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First DNA-based Records of New Alien Freshwater Species in the Republic of Georgia

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Abstract: The aim of the present study was to check the identity of a newly recorded freshwater crab and a goby species using DNA barcoding. The specimens were collected from the fresh waters of the western and eastern areas of Georgia. DNA barcodes (CO1) were obtained from samples, which turned out to belong to *Rhithropanopeus harrisii* (Decapoda: Panopeidae) and *Rhinogobius lindbergi* (Gobiiformes: Oxudercidae), detected in Georgia for the first time. Our results demonstrated the usefulness of the molecular methods for the identification of new species introductions that can play an important role in the early detection and the timely decision making. The results encourage the increase of taxon sampling and DNA barcode libraries development in order to effectively deal with the biodiversity inventory and monitoring.

Key words: *Rhinogobius lindbergi*, *Rhithropanopeus harrisii*, Georgia, new records, freshwater.

Introduction

The Ponto-Caspian region is one of the main sources of the worst invasive species worldwide (Bij de Vaate et al. 2002). On the other hand, the region is presumably a recipient of many alien species, some of which can have significant effect on the structure and functioning of the local ecosystems (Gozlan et al. 2010, Zhulidov et al. 2018). However, due to the ethno-cultural heterogeneity and constant political oscillations in the region, the biodiversity research, documentation and communication is apparently insufficient for the timely identification of invasive species and the establishment of effective monitoring programmes (Mumladze et al. 2020). For instance, only two alien invertebrate species, the gastropod *Ferrissia californica* (Rowell, 1863) and the bivalve *Mytilopsis leucophaeata* (Conrad, 1831) have been reported from the Georgian inland waters during the last two decades (Vinarski & Palatov 2018, Mumladze et al. 2019a). Such an unusual low number of fresh/ brackish water aliens in Georgia is most probably due to the scarcity of observation/ research activities rather than the rare invasion events.

In the recent years, the DNA barcoding has emerged as a tool for the rapid evaluation of species identification along with the traditional taxonomic expert-based approach. Since the seminal paper of Paul Hebert (Hebert et al. 2003), the DNA barcoding gained increasing importance in the species identification and biodiversity exploration, having many different applications. The DNA barcoding has already been recognised as an effective aid in

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the early detection of the alien species even without having the respective taxonomic expertise (Kvist et al. 2018). The only requirement for using the DNA barcoding in the detection of alien species is the availability of relevant DNA barcode libraries that can be used to evaluate the sequence identity. To this end, the publicly available DNA data in the large databases such as BOLD systems (Ratnasingham & Hebert 2007) and GenBank (Agarwala et al. 2018) is already sufficient to effectively diagnose most of the widespread non-native species for the regions with limited barcode coverage (Weigand et al. 2019).

The aim of the present study was to provide evidence of two new alien freshwater species: the crustacean *Rhithropanopeus harrisii* (Gould, 1841) and the fish *Rhinogobius lindbergi* Berg, 1933, for the Republic of Georgia based on the DNA barcoding approach (i.e. using traditional and the reverse taxonomic approach *sensu* Markmann & Tautz 2005).

Materials and Methods

The data reported in this article are based on the by-catch material collected during 2016–2019 for a project studying the freshwater molluscs of Georgia (Mumladze et al. 2019b).

Within the fieldwork campaigns, the samples of benthic macroinvertebrates were collected using a kick-net and hand-collecting. The samples were fixed in 96% alcohol and sorted afterwards. The large macroinvertebrates (such as crabs and some species of molluscs) and accidentally collected fish were preserved separately. One of the investigated locality (N42.116841, E41.702946; at the sea level) is in the westernmost coast of Lake Paliastomi near the ranger station (Fig. 1). The water in Lake Paliastomi is brackish and its salinity (ranging from 1 to 10‰ or more) is increasing towards the sea due to the regular water exchange. Crabs were collected in this locality for the first time in September 2016 and then in July 2018 and August 2019. In all sampling cases only one single species, apparently different from the native *Potamon* species, was recorded. The second locality, where similar crabs were collected, was the left tributary of the Kulevi River (N42.258500, E41.644500, altitude of 2 m a.s.l.), northwards from Lake Paliastomi. For the preliminary identification of the crab specimens, we used Jensen (2010) and Zaitsev & Öztürk (2001).

