1).

DNA was isolated using columns from the Syngen DNA mini Kit, following the manufacturer's instructions. Qualitative and quantitative assessment of the extracted DNA was conducted by measuring light absorbance using the NanoDrop 2000 UV-VIS spectrophotometer (Thermo Fisher Scientific Inc.) and electrophoretic separation on a 1.5% agarose gel (Basica GQT Prona agarose and TBE Fermentas buffer). Polymerase chain reaction (PCR) was conducted in a total volume of 25·1E–6 dm³ using a Promega kit (GoTag G2 Green MasterMix). Approximately 650-bp fragments were amplified from the 5' region of the mitochondrial cytochrome oxidase subunit 1 (COI) gene (COI gene, partial cds, mitochondrial) using primers designed by WARD *et al.* [2005]: FishF: 5'TCAACCAACCACAAAGACATTGGCAC3' and FishR: 5'ACTTCAGGGTGACCGAAGAATCA-GAA3'.

DNA was isolated from a total of 339 samples; all isolates were used in the PCR; 85 selected samples were bidirectionally sequenced using Sanger method (Genomed, Poland). Sequencing results were analysed and aligned using the BLAST-N software in order to identify the species. The obtained sequences were compared with the sequences available in the National Center for Biotechnology Information (NCBI) database listed in Table 2.

RESULTS

In the above analysis, sequences of 652 bp were obtained and compared with reference sequences in the NCBI database.

100% genetic similarity was found for the sequences from the three Gobiidae species verified with the Geneie Prime program.

No point mutations were found in the COI gene in mitochondrial DNA. In most cases (82/85), molecular analysis allowed classifying the analysed species as round goby. In 2/85 cases, there was a 100% similarity to the sequences of monkey goby. In one case, the identified species was tubenose goby (Tab. 3). Whereas round goby individuals were found at all sampling locations, monkey goby was found in Poland and Bulgaria, and tubenose goby in Bulgaria only (Tab. 4).



Fig. 1. Map of Europe with marked locations (1–17) of the collected individuals whose samples were sequenced; source: own elaboration

Table 1. Sites of sample collection for molecular assays; source: own study

Station	Geographic location	Country	Number of collected isolates	Year of collection
1	Eru Bay	Estonia	21	2017
2	Yantra River at Dolna Oryahovitsa	Bulgaria	10	2017
3	Iskar River at Iskar	Bulgaria	10	2017
4	Danube River at Yasen	Bulgaria	10	2017
5	Black Sea at Varna	Bulgaria	13	2017
6	Albert Canal	Belgium	30	2017
7	Salzhaff (basin) near the island of Poel, Bay of Wismar	Germany	29	2017
8	Peenestrom (strait)	Germany	19	2017
9	Baltic Sea, Bay of Greifswald at Spandowerhagen	Germany	9	2017
10	Baltic Sea, Rügen, Sassnitz	Germany	13	2017
11	Gummlin	Germany	10	2017
12	Lake Dąbie	Poland	30	2017
13	Bay of Puck	Poland	30	2017
14	Vistula River estuary	Poland	9	2017
15	Rhine River at Kaiseraugst	Switzerland	30	2018
16	Szczecin Lagoon	Poland	42	2018
17	Sea of Azov	Ukraine	24	2017

Explanation: station numbers as in Fig. 1.
Source: own elaboration.

Table 2. The sequences used for comparison with the sequences obtained in this work

Species	Access number (NCBI)
Round goby (<i>Neogobius melanostomus</i>)	KX247280 KX145236 KX145135 KX145056 EU524154
Tubenose goby (<i>Proterorhinus marmoratus</i>)	KJ554161 KX145466 KX145131 KX145074 KX145072
Monkey goby (<i>Neogobius fluviatilis</i>)	KM373653 FJ526804 KM373678 KM373655 KJ553686

Source: own elaboration.

