First DNA barcoding based record of *Echinogammarus trichiatus* (Martynov, 1932) (Crustacea, Gammaridae) in Belarus

Tatsiana Lipinskaya1,*, Adriana Radulovici2 and Andrei Makaranka1

1Scientific and Practical Center of the National Academy of Sciences of Belarus for Bioresources Akademicheskaya Str., 27, Minsk, 220072 Belarus
2Centre for Biodiversity Genomics, University of Guelph, Guelph, Canada

Author e-mails: tatsiana.lipinskaya@gmail.com (TL), aradulov@uoguelph.ca (AR), amakarenko198989@mail.ru (AM)

*Corresponding author

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Abstract

A new alien Ponto-Caspian amphipod species *Echinogammarus trichiatus* (Martynov, 1932) was identified in the Belarusian part of the Dnieper River using DNA barcoding. This species expands the national checklist of Ponto-Caspian amphipods to nine species. We found individuals of both sexes, including ovigerous females and juveniles. The structure of *E. trichiatus* population and low abundance suggest that it is still being established. We conclude that further expansion of *E. trichiatus* into Belarusian waters is possible.

Key words: alien species, Amphipoda, distribution, molecular techniques, Dnieper River

Introduction

Rapid and accurate species identification is critical for detecting alien species as well as for control and eradication programs. DNA barcoding as proposed by Hebert et al. (2003) is now widely used for species identification. A standard short nucleotide sequence of a 658 base-pair region in the mitochondrial cytochrome c oxidase 1 gene (COI) has been used for identification of crustaceans since the early 2000s (Lefèbure et al. 2006; Costa et al. 2007). This method can be more accurate and informative especially when attempting to identify immature specimens or when morphological features are not evident or damaged.

The spread of alien species of Ponto-Caspian origin to the Belarusian territory was caused by construction of several dam reservoirs on the Dnieper River in Ukraine and by intentional introduction of amphipods as food source for fish (Mordukhai-Bolotovskoi 1964; Karatayev et al. 2008; Semenchenko et al. 2009). There are three interbasin canals connecting the Dnieper River with several rivers from the Baltic Sea basin, therefore Belarus is a transit zone through which Ponto-Caspian species spread to the Baltic region (Bij de Vaate et al. 2002; Karatayev et al. 2008; Jazdzewski et al. 2002). The Dnieper River with its large tributary, Pripyat River, is a substantial part of the Central European invasion corridor (Bij de Vaate et al. 2002) and it plays an important role in the movement of alien aquatic species to Central Europe. According to several recent articles (Semenchenko et al. 2009; Semenchenko et al. 2013; Semenchenko et al. 2016), eight alien species of amphipods are established in the water bodies of Belarus.

Here, we identify the previously overlooked alien species *Echinogammarus trichiatus* Martynov, 1932, formerly known as *Chaetogammarus ischnus major* Carausu, 1943 and *Chaetogammarus tenellus major* Carausu, 1943, and placed in the genus *Tricho-gammarus* by Hou and Sket (2015), using DNA barcoding.

Material and methods

Samples were collected in May and September 2016 by hand net (ISO 7828) and hand-picked from submerged...
logs and other natural and artificial substrates floating or submerged in the water. Crustaceans were immediately fixed in cold 96% ethanol and stored on ice for return to the laboratory. Additional samples from a previous collection (August 2010) were also used. The keys of Cărăuşu et al. (1955), Morduchai-Boltovskoy (1969) and Dobson (2013) were used for species identification. Specimens were stored in the fridge until subsampling for DNA extraction. All details regarding taxonomy and vouchers can be found on the Barcode of Life Datasystem (BOLD) website (Ratnasingham and Hebert 2007) in the “Echinogammarus” from Belarus” dataset (DS-BELCRUST) section. The samples collected in 2010 were processed in 2015 during the Global Taxonomy Initiative Training Course, whereas the sequencing of samples collected in 2016 was obtained in 2017 as a part of inventory of alien species list using DNA barcoding.

DNA was extracted from one or two pereopods of amphipods using an automated silica-based protocol with glass fiber filtration plates (Ivanova et al. 2006). The 658-bp barcode region of the mitochondrial cytochrome c oxidase subunit 1 (COI) gene (Hebert et al. 2003) was amplified using crustacean primer set (CrustDR1 / CrustDF1) (Steinke et al. 2016) and Lepidoptera and Folmer primer cocktail (C_LepF0f / C_LepF0r, 1:1) (Hernández-Triana et al. 2014). Polymerase chain reaction was performed in a 12.5 μL volume containing 6.25 μL 10% trehalose, 2μL ddH2O, 1.25 μL 10× PCR buffer, 0.625 μL MgCl2 (50 mM), 0.125 μL of each primer (10 μm), 0.0625 μL dNTPs (10 mM), 0.06 μL Platinum Taq polymerase (5 U/μL) and 2 μL DNA template (Ivanova and Grainger 2007a). The thermocycling regime used for all reactions 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 ºC for 1 min, followed by 35 cycles of 94 °C for 40 s, 51 °C for 40 s and 72 ºC for 1 min, and a final step of 72 °C for 5 min. Bidirectional sequencing was done using BigDye Terminator v3.1 sequencing kit as described in the Canadian Center for DNA Barcoding (CCDB) sequencing protocols (Ivanova and Grainger 2007b).

