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Discrimination between invasive Ponto-Caspian gobies using a PCR-RFLP method

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Summary

Accurate identification of invaders, and especially their juveniles and eggs, is a difficult task if several morphologically similar species co-occur. The aim of the study was to develop and test a rapid and cost-effective procedure for identification of five species of invasive gobies occurring in the middle Danube basin, namely round goby *Neogobius melanostomus*, bighead goby *Ponticola kessleri*, monkey goby *Neogobius fluviatilis*, racer goby *Babka gymnotrachelus* and tubenose goby *Proterorhinus semilunaris*. First, a 708 bp fragment of the cytochrome oxidase I gene was amplified and sequenced for representative samples of these five species. Appropriate sequences of the five species available in public databases were used for *in silico* analysis. A digestion of the amplified fragment with the *Bfa*I enzyme was found to be suitable for the species identification, as it showed unique restriction patterns for each species. The technique was also successfully applied for fish remains from burbot *Lota lota* stomachs. Thus the technique could be a useful tool in monitoring biological invasions, especially by identifying specimens that could not be determined on the basis of morphological features. The results demonstrate that the PCR-RFLP method may in some cases be more reliable for species identification than a standard DNA sequencing.

Introduction

The flock of Ponto-Caspian 'neogobiins' includes around 24 species, five of which, namely tubenose goby *Proterorhinus semilunaris* (Heckel 1837), round goby *Neogobius melanostomus* (Pallas 1814), racer goby *Babka gymnotrachelus* (Kessler 1857), bighead goby *Ponticola kessleri* (Günther 1861) and monkey goby *Neogobius fluviatilis* (Pallas 1814) exhibit invasive behaviour (Neilson and Stepien, 2009). Within recently past decades, these species have spread upstream in rivers far from their original range and penetrated via canals to other Eurasian basins (Kottelat and Freyhof, 2007). Moreover, they were accidentally introduced via ships and bait release (Anhelt et al., 1998; Neilson and Stepien, 2009). Such translocations have caused colonisation of considerably distant water systems, such as the North American Great Lakes. At present, these five species co-occur in the Middle Danube basin (Koščo et al., 2010). Co-occurrence of some of these species has been also reported for other invaded systems, such as the Rhine, Vistula, upper Dnieper and the Great Lakes (Kottelat and Freyhof, 2007; Neilson and Stepien, 2009; Borcherding et al., 2011).

In a new environment the invasive 'neogobiins' are able to rapidly increase their densities with deleterious effects on ecosystems (Stepien and Tumeo, 2006; Kornis et al., 2012). They cause substantial changes to the local fauna through food and space competition, and predation on invertebrates, fish eggs and fry. On the other hand, the gobies were found to be an important prey source for native predators (Sloboda et al., 2010; Madenjian et al., 2011; Kornis et al., 2012 and citations therein; Pąchocki et al., 2012). A goby population could thus be controlled by an increased number of predators (Madenjian et al., 2011). Relationships of native predators and non-native prey seem to be important questions to resolve for understanding ecological mechanisms and consequences of biological invasions. Accurate identification of prey species found in the digestive tract can be a useful tool in future surveys. However, this seems to be quite difficult, because the consumed prey is in various stages of degradation and the characteristic morphological traits can be affected or destroyed.

It is assumed that, along with the active migration and inadvertent transport of larvae in ballast water of ships and by eggs attached to ship hulls (Anhelt et al., 1998), a downstream drift of the earliest stages is another means of 'neogobiins' spread (Kornis et al., 2012). Nevertheless, the way of spreading may differ in particular cases, and the hypotheses of the 'neogobiins' invasion routes are not yet sufficiently supported. A correct identification of early-life stages could help solve these complex questions. The identification of 'neogobiin' larvae and juveniles has been based mainly on morphological features (Koblickaya, 1981). However, such identification requires considerable experience, is substantially time-consuming and also very difficult in the eggs and smallest larvae, especially those damaged during sampling. A current effort is to standardize the species identification using a DNA sequencing of the 5′ fragment of mitochondrial cytochrome oxidase I gene. For this purpose, reference data for species are collected within the Barcode of Life Database (http://boldsystems.org, Ratnasingham and Hebert, 2007). The database already includes reference sequences of two invasive 'neogobiins' (round goby and tubenose goby). Nevertheless, although the standard sequencing is a reliable technique for determining particular species, it has some requirements and limitations related to sequencing machine handling, costliness, and the quality of resulting data. In recent years, next-generation sequencing techniques have been developed. These approaches appear to be able to identify species from large mixed-species samples. However, these techniques are not available for most fish ecologists and other researchers.

