Rapid Communication

First record of an established marbled crayfish *Procambarus virginalis* (Lyko, 2017) population in Estonia

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Abstract

Invasive marbled crayfish *Procambarus virginalis* (Lyko, 2017) is spreading alarmingly fast across European countries and beyond. Early maturation, parthenogenetic reproduction mode and high growth rate contribute to a high potential invasiveness. Marbled crayfish can pose severe effects on native communities impacting the native crayfish populations being carrier of the crayfish plague disease caused by *Aphanomyces astaci*. Here we report the first record of marbled crayfish in Estonia. In total, 104 individuals were found in the artificially warm outflow channel of the cooling system of Balti Power Plant, entering to the water reservoir of the River Narva. Molecular analyses confirmed the morphological identification of captured specimens as a marbled crayfish. Four out of six marbled crayfish individuals exhibited the presence of crayfish plague agent, though at very low level. This suggests that marbled crayfish can potentially be a new vector of crayfish plague in Estonian freshwater ecosystems containing native noble crayfish *Astacus astacus* populations. Monitoring and eradication actions are urgently needed not only in the outflow channel where the species was found but in the whole water reservoir and River Narva itself.

Key words: invasive crayfish, NICS, crayfish plague, reservoir, artificial refuge trap, mitochondrial DNA

Introduction

Freshwater ecosystems are nowadays experiencing high pressure from invasions of introduced non-native species. Non-native crayfish species represent one of the most well-known example of freshwater invaders that are threating native crayfish species (Kouba et al. 2014) and freshwater ecosystems (Rodríguez et al. 2005). Recently, attention of researchers has been focused on the fast and uncontrollable spread of the marbled crayfish *Procambarus virginalis* (Lyko, 2017). It is a parthenogenetic all-female species, which was formerly described as *Procambarus fallax* f. *virginalis* and was found first in German and Austrian aquarium trade in the mid-1990s (Lukhaup 2001; Scholtz et al. 2003). Its rapid parthenogenetic