DNA barcodes were compared with other COI sequences of Echinogammarus species available in BOLD (Table 1) and the most probable to be found in Belarus. A phylogenetic tree was constructed in MEGA 6 (Tamura et al. 2013) using the Neighbor-Joining method (Saitou and Nei 1987) based on the p-distance (Nei and Kumar 2000) with a bootstrap test performed on 10000 replicates (Felsenstein 1985). New sequence of Gammarus lacustris Sars, 1863 was used as an outgroup. Mean Kimura 2-parameter (K2P) genetic distance between E. trichiatus sequences and standard error (SE) were also calculated in Mega 6 (Kimura 1980).

### Results

The only location, where E. trichiatus is established in Belarus (Figure 1), is in the Dnieper River near Nizhnie Zhary vill. (51º17′40.5″N; 30º34′21.1″E). The sampling site is characterized by a sandy bottom with numerous empty shells of Lymnaeidae species and submerged beams (Figure 2). Chemical parameters were as following: pH 8.5, dissolved oxygen 8.5 mg O2/l, conductivity 173 μS.

The analysis of our own data and existing literature revealed that this species was accidentally unnoticed and omitted from the checklist of non-native benthic macroinvertebrates in the Dnieper River basin published by Semenchenko et al. (2016).

**Species confirmation – standard DNA barcode sequencing**

Due to the uncertainty regarding morphological identification of E. trichiatus, the specimens collected in 2010 were checked using molecular techniques in 2015. Only one sequence of E. trichiatus was derived (BOLD sample ID: SPCNAS-GTI0178, GenBank accession – MF629684). Eight specimens collected in 2016 and identified morphologically as E. trichiatus were analyzed. Seven sequences of E. trichiatus were derived (BOLD sample ID: SPCB-ET001 – SPCB-ET003 (Genbank MF629709 – MF629711), SPCB-ET005 – SPCB-ET008 (GenBank MF629713 – MF629715, MF629717)). These sequences clustered with BOLD sequences of E. trichiatus from Ukraine, Romania, Belgium, Netherlands, Germany, and Turkey (Table 1; Figure 3) in a single clade with bootstrap support of 100%. The mean K2P distance between 15 compared sequences of E. trichiatus was 0.0041 (± 0.0021 SE).

One sequence (BOLD sample ID SPCB-ET004) belonged to E. ischnus due to misidentification. This sequence clustered with 100 % value within one clade with BOLD obtained sequences of E. ischnus from Ukraine, Belarus, Romania, Netherlands, Canada, and Russia (Table 1; Figure 3). The mean K2P distance between 46 compared sequences of E. ischnus was 0.0164 (± 0.0053 SE). The mean genetic distance between specimens of each species was below 0.03 upper threshold for the intraspecific genetic distance defined by Hebert et al. (2003) and Costa et al. (2009). The mean interspecific genetic distance between the two species was 23.54 %.

### Discussion

The native distribution region of E. trichiatus includes the Black Sea, the Azov Sea, their deltas as well as the coastal lakes of Bulgaria and Romania.
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**Table 1.** Cytochrome oxidase I (COI) sequences of *Echinogammarus ischnus* and *Echinogammarus trichiatus* used in the study. Samples from Belarus are in bold. Localities with approximate coordinates are marked by asterisk (*).

<table>
<thead>
<tr>
<th>GenBank accession</th>
<th>BOLD sample ID</th>
<th>Locality</th>
<th>Latitude, N</th>
<th>Longitude, E</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Echinogammarus ischnus</strong> (Stebbing, 1899)</td>
<td>MF629685–MF629699, SPCB-EI001–SPCB-EI005</td>
<td>Dnieper River, Rechica town, Belarus</td>
<td>52.3221</td>
<td>30.525</td>
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<tr>
<td></td>
<td>MF629716, SPCB-EI025</td>
<td>Pripyat River, Mozyr town, Belarus</td>
<td>52.0244</td>
<td>29.3208</td>
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<tr>
<td><strong>MF629680–MF629683</strong></td>
<td>SPCNAS-GTI 0099, SPCNAS-GTI 0100, SPCNAS-GTI 0102, SPCNAS-GTI 0106</td>
<td>Muchavce River, Brest city, Belarus</td>
<td>51.2833</td>
<td>30.5833</td>
</tr>
<tr>
<td><strong>Echinogammarus trichiatus</strong> (Martynov, 1932)</td>
<td>MF629709–MF629711, MF629713–MF629715, MF629717</td>
<td>Dnieper River, Nizhnie Zhary vill., Belarus</td>
<td>51.2946</td>
<td>30.5725</td>
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<tr>
<td></td>
<td>KR007308.1–KR007309.1</td>
<td>Lake Durusu, Turkey*</td>
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<td></td>
<td>AY529051</td>
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<tr>
<td></td>
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<td>Dniester Liman, Ukraine</td>
<td>46.2987</td>
<td>28.3205</td>
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<td>KM024679</td>
<td>The Netherlands*</td>
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<td></td>
<td>KT075260.1</td>
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<td>6.7368</td>
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<tr>
<td></td>
<td>KM009053.1</td>
<td>Meuse River, Belgium*</td>
<td>50.7110</td>
<td>5.6830</td>
</tr>
</tbody>
</table>