Table 1
Samples and sequences of invasive Ponto-Caspian 'neogobiins' and burbot. Includes accession numbers of sequences downloaded from GenBank or BOLD (in bold) databases, number of sequences used for *in silico* analysis (N_{SEQ}) and number of individuals analysed using the PCR-RFLP method (N_{RFLP})

Species	Location	Latitude	Longitude	Accession No.	N_{SEQ}	N_{RFLP}	Reference
Monkey goby	Danube R., Prahovo, Serbia	44.297	22.582			8	5
Neogobius	Danube R., Tekija, Serbia	44.682	22.404			8	5
fluviatilis	Danube R., Belgrade, Serbia	44.840	20.424			8	5
	Danube R., Tutrakan, Bulgaria	44.055	26.624			8	5
	Ipel' R., Chl'aba, Slovakia	47.833	18.830		7	5	5
	Hron R. and Danube R. confluence, Slovakia	47.818	18.741		3	3	5
	Danube R., Vilkove, Ukraine	45.394	29.587	FJ526804	1		1
	Sea of Azov, Molochnyi, Ukraine	46.656	35.279	FJ526805	1		1
	Ozero Manych, Prujitnoe, Russia	46.016	43.448	FJ526808	1		1
	Volga R., Volgograd, Russia	48.871	44.660	FJ526806	1		1
	Chernozemelskii Canal, near Elista, Russia	46.272	45.615	FJ526807	1		1
Bighead goby	Danube R., Prahovo, Serbia	44.297	22.582			8	5
Ponticola kessleri	Danube R., Tekija, Serbia	44.682	22.404			8	5
	Danube R., Radvaň, Slovakia	47.745	18.363		10	5	5
	Hron R., Kamenica, Slovakia	47.827	18.720		10	5	5
	Ipel' R., Chl'aba, Slovakia	47.833	18.830		9	5	5
	Morava R., Vysoká, Slovakia	48.316	16.899		7	5	5
	Danube R., near Chl'aba, Slovakia	47.822	18.781		10	5	5
	Danube R., Dobra, Serbia	44.638	21.909	FJ526825	1		1
	Dniester R., Yampil, Ukraine	48.235	28.293	FJ526823	1		1
	Simferopol Reservoir, Simferopol, Ukraine	44.922	34.156	FJ526824	1		1
Round goby	Danube R., Prahovo, Serbia	44.297	22.582			8	5
Neogobius	Danube R., Tekija, Serbia	44.682	22.404			8	5
melanostomus	Ipel' R., Chl'aba, Slovakia	47.833	18.830		8	1	5
	Morava R., Vysoká, Slovakia	48.316	16.899		6	1	5
	Danube R., Radvaň, Slovakia	47.745	18.363		14	2	5
	Hron R., Kamenica, Slovakia	47.827	18.720		10	1	5
	Dyje R., Lanžhot, Czech Rep.	48.693	16.919	HQ960511, **IFCZE773-11**, **IFCZE795-11**, **IFCZE879-11** – **IFCZE881-11**	6		2
	Morava R., Lanžhot, Czech Rep.	48.647	16.970	**IFCZE715-11**, **IFCZE772-11**	2		2
	Lake Ontario, ON, Canada	43.450		EU524919	1		2
	Fleuve St-Laurent, QC, Canada	46.767	−71.233	EU524920	1		2
	Georgian bay, ON, Canada	45.585	−81.311	EU524154	1		2
	St. Clair R., McLoad creek, Canada	42.519	−82.374	EU524155	1		2
	Fleuve St-Laurent, QC, Canada	46.782	−71.238	EU524156	1		2
	Unknown			HQ909479, HQ909495	2		3
	Black Sea, Sevastopol, Ukraine	44.604	33.541	FJ526800	1		1
	Kerch Strait, Kerch, Ukraine	45.358	36.476	FJ526803	1		1
	Volga R., Svetli Yar, Russia	48.485	44.785	FJ526802	1		1
	Caspian Sea, Nabran, Azerbaijan	41.837	48.620	FJ526801	1		1
	Dnieper R., Kiev, Ukraine	50.270	30.300	FJ526799	1		1
Racer goby	Danube R., Prahovo, Serbia	44.297	22.582			8	5
Babka	Danube R., Tekija, Serbia	44.682	22.404			8	5
gymnotrachelus	Ipel' R., Chl'aba, Slovakia	47.833	18.830		5	1	5
	Unknown			HQ909491	1		3
	Kanev Reservoir, Kiev, Ukraine	50.270	30.300	EU444694	1		1
	Dniester R., delta, Bilyayivka, Ukraine	46.468	30.217	FJ526820	1		1
	Dnieper R., Kiev, Ukraine	50.270	30.300	FJ526821	1		1
	Tyligul Estuary, Ukraine	46.471	30.735	FJ526822	1		1
Tubenose goby	Hron R., Kamenica, Slovakia	47.827	18.720		8	5	5
Proterorhinus	Ipel' R., Chl'aba, Slovakia	47.833	18.830		8	5	5
semilunaris	Hron R., Vozokany, Slovakia	48.008	18.667		3	3	5
	Hron R., Kamenín, Slovakia	47.881	18.661		3	3	5
	Morava R., Tvrdonice, Czech Rep.	48.741	17.027	HQ961006	1		2
	Dyje R., Podhradí, Czech Rep.	48.889	15.649	**IFCZE755-11** – **IFCZE758-11**	4		2
	Dyje R., Dyjakovice, Czech Rep.	48.754	16.295	**IFCZE759-11**	1		2
	Dyje R., Bulhary, Czech Rep.	48.826	16.770	**IFCZE864-11**, **IFCZE865-11**, **IFCZE867-11**	3		2
	Stará Dyje R., Poštorná, Czech Rep.	48.762	16.860	**IFCZE866-11**	1		2
	Lake St. Clair, Mitchell Bay, Canada	42.344	−82.432	EU524306	1		2
	Lake St. Clair, Mitchell Bay, Canada	42.372	−82.420	EU524307	1		2