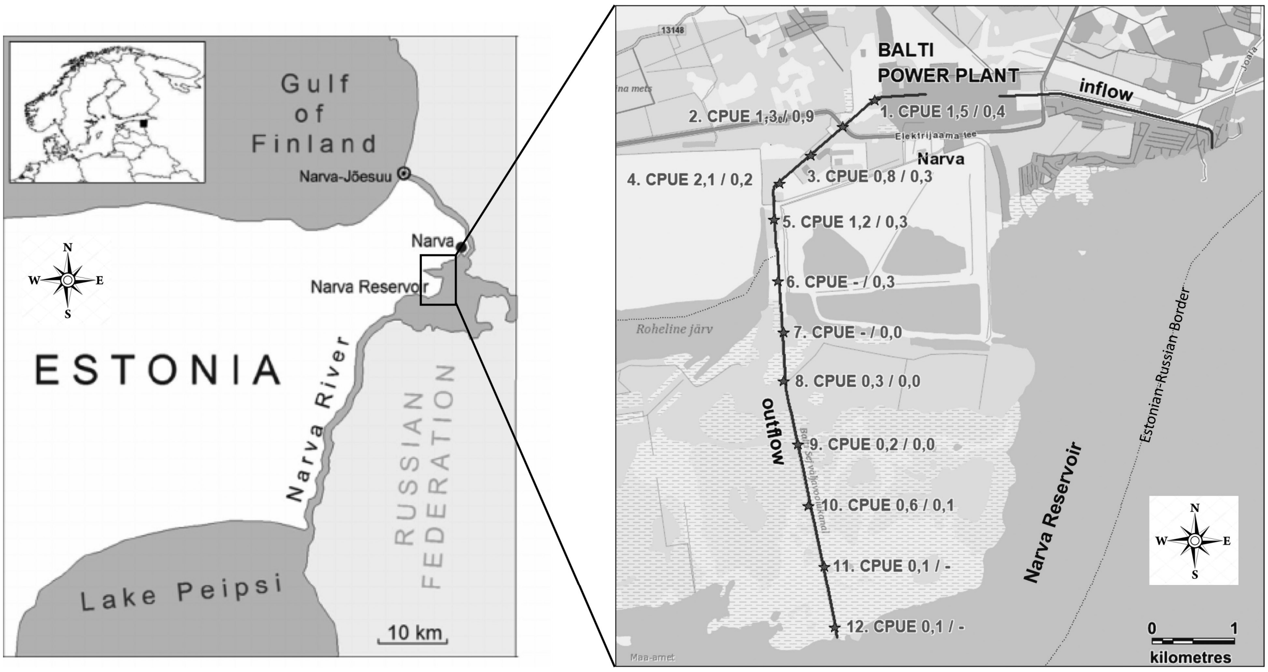

Figure 1. Fishing sites in the water system of Narva Reservoir. Stars indicate the 12 sites along the channel where test fishing was performed. In each site CPUE of sampling in May and June 2018 is reported, first and second values respectively. First record of the six marbled crayfish occurred in the site nine in 2017.

reproduction, high adaptability to new habitat and harsh environmental conditions (Kaldre et al. 2015; Veselý et al. 2017) along with irresponsible behavior of people involved in the aquarium pet trade who increase the risk of invasion (Chucholl 2014; Patoka et al. 2017), place this crayfish species among the potentially most dangerous freshwater invaders (Nentwig et al. 2018). Besides effects that marbled crayfish can have on native aquatic communities due to its high competitive abilities (Vodovsky et al. 2017), it can also severely affect native species by transferring the lethal crayfish plague disease, caused by an oomycete *Aphanomyces astaci* (Schikora, 1906). Number of records about spreading of marbled crayfish across the European countries and beyond (Jones et al. 2009; Kouba et al. 2014; Lőkkös et al. 2016; Patoka et al. 2016; Lipták et al. 2017) are alarmingly increasing. In this paper, we report the first record of invasive marbled crayfish in Estonia, where, together with the already existing populations of invasive signal crayfish *Pacifastacus leniusculus* (Dana, 1852), and spiny-cheek crayfish *Faxonius limosus* (Crandall and De Grave, 2017), it is likely to pose additional threats to native communities in the Estonian freshwater ecosystems, particularly to the native noble crayfish *Astacus astacus* (Linnaeus, 1758).

Materials and methods

Sampling

Field sampling of biota, including macroinvertebrates, was carried out in September 2017 in Narva Reservoir and in the outflow channel of the cooling system of the Balti Power Plant (Figure 1). When the sampled

