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First record for an alien oriental river prawn Macrobrachium nipponense(De Haan, 1849) (Decapoda: Palaemonidae) distribution in the lower Danube, Bulgarian part confirmed with DNA barcoding

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First record for an alien oriental river prawn *Macrobrachium nipponense* (De Haan, 1849) (Decapoda: Palaemonidae) distribution in the lower Danube, Bulgarian part confirmed with DNA barcoding

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Abstract

The alien shrimp *Macrobrachium nipponense* (De Haan, 1849), known as the oriental river prawn, has been identified in the Bulgarian part of the Lower Danube for the first time, based on morphological characteristics and DNA barcoding. Four specimens were caught in October 2023 in the Danube River near Pozharevo island (422.2 rkm). The diagnosis of the species was based on the number of the teeth on the dorsal and ventral side of the rostrum, the large size of the second pair of pereopods, and the presence of the hepatic spine. A fragment of cytochrome c oxidase subunit I (COI) gene of mitochondrial DNA was sequenced to supplement species identification. Phylogenetic analysis has revealed a high genetic similarity (over 99%) between the Bulgarian sample and samples from various geographic locations (Ukraine, China, Bangladesh, Iran, Iraq, and Russia). The record of the oriental river prawn confirms its wide expansion and successful naturalization in the Lower Danube region. The invasion of the species will probably affect the structure of benthic macroinvertebrate communities and represent a high risk to native species, which fact imposes the need for long-term monitoring of species' impact on Danube River ecosystems.

Keywords: Danube River basin, invasive species, shrimp, palaemonids

Introduction

Climate change has amplified the threat posed by aquatic invasive species, which act as potential disruptors of biodiversity and ecosystem functioning (Nekrasova et al. 2024). In many cases, biological invasions result from intentional introductions, mainly related to commercially important species used in aquaculture (Thresher and Kuris 2004). Invasive species have become a major problem for ecosystems worldwide due to their impacts on native biodiversity and ecosystem functioning (Gallardo et al. 2015).

The oriental river prawn, *Macrobrachium nipponense* (De Haan, 1849), is a widely distributed Indo-Pacific species that inhabits estuaries and freshwater environments (Chen et al. 2017; Zheng et al. 2019). It is among the most widely cultured freshwater prawns in the world (Kurguru et al. 2019). Seven global centers of species distribution have been identified: the native range in East Asia, Northern, Western and Eastern Europe, the Irano-Turanian region, and North, and South America (Nekrasova et al. 2024). In Europe, introduction of this species

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have been recorded since the 1980s in Belarus, Moldova, and Ukraine (Vladimirov et al. 1989; Alekhnovich and Kulesh 2001). The prawns are increasingly found in European inland waters. Successful adaptation and favorable climatic conditions in recent years have contributed a significant rise in East Asian river prawn populations in the Danube River (Bushuiev et al. 2023). From 2019 to 2021, *Macrobrachium nipponense* has become a common component of the benthic fauna in the Danube Delta, spanning both its Ukrainian and Romanian regions (Son et al. 2013, 2020; Surugiu 2022; Zhmud et al. 2022).

The Danube River and its floodplain are considered an important part of the South-European Aquatic Invasion Corridor, linking the Black Sea Basin with the North Sea Basin via the Danube–Main–Rhine Canal, and forming a key part of the European invasion network (Panov et al. 2009, according to Trichkova et al., 2022). Invasive alien species (IAS) are recognized as one of the main threats to aquatic biodiversity in the Danube River basin (Csányi et al. 2021). Among various invasive species, freshwater decapods exhibit a particularly strong invasive potential (Holdich et al. 2009; Strauss et al. 2012; James et al. 2016). The Bulgarian section of the Lower Danube has long served as an entry point fornew species trough natural dispersal across borders and many invasive crayfish species have found suitable habitats and established abundant populations in the tributaries (Trichkova et al. 2017; Trichkova et al. 2022). These species can also spread (passively or actively, facilitated by human activities) into the inland waters of Bulgaria.

So far, two invasive species have been found in the Bulgarian section of the Danube River: the Chinese mitten crab, *Eriocheir sinensis* H. Milne Edwards, 1853, and the spiny-cheek crayfish, *Faxonius limosus* (Rafinesque, 1817) (Trichkova et al. 2017, 2022). The discovery by local fishermen in the Ruse region of large shrimp of an unknown species attacking their fish caught the interest of the local public, leading to publications on YouTube and the local press.