Table 1
(continued)

Species	Location	Latitude	Longitude	Accession No.	N_{SEQ}	N_{RFLP}	Reference
	Lake Erie, Rose Beach, Canada			EU524305, EU524308-EU524310	4		2
	Lake Superior, MI, USA	46.667	−92.200	EU444690	1		1
	Lake St. Clair, MI, USA	42.594	−82.803	EU444674	1		1
	Danube R., Dobra, Serbia	44.638	21.909	EU444677	1		1
	Kurchurgan Reservoir, Hradenytsi, Ukraine	46.100	30.200	EU444686	1		1
	Cape Malyi Fontan, Odessa Bay, Ukraine	46.450	30.767	EU444683	1		1
	Simferopol Reservoir, Simferopol, Ukraine	44.922	34.156	EU444691	1		1
Burbot	Morava R., Vysoká, Slovakia	48.316	16.899		1	1	5
Lota lota	Fleuve St-Laurent, QC, Canada	45.400	−73.900	EU524757	1		2
	Lake Erie, ON, Canada	42.146	−81.479	EU524756	1		2
	Lake Erie, ON, Canada	42.150	−81.517	EU524755	1		2
	Lake Erie, ON, Canada	42.183	−81.517	EU524754	1		2
	Lake Erie, ON, Canada	42.229	−81.562	EU524753	1		2
	Lac Duparquet, QC, Canada			EU524749 – EU524752	4		2
	Digdegaush lake, NB, Canada			EU524746 – EU524748	3		2
	Goose Creek, MB, Canada	58.663	−94.167	**DSFCH080-08**	1		4
	Lužnice R., Holicky, Czech Rep.	49.024	14.823	HQ960501	1		4
	Svratka R., Jimramov, Czech Rep.	49.633	16.225	HQ960605	1		4
	Mostiště hatchery, Czech Rep.	49.376	16.015	HQ960657	1		4
	Melounka, Všestary, Czech Rep.	50.199	15.777	HQ960759	1		4
	Orlice R., Albrechtice, Czech Rep.	50.140	16.068	HQ960761	1		4
	Lučina R., Žermanice, Czech Rep.	49.716	18.294	HQ960860, HQ960861	2		4
	Morava R., Tvrdonice, Czech Rep.	48.741	17.027	HQ961003, HQ961004	2		4
	Ohře R., Jinřichov, Czech Rep.	50.106	12.396	HQ961083 – HQ961085	3		4
	Dyje R., Lanžhot, Czech Rep.	48.693	16.919	HQ961089	1		4
	Teplá Vltava, Horní Vltavice, Czech Rep.	48.953	13.757	HQ960500	1		4
	Šlajza hatchery, Kroměříž, Czech Rep.	49.313	17.378	HQ960596	1		4
	Odra R., Bernatice, Czech Rep.	49.627	17.943	HQ960862	1		4
Prey of burbot	Morava River, Vysoká, Slovakia	48.316	16.899			6	5
Gobiid larvae	Morava River, Vysoká, Slovakia	48.316	16.899			15	5