**Figure 1.** Map of Belarus with indication of the sampling location of *E. trichiatus* records.
Figure 2. The Dnieper River near Nizhnie Zhary vill. (Belarus) where E. trichiatus was sampled (photo by Aliaksei Mileika)

Figure 3. Neighbor-joining tree based on COI p-distances for sequences of Echinogammarus ischnus and Echinogammarus trichiatus. Gammarus lacustris was used as an outgroup. Arrows indicate sequences coming from the Dnieper River obtained in this study. Numbers above branch represent bootstrap values.
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(Cărăuşu et al. 1955; Mordukhaj-Boltovskoj 1969). The first record of this species outside the native area was made by Weinzierl et al. (1997) in the upper part of the Danube River in Germany. Subsequently, *E. trichiatus* has spread in German waterways and navigation canals due to human-mediated transport (Tittizer et al. 2000; Podraza et al. 2001; Bernerth and Stein 2003; Eggers 2005; Hirt and Schödel 2005; Müller and Eggers 2006). This species has also been found in Slovakia and Hungary (Borza 2009), France (Labat et al. 2011), Belgium (Boets et al. 2012), the Netherlands (Duijs 2011; Tempelman 2011), Poland (Rachalewski et al. 2013), and recently in Turkey (Rewicz et al. 2016). The easternmost record came from the lowest flow of the Caucasian river Khosta (Dedju 1967), which is the *locus typicus* of this species (Martynov 1932).

In Belarus, few specimens of *E. trichiatus* were sampled for the first time in 2008 by Vezhnovetz V. (unpublished data) and identified as a subspecies of *Chaetogammarus ischmus* (Stebbing, 1899). Due to this misidentification, *E. trichiatus* was not included into the first national checklist published in 2009 (Semenchenko et al. 2009). Afterwards, four specimens of *E. trichiatus* were found at the same location in 2010 and their identification was checked by DNA barcoding in 2015 (results presented in this study). Data on alien amphipods distribution, including *Ch. ischmus* with two subspecies *Ch. ischmus behnindi* Martynov, 1919 and *Ch. ischmus major* (= *E. trichiatus*) Cărăusu, 1943, was published recently (Makarenko and Vezhnovetz 2014). The material for the above-mentioned study was sampled in 2011–2013. At the beginning of 2015, another article was published by Makarenko (2015), where *E. trichiatus* was mentioned as a separate species and morphological data for this species were provided. This taxonomic confusion has been discussed in depth by Rachalewski et al. (2013).

It is known that *E. trichiatus* was introduced in the Kiev reservoir early in 1998 (Grigorovich et al. 2002) and it was mentioned for the Kremenchuk and Dniproderzhynsk reservoirs (Rachalewski et al. 2013) in Ukraine. As described above, this species was found in Belarus only in one location situated very close to the Kiev reservoir. We can conclude that dispersal of *E. trichiatus* into the Belarusian part of Dnieper River is the result of natural spread upstream. However, it is not clear why this species was found only at one site since 2010.

Individuals of both sexes, including ovigerous females and juveniles were found in the River Dnieper. It should be noted that only few specimens were caught by hand net while most of them were found on submerged logs near a place where boats are usually floated and lifted by fishermen. Taking into consideration its high viability outside the water bodies for six days between mussel layers (Boets et al. 2012), *E. trichiatus* could be unintentionally spread by overland transportation of fishing boats and nets. The structure of its population and low abundance suggests that this species is still establishing and further expansion of *E. trichiatus* into Belarusian waters is possible.

In conclusion, the presence of *Echinogammarus trichiatus* was confirmed by DNA barcoding in the Belarusian part of the Dnieper River at least since 2010. *Echinogammarus trichiatus* expands the national checklist of Ponto-Caspian amphipods to 9 species. All the Ponto-Caspian species known from Belarus occur in this locality of the Dnieper River making it a regional hotspot for alien amphipods.

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**References**