Small goby fish (family Oxudercidae, subfamily Gobionellinae) were collected on 28 April 2017 in an intermittent brook (the Ozaani Stream) located in the eastern Georgia (N41.560567, E45.993493, altitude of 445 m a.s.l.). The collected goby specimens were very small (max total length of 54 mm) and it was impossible to match any of the species known for the region according to Kottelat & Freyhof (2007) and Ninua et al. (2013).

The crab and goby samples were submitted to the Scientific and Practical Center for Bioresources of the National Academy of Sciences of Belarus for DNA barcoding.

For the barcoding purposes, DNA was extracted from one or two pereiopods of the crabs and fin-clip muscles of the fish, using a spin column based DNA Preparation Kit (Jena Bioscience™). The 658-bp barcode region of the mitochondrial cytochrome *c* oxidase subunit 1 (COI) gene (Hebert et al. 2003) was amplified using Lepidoptera and Folmer primer cocktail (C_LepFolF / C_LepFolR, 1:1) (Hernández-Triana et al. 2014) for the crabs, and fish primers (L6468/H7696) (Thacker 2003) for the gobies. The polymerase chain reaction was performed in the solution totalling 25 µl: 200 µM of each dNTP, 0.5 µM of each primer, 1.5 mMol of MgCl2, Taq Buffer "A" (65 mMTris-HCl, 16.6 mM (NH4)2SO_4, 0.02% Tween 20 at a pH of 8.8) 0.5 units and with *Taq* polymerase, approximately 100 ng DNA matrix (2 µl). The thermocycling regime used for the crabs was the following: initial denaturation at 94°C for 1 min, 5 cycles of 94°C for 40 s, 45°C for 40 s and 72°C for 1 min, followed by 35 cycles of 94°C for 40 s, 51°C for 40 s and 72°C for 1 min, and a final step of 72°C for 5 min. The thermocycling regime used for the gobies was the following: 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. Bidirectional sequencing was done using a BrilliantDye™ Terminator v3.1 Cycle Sequencing Kit (Life Technologies, 2018). The sequences obtained thereafter were submitted to the Barcode of Life Data System (BOLD) under the project INSGE. The sequence analysis tools available in the BOLD systems were used to identify the specimens by sequences, and calculate the sequence divergence statistics (based on uncorrected *p* distance) (Srivathsan & Meier 2012). We also downloaded the available sequences of the most closely related species (sequences within the same Barcode Index Number – BIN) and the other congenerics in order to construct phylogenetic trees. The sequences from Projecto-Garcia et al. (2010) and Simakova et al. (2017) were used for the crabs, while the sequences from Xie et al. (2015), Yamasaki et al. (2015) and Chang et al. (2017) were used for the fish. The sequence alignments and neighbour-joining trees

Fig. 1. Map of Georgia with sampling localities and habitats: 1 – left tributary of the Kulevi River (*R. harrisii*); 2 – Lake Paliastomi (*Rhithropanopeus harrisii*); and 3 – the Ozaani Stream (*Rhinogobius lindbergi*).

were constructed and illustrated by MEGA X software using default parameters (Saitou & Nei 1987, Kumar et al. 2018).

Results

Crabs: Two specimens of the crab were successfully sequenced and the BOLD identification tool recovered 100% identity of our samples (BOLD System ID: NEOCR015-18; NEOCR025-19) with the species *Rhithropanopeus harrisii* (Gould, 1841) (Fig. 2), belonging to the BIN (barcode index number): AAA2223. A comparison of sequences from the Georgian specimens to other samples available in the BOLD/GeneBank databases from the North-Western Black Sea and Europe revealed that the Georgian specimens belong to the most widespread *R. harrisii* lineage (Haplotype A *sensu* Projecto-Garcia et al. 2010) distributed in the region with uncorrected *p*-distance near zero (Fig. 3). The morphology of the Georgian *R. harrisii* specimens also perfectly matched the descriptions provided in the literature (Zaitsev & Öztürk 2001, Jensen 2010). In particular, the species could easily be distinguished from the other potentially co-occurring crab species by having three pronounced teeth on the fore lateral edges of the carapace and a slightly protruded (almost straight) anterior margin of the carapace (between eyes) with an indistinct median notch (Fig. 2).