References: 1. Neilson and Stepien (2009), 2. Hubert et al. (2008), 3. Thacker et al. (2011), 4. iBOL (http://ibol.org), 5. this study.

The aim of our work was to develop an effective molecular technique for distinguishing the five species of invasive gobies in the middle Danube basin. We especially aimed for a quick and cost-effective identification of a large number of specimens within a short time. For this purpose, a polymerase chain reaction (PCR) of the 708 bp fragment of mitochondrial cytochrome oxidase I gene followed by a restriction fragment length polymorphism (RFLP) method was chosen with the intention of applying the technique on larvae, juveniles and egg specimens that are difficult to identify based on morphological features. The technique was also tested for partially degraded prey specimens from stomachs of burbot (*Lota lota* L.).

Materials and methods

Adult individuals of the five species of gobies were sampled by electrofishing. Fin clips were fixed in 98% ethanol. Drift was obtained using a drift net (mesh size 0.5 mm) and preserved in 60–70% ethanol to avoid shape and colour changes. Over the several weeks after sampling, fish larvae were selected, divided into groups and stored in 98% ethanol. The burbot population was sampled by electrofishing whereby whole individuals were fixed in 4% formaldehyde. Fish remains were removed from the burbot stomachs and transferred to 98% ethanol within 2 days after sampling. Details on sampling sites and numbers of specimens are given in Table 1.

Genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, CA) according to the manufacturer's instructions. Extracted DNA was eluted in 150 µl in two (100 and 50 µl) steps. For the RFLP analysis, the 708 bp fragment of cytochrome oxidase I (COI) gene was amplified using the primers FF2d and FR1d (Ivanova et al., 2007). The PCR was performed in a total volume of 25 µl. The reaction mix contained 1× PCR buffer, 0.5 mM each primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.625 U of GoTaq DNA polymerase (Promega, Madison, WI) and 50–150 ng of DNA. Initial PCR denaturation was at 94°C for 5 min, 35 cycles at 94°C for 30 s, 52°C for 30 s and 72°C for 1 min, and final extension at 72°C for 10 min. Amplified fragments of representative specimens were sequenced on the ABI Prism 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences were revised and aligned using BioEdit version 7.0.9 (Hall, 1999). Moreover, appropriate sequences of the five gobiid species and burbot available in NCBI GenBank (http://www.ncbi.nlm.nih.gov/genbank) and BOLDSYSTEMS (http://boldsystems.org) databases were downloaded for *in silico* RFLP analysis (Table 1).