Table 3. Gobiidae species identification in each location

Location	Country	Species	Samples
Eru Bay	Estonia	round goby (<i>Neogobius melanostomus</i>)	5/5
Iskar River	Bulgaria	round goby (<i>Neogobius melanostomus</i>)	3/5
Iskar River	Bulgaria	monkey goby (<i>Neogobius fluviatilis</i>)	2/5
Danube River at Yasen	Bulgaria	round goby (<i>Neogobius melanostomus</i>)	5/5
Black Sea at Varna	Bulgaria	round goby (<i>Neogobius melanostomus</i>)	5/5
Yantra River at Dolna Oryahovitsa	Bulgaria	round goby (<i>Neogobius melanostomus</i>)	4/5
Yantra River at Dolna Oryahovitsa	Bulgaria	tubenose goby (<i>Proterorhinus marmoratus</i>)	1/5
Albert Canal	Belgium	round goby (<i>Neogobius melanostomus</i>)	5/5
Oder River estuary	Poland	round goby (<i>Neogobius melanostomus</i>)	5/5
Bay of Puck	Poland	round goby (<i>Neogobius melanostomus</i>)	5/5
Vistula River estuary	Poland	round goby (<i>Neogobius melanostomus</i>)	4/5
Vistula River estuary	Poland	monkey goby (<i>Neogobius fluviatilis</i>)	1/5
Szczecin Lagoon	Poland	round goby (<i>Neogobius melanostomus</i>)	5/5
Peenestrom (river)	Germany	round goby (<i>Neogobius melanostomus</i>)	5/5
Baltic Sea, Bay of Greifswald at Spandowerhagen	Germany	round goby (<i>Neogobius melanostomus</i>)	5/5

cont. Tab. 3

Location	Country	Species	Samples
Baltic Sea, Rügen, Sassnitz	Germany	round goby (<i>Neogobius melanostomus</i>)	5/5
Salzhaff (basin) near the island of Poel, Bay of Wismar	Germany	round goby (<i>Neogobius melanostomus</i>)	5/5
Gummlin	Germany	round goby (<i>Neogobius melanostomus</i>)	5/5
Rhine River at Kaiser-augst	Switzerland	round goby (<i>Neogobius melanostomus</i>)	5/5
Sea of Azov	Ukraine	round goby (<i>Neogobius melanostomus</i>)	5/5

Source: own study.

Table 4. Species identified in molecular analysis with the site of collection

Species	Cases	Countries	Location
Round goby (<i>Neogobius melanostomus</i>)	81/85	Poland, Bulgaria, Belgium, Estonia, Germany, Switzerland, Ukraine	all locations
Monkey goby (<i>Neogobius fluviatilis</i>)	3/85	Poland, Bulgaria	Vistula River estuary, Iskar River
Tubenose goby (<i>Proterorhinus marmoratus</i>)	1/85	Bulgaria	Yantra River at Dolna Oryahovitsa

Source: own study.

DISCUSSION

Population increase and rapid colonisation of major European river systems by round goby is due to increased freight activity resulting from globalisation [COPP *et al.* 2005], as well as due to the high adaptability of the species to new environmental conditions and its biological characteristics. The latter are associated with a wide range of natural occurrence in different environmental conditions (including a wide range of water salinity, temperature and oxygen level). As reported by MOSKALIKOVA [1996], round goby is present in waters of a salinity of up to 40.5 ppm, although KARSOTIS *et al.* [2012] reported a slightly lower value (25 ppm) along with a temperature range of -1 to 30°C. The species is tolerant to oxygen deficits in water, with the lethal oxygen level in water of 0.4–1.3 mg·dm⁻³ [CHARLEBOIS *et al.* 1997]. Moreover, increased abundance of round goby in new locations is facilitated by favourable reproductive characteristics. Long reproduction period (April to September) in a wide water temperature range (9–26°C) [CHARLEBOIS *et al.* 1997], rapid sexual maturity, particularly in the colonised areas [MACINNIS, CORKUM 2000], much larger size of eggs than in other Gobiidae [KORNIS *et al.* 2012] and males taking care of laid eggs [MEUNIER *et al.* 2009], all allow survival and growth of the population despite its relatively low fertility [TOMCZAK, SAPOTA 2006]. Therefore, the occurrence

of round goby in new locations may be associated with changes in the functioning of many aquatic ecosystems [KORNIS *et al.* 2012; MOSKALIKOVA 1996], especially because the species is widespread in Europe, forming stable non-indigenous populations [CHARLEBOIS *et al.* 1997, OJAVEER 2006, VAN BEEK 2006]. These changes are caused by the predatoriness and aggressive behaviour of this species [MEUNIER *et al.* 2009], as well as competition for food and habitat with native species, including protected ones [POOS *et al.* 2010]. For example, as reported by LAUER *et al.* [2004], the invasion of round goby in the Laurentian Great Lakes caused a sharp decrease in the catches of mottled sculpin and Johnny darter. A similar negative effect of the occurrence of round goby has been observed in the Baltic Sea for eelpout, European eel, perch, roach and vimba bream, as reported by SKÓRA and RZEŹNIK [2001]. This could be associated with an indirect effect of round goby on ichthyofauna, particularly species with a similar composition of the diet, by feeding on and thus limiting their sources of nutrients [KARLSON *et al.* 2007]. As a consequence, loss of biodiversity of benthic organisms in locations colonised by round goby is observed [KRAKOWIAK, PENNUTO 2008]. It should also be mentioned that round goby can hybridise with other invasive species of fish of the Benthophilinae subfamily, although extremely rarely in natural settings [LINDNER *et al.* 2013]. However, in anthropogenically changed rivers which are the main habitats of invasive fish, this phenomenon occurs much more frequently [ARNOLD 1997]. As suggested by PETT [2004], hybridisation of invasive and native species in ecosystems cannot be excluded. Hybrids may again become a novel invasive lineage with unique adaptations facilitating the invasion of novel areas [NOLTE *et al.* 2005]. It is also of importance for native organisms inhabiting areas colonised by round goby that this invasive species is a vector for pathogens and parasites [KVACH, STEPIEN 2008].