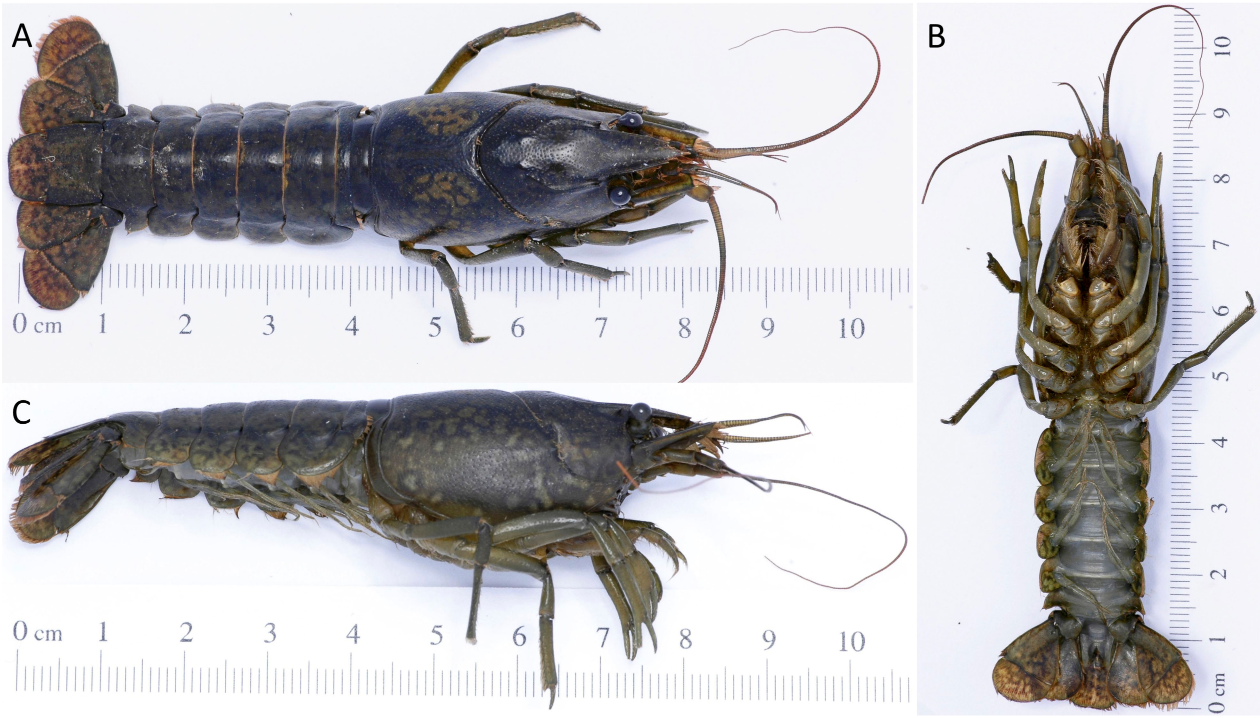

Figure 2. Marbled crayfish specimen found in cooling system channel of Narva Reservoir. Dorsal (A), ventral (B) and lateral view (C). Photos by Timo Ruokonen (A, B, C).

macroinvertebrates were identified in laboratory, six crayfish specimens (Figure 2) were found among samples from the middle part of the channel, which were identified as a marbled crayfish based on their morphological characteristics. Tissue samples were collected for subsequent DNA analyses of species identity confirmation and of the presence/absence test of the crayfish plague agent.

In May and June 2018, further sampling was carried out along the whole channel in order to assess population density of the newly detected non-native crayfish species (Supplementary material Table S1). One hundred cylindrical crayfish traps with 10 mm mesh size ("Mjärde Lini") were placed as separate lines consisting of 10 traps per line in each of the 12 sites selected in the channel (Table S1) and kept in the water overnight. Frozen cyprinid fish were used as bait. For every trapping session at each site, capture per unit effort (CPUE) was calculated per line, which was consisting of 10 traps each (except in the sites 4 in May and 5 in July where number of traps were 9 and 8, respectively) (Figure 1).

In addition to the Mjärde Lini traps, which predominantly capture adult crayfish, 20 artificial refuge traps (ART) (Green et al. 2018), which also catch small crayfish individuals, were used. The ARTs used in this study consisted of ten plastic tubes of 15 to 20 mm diameter and 120 mm long that were attached to a metal baseplate. Four test lines consisting of five ARTs each were placed into study sites 1, 3, 8 and 10 in May and kept in the channel for 20 days.

Genetic identification of marbled crayfish

The identity of the crayfish species was investigated by sequencing of a fragment of the mitochondrial gene for the cytochrome oxidase subunit I (COI) in six crayfish individuals found in 2017, using the universal primer pair LCO1490/HCO2198 (Folmer et al. 1994). A segment of leg of each captured crayfish was dissected to obtain muscle tissue, from which the genomic DNA was subsequently extracted using the NucleoSpin® Tissue kit (MACHEREY-NAGEL). PCR reactions (15 µl) contained 1× Type-it Multiplex PCR Master Mix (QIAGEN, Germany), 200 nM of forward and reverse primer, and ~ 50 ng of DNA template. The amplification program consisted of an initial denaturation step of 5 min at 95 °C, 35 cycles of 30 s at 95 °C, 90 s at 55 °C and 30 s at 72 °C, and a final extension for 30 min at 60 °C. The PCR products were purified using Exonuclease I (New England Biolabs) and Shrimp Alcaline Phosphatase (Thermo Fisher Scientific) and sequenced in both directions using the BigDye v. 3.1 Terminator kit (Thermo Fisher Scientific) and Applied Biosystems 3130xl Genetic Analyzer (Thermo Fisher Scientific). DNA sequencing sample files were assembled and trimmed for primer sequences using Variant Reporter Software 2 (Applied Biosystems). This resulted in 658 bp partial COI sequences that were aligned using the software BioEdit v7.2.5 (Hall 1999). Their similarity to sequences in GenBank nucleotide database was estimated using the NCBI nucleotide basic local alignment search tool (BLAST) (https://blast.ncbi.nlm.nih.gov/).