The restriction enzymes, which are able to determine species-specific mutations, were determined. The enzyme *Bfa*I was found to show unique restriction patterns for each of

the species. Representative specimens of the five goby species and burbot were digested with this enzyme (Table 1). Five microlitre of the PCR product were incubated with 5 U of *Bfa*I and 1× NEB4 buffer (New England BioLabs, Hitchin, UK) in a total volume of 10 μl at 37°C overnight. The restriction products were separated on a 2% agarose gel with a 1 : 20 000 dilution of GoldView Nucleic Acid Stain (Guangzhou Geneshun Biotech Ltd., Guangzhou, China) and photographed under UV illumination. Applicability of the PCR-RFLP method was further tested on the samples of gobiid larvae and burbot prey (Table 1).

Results

The digestion with the enzyme *Bfa*I generated unique restriction patterns for each of the species (Fig. 1). All specimens of round goby were characterised by 454 and 254 bp fragments; bighead goby by 373 and 335 bp fragments; monkey goby by 316, 254 and 138 bp fragments; and specimens of racer goby by 324, 223, 93 and 69 bp fragments. Specimens of tubenose goby possessed two restriction patterns, the first of which consisted of five fragments (275, 223, 93, 60 and 57 bp) and the second of four fragments (275, 223, 150 and 60 bp) due to a substitution in position 318. The six patterns were clearly distinguished from each other using the 2% agarose gel electrophoresis. All larval individuals were successfully analysed and identified as round goby. All burbot sequences included three restriction sites generating 335, 223, 93 and 57 bp fragments. Six burbot individuals contained putative fish remains in their stomachs (Fig. 2). PCR amplification of the remains yielded a sufficient amount of product to perform the RFLP analysis. Three of the six specimens provided the pattern characteristic for round goby, one specimen showed the pattern of burbot, and the remaining two specimens had a combined pattern including fragments of both round goby and burbot (Fig. 2). On the contrary, sequences of these latter two specimens had ambiguous peaks disabling identification of the species (Fig. 3).

Discussion

The COI fragment seems to be a suitable marker for the discrimination of the five invasive 'neogobiins'. The *Bfa*I digestion of PCR product followed by the 2% agarose gel electrophoresis provides easily distinguishable patterns, unique for each of the species. Taking into account all new and previously published data on these five species, none of the restriction patterns is shared by different species. The marker thus could be considered species-specific. The advantage of this method is that it uses the universal primers developed for the DNA barcoding of fish and is therefore applicable in many laboratories without the need of additional primers and PCR optimisation. Moreover, the technique proved applicable to samples of burbot stomach contents stored in 4% formaldehyde.

Interactions between predators and non-native prey are important topics in ecological research. Non-native species have been shown to facilitate native predator population growth (Dijkstra et al., 2013), consequently the abundance of non-native prey decreases. It was estimated in the North

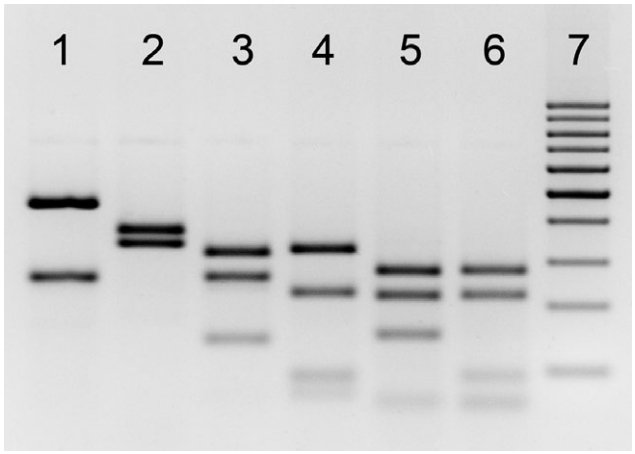

Fig. 1. Identification of five species of invasive gobies by *Bfa*I digestion of PCR amplified 708 bp fragment of cytochrome oxidase I gene. Patterns on 2% agarose gel correspond to round goby (line 1), bighead goby (line 2), monkey goby (line 3), racer goby (line 4), tubenose goby (lines 5 and 6) and 100 bp DNA ladder (line 7)