Round goby plays an important role in the trophic pyramid in basins in which it has formed stable populations. These fish transfer to higher trophic levels previously unavailable energy [KORNIS *et al.* 2012], but also heavy metals and PCBs (polychlorinated biphenyls) in contaminated areas [HOGAN *et al.* 2007; KWON *et al.* 2006]. This may contribute to increased concentrations of these substances in predatory fish exceeding consumption limits [Commission Regulation (EU) No. 420/2011; Legal Notice No. 66/2003].

Mitochondrial DNA analysis including the cytochrome B gene [GRABOWSKI *et al.* 2016] indicated the need to consider the population of racer goby (*Babka gymnotrachelus*) in Striwiąż separately. In contrast to the invasive population of this species in the Vistula River catchment area, the Striwiąż population is a special case indicating a double origin of Gobiidae in Poland. This poses a risk for legislative procedures in Poland, as one of the species is considered as foreign and invasive, and the other is not. In the study by BJORKLUND and ALMQVIST [2009], it was found that the most genetically diverse goby populations are those that are geographically distant, while populations inhabiting sites located close to each other do not show genetic differences. A study by STEPIEN and TUMEO [2006] indicated a relatively high genetic variability – as many as 17 haplotypes were identified among 64 analysed individuals of round goby. The authors indicated that the level of genetic variability basic on cytochrom B gene in round goby in native and colonised habitats was very similar, which suggests relatively numerous original populations and lack of

“bottlenecks”. The North Sea has been excluded as the original habitat of the round goby population occurring in the Great Lakes of Poland, as well as those introduced in Poland. The presence of hybrids of two species of goby (*Pomatoschistus minutus* and *P. lozanoi*) has also been confirmed [WALLIS, BEARDMORE 1980]. It can be presumed that local round goby populations in Poland potentially form hybrids with other Gobiidae species naturally present in Polish waters. In conclusion, the occurrence of the invasive round goby in the waters of Poland and Germany not only poses a threat for the native species, but also constitutes a potential source of undesired gene transfer and presents a risk for the genetic purity of non-invasive Gobiidae. It appears that the management of fish stocks should encompass not only protection of endangered species, but also active elimination of species presenting a risk for stable natural ecosystems. Genetic characterisation of such populations offers the opportunity to assess their genetic stability, which provides agencies managing the aquatic environment in EU countries with a tool for revising the legislation, including regulations relevant for updating the status of native and invasive species in European waters.

CONCLUSIONS

Sequence analysis of the COI gene fragment from the mitochondrial DNA 5' region in three species of goby *Neogobius melanostomus*, *Proterorhinus marmoratus* and *Neogobius fluviatilis* showed that despite of the absence of any point mutations, these species function in different geographic spaces. *N. melanostomus* were present in all sampling sites, *N. fluviatilis* only in Poland and Bulgaria, and *P. marmoratus* only in Bulgaria. Due to the lack of variability in the area of the analysed gene, it is difficult to determine the level of phylogenetic distances of the analysed species. Nevertheless, sequence analysis of the COI gene was used to identify the species due to their great morphological similarity. Determining of the occurrence of three analysed species is a key element to stop the spread of the invasive species in natural waters due to limited adaptation to environmental conditions. The aim of further research will therefore be to verify the differences at the nuclear DNA level and to establish the genetic differences allowing the identification and differentiation of invasive and non-invasive species within Gobiidae.

ACKNOWLEDGMENT

The authors of the paper thank Dr. Malte Dorow from the Institute of Fisheries, Mecklenburg-Vorpommern Research Center for Agriculture and Fisheries, Rostock (Germany) for assistance in obtaining material from Germany.

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