Detection of crayfish plague agent

The six marbled crayfish caught in 2017 were analysed for the presence of crayfish plague agent *A. astaci* by the quantitative TaqMan® MGB real-time PCR method using the protocol developed by Vrålstad et al. (2009) and an Applied Biosystems 7500 Fast Instrument. Two sub-samples were individually dissected from crayfish. The first tissue sample was dissected from the soft abdominal cuticle and the second from the uropod from each crayfish using sterile instruments. Tissues from each individual were placed in a single Eppendorf 1.5 mL tube in 96% ethanol and stored until analysis. DNA was isolated by NucleoSpin® Plant II kit. Based on their PCR forming units (PFU) values, samples were classified into semi-quantitative categories of pathogen load as proposed by Vrålstad et al. (2009).

Results

Sequencing of mitochondrial gene COI fragment confirmed the morphological identification of the crayfish captured in 2017 as a marbled crayfish. All obtained 658 bp COI fragments were identical to each other and either 100% (GenBank accession numbers LC228303, KT074364, KC107813, JF438007, HM358011, HM358010) or 99% (accession numbers KT074365, HM358012, HQ171456, HQ171455, HQ171453, HQ171458,

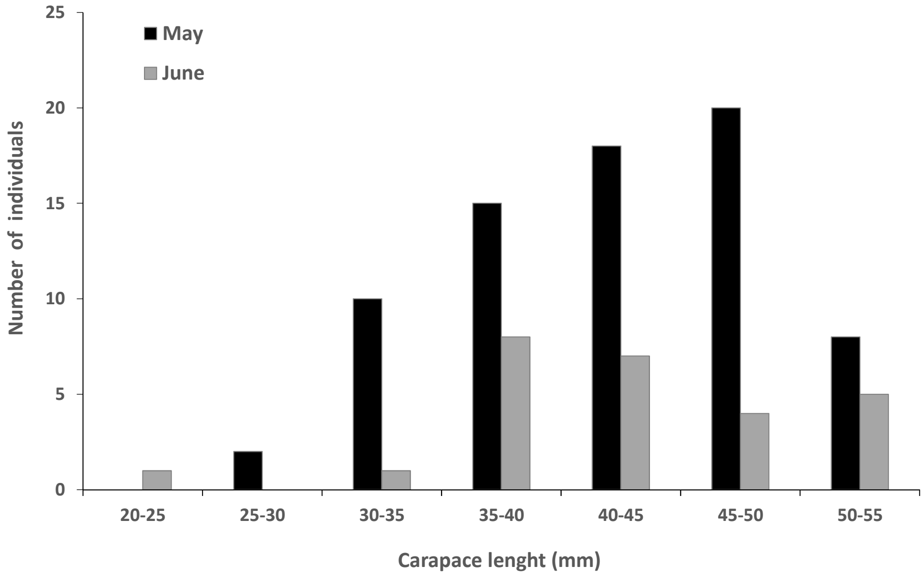

Figure 3. Size distribution as carapace length classes of sampled crayfish in May and June 2018 fishing sites.

HQ171454) identical to the sequences of *P. fallax*, the parental species of *P. virginalis,* in GenBank. According to Tulonen et al. (1998), the density of marbled crayfish population based on CPUE in the outflow channel of the Balti Power Plant, was considered sparse to moderate. In May 2018, 80 marbled crayfish between 23 to 54 mm in carapace length (CL: from the tip of the rostrum to the end of carapace) were caught (Figure 3) with a mean length of 40 mm ± 7 SD, while in June 2018 24 marbled crayfish between 21 to 51 mm in CL were caught (Figure 3) with a mean length of 42 mm ± 7 SD (Table S1). Eight individuals were longer than 51 mm in CL which makes them the largest marbled crayfish found in the wild so far. No specimens with eggs were found neither in May nor in June. With ARTs only two marbled crayfish were captured from sites 8, 10 with 21.4 and 30.5 mm CL respectively, and mean lenght of 25.9 mm ± 4.5 SD. However, six ARTs were lost. In this study, the use of ARTs proved to be not so effective as expected.

Based on their PCR forming units (PFU) values, samples from the soft abdominal cuticle of four marbled crayfish indicated the presence of crayfish plague agent *A. astaci* at a very low level (A1). Agent level of other samples was 0.

Discussion

After the first detection of six marbled crayfish in 2017, additional sampling in summer 2018 confirmed the presence of an established marbled crayfish population in the Balti Power Plant outflow channel in Narva Reservoir. Marbled crayfish is able to reproduce at small size due to the early maturation. Since traditional cylindrical traps are ineffective to catch small crayfish, artificial refuge traps (ARTs) were also used.