Fish: Three specimens of the fish were successfully sequenced and the BOLD identification tool recovered 18 sequences of the unidentified specimens (belonging to BIN ACB4145) most closely related (from 99.43% to 99.81% similarity based on uncorrected *p*-distance) to our specimens (BOLD System ID: NEOCR018-18; NEOCR030-19, NEOCR03-19). All of those additional sequences were not yet publicly known though originating from the Caspian Sea Basin. The same BIN also included sequences identified as *Rhinogobius brunneus* (Temminck & Schlegel, 1845) from the Nomura River (Japan) (Yamasaki et al. 2015), with high level of sequence similarity (99.22%) (Fig. 3). However, the morphological characters of our specimens resembled *R. lindbergi* Berg, 1933 (Fig. 2) according to the recent morphological treatment of several *Rhinogobius* taxa (Table 2 in Sadeghi et al. 2019). Namely, the lower number of transverse scales (9–11) and the absence of the predorsal scales distinguished our specimens from the typical *R. brunneus*. In addition, the 18-rays-in-pectoral fin has been ascribed to the Caspian lineage of *R. lindbergi*. Unfortunately, there were no DNA sequences available in the BOLD Sytems or the GenBank identified as *R. lindbergi,* which made it impossible to compare directly the newly obtained sequences.

Discussion

The crab and fish species reported here most probably are not very recent introductions for Georgia, but rather were overlooked in the past. The lack of biodiversity research in the last few decades in the country is presumably the reason why these

Fig. 2. The newly recorded species in Georgia: A – *Rhithropanopeus harrisii* (N42.116841, E41.702946); B – *Rhinogobius lindbergi* (N41.560567, E45.993493). Scale bar – 1 cm.

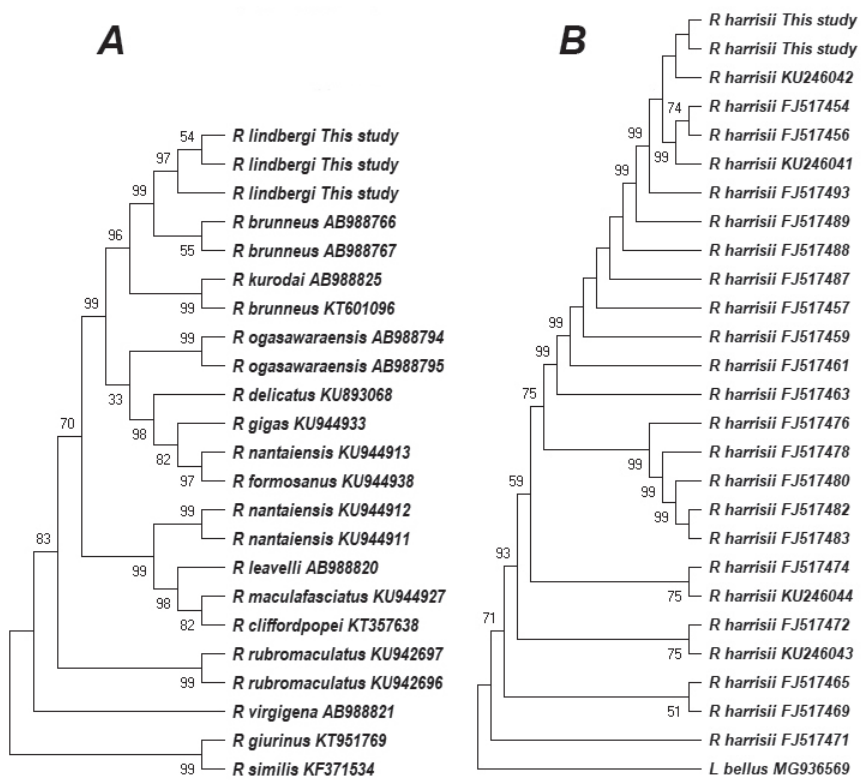

Fig. 3. Unscaled neighbour-joining phylogenetic trees reconstructed using CO1 gene fragments. A – *Rhinogobius* species; B – *Rhithropanopeus harrisii* [*Lophopanopeus bellus* (Stimpson, 1860) were used as an outgroup].

species have not been recorded before (Mumladze et al. 2020).