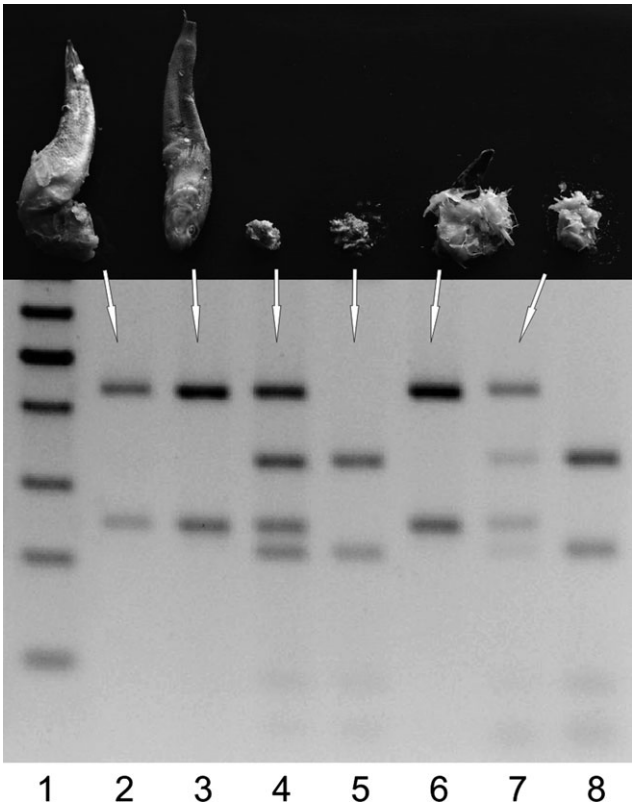

Fig. 2. Identification of fish remains from stomach content of burbot (lines 2–7) using the PCR-RFLP technique. The specimen of burbot (line 8) was used as a reference. Line 1 represents a 100 bp DNA ladder

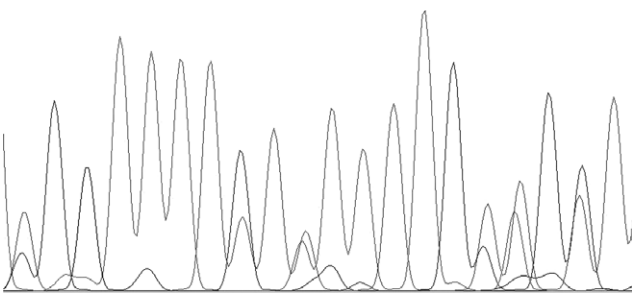

Fig. 3. Fragment of a capillary electrophoresis chromatogram resulting from DNA sequencing of a burbot prey specimen. Two peaks in the single positions disable the species identification. Specimen corresponds to line 4 in Fig. 2

American Lake Erie that burbot is annually able to eliminate as much as 61% of the round goby stock (Madenjian et al., 2011). In the Middle Danube basin, burbot represents the most significant native fish predator preying on gobies in habitats where they co-occur (personal observations). Extensive studies of interactions between native piscivorous predators and invasive gobies are planned for the near future; for such surveys the identification of prey species is essential. However, morphological differences are often not sufficient for the unambiguous determination of the species; identification is particularly difficult once in the predator's stomach due to a certain degradation of prey. Therefore, molecular methods are necessary for accurate species determination. Standard DNA sequencing has many advantages compared to other diagnostic methods; however, it is unsuitable for analysing specimens where the DNA of two or more species is mixed, because an overlap of fluorescent signals disables the detection of particular nucleotides. This is the case in the contamination of prey DNA in the predator's stomach by DNA of the predator or by cross-contamination of DNA by the different prey items. Application of the PCR-RFLP technique enables the identification of the prey species in a predator's stomach contents. This study shows that along with the patterns characteristic for the particular prey species, a common pattern determining both the prey species and the predator can be identified in one reaction.

The PCR-RFLP technique is a powerful diagnostic tool enabling rapid identification of the species without the need of a sequencer or other sophisticated machine, the application of which is too expensive for analysing large numbers of specimens. Regarding the potential to ascertain 'neogobiins' biology and ecology, the technique could be useful for studying biological invasions as well as for conservation and management applications.

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