However, our results showed unexpectedly low CPUE of ARTs as only two crayfish were caught by 14 traps.

Higher CPUE from both May and June sampling were recorded in the first five study sites near to the Balti Power Plant (Figure 1 and Table S1), where likely warmer temperature and habitat were more suitable for crayfish. Other sites, more far from Power Plant, were less suitable most probably due to muddy bottom, lesser availability of shelter and colder water.

Outflow channel of cooling water system of electric power plant may indeed represent a particularly good habitat for marbled crayfish where water temperature registered in the period from autumn to spring was 8–10 °C higher than in the River Narva and Narva Reservoir, hence likely facilitating the establishment of marbled crayfish population. However, a study by Kaldre et al. (2015) indicated that marbled crayfish can survive even at low temperatures (below 6 °C for six months) and, thus, might be able to survive through the winter in North European countries. Therefore, we think that marbled crayfish might be able to survive not only in the warm water channel but also in the reservoir and/or in the river where water temperature is lower during the winter. Thus, further monitoring of the whole Narva water system is strongly warranted.

It is very likely that the marbled crayfish has been introduced into the Narva water system (river and reservoir) by humans. Natural immigration of this species is unlikely because there are no data about the presence of marbled crayfish in vicinity neither in Estonia nor in Russia so far (including nearby River Narva and Narva reservoir). The main way of introduction of marbled crayfish is the commercial aquarium trade (Uderbayev et al. 2017; Vodovsky et al. 2017). Until recently, marbled crayfish were also sold in aquarium shops in Estonia and through websites for aquarists. High parthenogenetic reproduction of marbled crayfish facilitates their overcrowding in aquariums, which may lead the owners to release the excess animals into local waterbodies. We may assume that this was the introduction pathway of the marbled crayfish population in the outflow channel of electric power plant in Estonia. As River Narva and Narva reservoir are Estonian/Russian border water bodies, this might have occurred either from Estonian or Russian side. Even though European Commission Regulations (EU 2016) prohibit breeding, commercial production, import and keeping of marbled crayfish in the European countries, the number of new records of marbled crayfish from EU countries is increasing quickly. It has been already reported in Italy (Vojkovská et al. 2014), Slovakia (Janský and Mutkovič 2010), Czech Republic (Patoka et al. 2016), Netherland (Kouba et al. 2014), Sweden (Bohman et al. 2013), Germany (Chucholl et al. 2012), Romania (Pârvulescu et al. 2017), Ukraine (Novitsky and Son 2016), Croatia (Samardžić et al. 2014), and Hungary (Weiperth et al. 2015; Lőkkös et al.

2016). However, according to the regulation, aquarium shops are allowed to exhaust their stock of invasive alien species of Union concern following the entry into force of the Regulation, so that the species could have been sold until 2018, potentially increasing any release in nature. A single release of just single marbled crayfish individual into nature might be the potential seed for a new population (Jones et al. 2009). We believe that more public awareness of potential negative effects of marbled crayfish on freshwater ecosystems is strongly needed, especially among people involved in the aquarium pet trade (Patoka et al. 2016), in order to avoid further uncontrollable spreading of this invasive crayfish species.

In the last ten years, two invasive crayfish species (signal crayfish and spiny-cheek crayfish) have been already reported in Estonia. The new record of another invasive crayfish species, the marbled crayfish, may bring further menace for the already threatened noble crayfish, the only native crayfish species in Estonia. The non-indigenous crayfish species may be the latent carriers of the crayfish plague, which has the most devastating effect on the noble crayfish stocks. In our study, four marbled crayfish individuals indicated a very low presence of *A. astaci* of the agent at level A1. According to Vrålstad et al. (2009), the animals with agent level 0 and 1 are considered uninfected. However, Vrålstad et al. (2009) noted also that symptom free carrier crayfish typically have much lower levels of agent DNA (from A1–A3) than infected native species. Therefore, the status of the marbled crayfish found in outflow channel of the Balti Power Plant as a carrier of *A. astaci* remains ambiguous and more extended studies are needed to confirm this.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Coordinates in Decimal Degrees (DD) of each sampling site in 2018 and its respective number of traps, number of marbled crayfish trapped and Catch Per Unit Effort (CPUE).

This material is available as part of online article from:
http://www.reabic.net/journals/bir/2019/Supplements/BIR_2019_Ercoli_etal_Table_S1.xlsx