According to the literature *R. harrisii* was first recorded in the Black Sea area in 1939 (Makarov 1939 as cited in Zaitsev & Öztürk 2001). Subsequently, *R. harrisii* spread widely in the Black Sea low saline waters and most probably reached the Georgian coast well before 2016. The ballast water and ship hull fouling are considered as means of its introduction (Jensen 2010), though it is not known whether any repeated introduction took place in the past. While there are a number of *R. harrisii* haplotypes in Europe, only two of them are detected in the Black Sea (Simakova et al. 2017, this study), indicating that the populations might be spread from the Liman estuary (the North-Western Black Sea) since its original introduction in 1939.

Very few studies quantifying the impact of *R. harrisii* on the local ecosystems are available to date. In Texas the crab has become very abundant in almost all freshwater reservoirs and is reported to foul PVC intakes in the lakeside homes and clog the cooling system of a nuclear power plant in Glenrose, as well as being a resource competitor in an invaded system (Patton et al. 2010). Payen & Bonami (1979) also identified *R. harrisii* as a carrier of the white spot baculovirus, which causes disease in the penaeid prawn species and the blue crab (*Callinectes sapidus*). In both Caspian and Black Sea basins, where it has reached high densities, *R. harrisii* is considered as useful and additional food source for fish such as the endangered sturgeons (Zaitsev & Öztürk 2001). However, in the Georgian Black Sea offshores where the native crab *Potamon ibericum* (Biberstein, 1808) and the crayfish *Astacus colchicus* Kessler, 1876 occur (Parvizi et al. 2019, Bláha et al. 2020), *R. harrisii* might be a significant competitor for feeding resources and shelter. It should be mentioned that no native crabs were observed during our sampling campaigns in Lake Paliastomi.

The genus *Rhinogobius* includes more than 60 species widely distributed in Eastern Asia (Froese & Pauly 2019). Some of these species are believed to be introduced in freshwater systems of Mongolia (Neely et al. 2008), Kazakhstan (Kopylets & Dukravets 1981), Russia (Bogutskaya & Naseka 2002), Iran (Esmaeili et al. 2017, Eagderi et al. 2018, Sadeghi et al. 2019), etc., due to accidental introduction with the economically important cyprinid fish. Systematics of *Rhinogobius* still seems to be highly speculative, at least for some species groups. In addition, no extensive DNA barcode library exists in BOLD for all putative species. Our specimens of *Rhinogobius* were closely related to the *R. brunneus* sequences as belonging to the same BIN (ACB4145). However, the identity of the BOLD specimens, as well as the taxonomic status of the Middle Asian *Rhinogobius* remains widely dubious. For instance, until recently the goby species *R. similis* has been considered to be an introduced species in the South Caspian Sea Basin (Esmaeili et al. 2017). Later on, Sadeghi et al. (2019) based on morphological taxonomic review, showed that *R. lindbergi* (a native species for the Amur River) instead of *R. similis* is widely introduced species in Iran and elsewhere in the Caspian Sea basin. Georgian specimens also support the conclusions of Sadeghi et al. (2019). On the other hand, no barcodes for *R. lindbergi* (as well as some other closely related taxa) exist in BOLD which makes impossible to unambiguously match the specimens to the taxonomic names. Accordingly, further investigation is needed to solve the taxonomic ambiguities related to *Rhinogobius* species in the Caspian Sea Basin.

Although there are some warnings with regard to the potential effect of *R. lindbergi* on the native fish fauna (Neely et al. 2008), still no published evidence exists about its negative impact. The Ozaani Stream is a very small and heavily disturbed river with no other fish detected during our sampling. Hence, it is unlikely to distinguish any effect of *R. lindbergi* from the direct anthropogenic impact on this stream. On the other hand, reaching such a small and remotely located river is possible only if the species exists in the other rivers, such as the Alazani River (the Ozaani Stream being its right tributary), which is a tributary of the Kura River. Cosequently, it may be assumed that the distribution of this species in Eastern Georgia is much wider.

The importance of the DNA barcoding in detection of alien species or identification of putative new species is indisputable. The development of the DNA barcode library is also crucial in terms of the effective use of barcoding applications (Weigand et al. 2019). In the present work we showed an effectiveness of the available DNA barcode library (to identify the invasive crab species) and also faced problems related to the availability of incomplete barcode database and/or unsolved systematics of specific fish taxon. Since the large amount of alien species originate from the Asian and Ponto-Caspian parts of the world, we encourage to continue developing DNA barcode libraries particularly targeting these and other less studied regions.

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