The aim of this paper is to describe the first record of the invasive shrimp *Macrobrachium nipponense* (De Haan, 1849) in the lower Danube River, in the Bulgarian section, based on morphological and molecular evidence.

Materials and methods

Sampling

Four shrimp specimens were collected on 22. 09. 2024 from two points on the Danube River at 422.2 rkm, near Pozharevo Island (44° 4' 14.92" N; 26^0 44’45.36’’E) during our ongoing project activities and observations. The collected specimens were immediately preserved in 96% ethanol for further morphological examination and molecular analysis. The sampling area (Figure 1) is characterized by a hard gravel bottom, without large stones, at a depth of up to 50 cm. It is overgrown with the underwater leaves of European Water Plantain (*Alisma plantago-aquatica*) in the shallower parts, while the deeper parts lack vegetation. The water is clear with a slow current.. Sampling was conducted along two transects, each 100 m long,covering 300 square meters per transect. The first transect was at a depth of 20-50 cm in a vegetated area,and the second was ata depth of 50–100 cm in an area without vegetation. Sampling was done using an electrofisher set (SAMUS-725G) with the following parameters: output voltage 550-600 V, volt-impulse aperiodic up to 1000 V, output power 650 watts, output frequency 65 Hz, output duration 30 microseconds, and a hand net with a 5 mm mesh. Short electrical pulses were applied every 5 meters, and the net was then used to scrape plant leaves for collecting the specimens.

Figure 1. Habitats of *Macrobrachium nipponense* on Pozharevo Iceland (Danube River).

Morphological measurements

The bogy length was measured using IP67 digital display Vernier calipers (Mitutoyo) with an accuracy of 0.01 mm.

DNA extraction and PCR amplification

A sample was preserved in 96% ethanol at 4 °C. Genomic DNA was isolated from the muscle tissue of the abdomenusing the NucleoSpin Tissue kit for DNA from cells and tissue (Machery-Nagel, Germany). A fragment of the COI (619 bp) gene was amplified through conventional polymerase chain reaction (PCR) using two primers: LCO1490 (5' GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al. 1994). The PCR was carried out in a reaction volume of 50 µl, containing 1 µl of each primer, 25 µl of the mastermix (MyTaqTM HS Mix), and 2 µl of the target DNA. The PCR conditions were as follows: 95 °C for 1 min, 95 °C for 1 min, 40 °C for 1 min, 72 °C for 1 min 30 sec (30 cycles), and a final extension at 72 °C for 7 min. Quality control of the PCR product was performed by electrophoresis on a 1% agarose gel. DNA sequencing was conducted by Macrogen Europe B.V. The obtained sequence was submitted to GenBank under the accession number PQ565563.

Phylogenetic analysis

For phylogenetic analyses, the sequence of the LD1 isolate was aligned with sequences of *Macrobrachium nipponense* from different geographic locations and of *Macrobrachium japonicum* and *Litopenaeus vannamei* (used as outgroups) obtained from the GenBank database (Clark et al. 2016) (Supplementary Table 1). Sequence alignment was conducted with MUSCLE (Edgar 2004) using default settings in MEGA X (Kumar et al. 2018). The final dataset used for phylogenetic analyses included 21 sequences (partial COI) with a total of 669 positions. All sites were used for analyses. Phylogenetic relationships were determined using the Maximum Likelihood (ML) method in MEGA X (Kumar et al. 2018) and the Bayesian Inference (BI) method in MrBayes v.3.2 (Ronquist et al. 2012). For BI, four Markov chain Monte Carlo (MCMC) chains were run for 1,000,000 generations, sampling every 100 generations, with the first 25% of burn-in trees discarded. Bootstrap support values for ML analyses were estimated using 1,000 replicates. The TN93+I substitution model was used for ML, selected based on the lowest Bayesian Information Criterion (BIC) scores with default settings in MEGA X (Kumar et al., 2018). For BI, the GTR+I model was selected based on the lowest Akaike Information Criterion (AIC) score using MrModeltest2 (Nylander 2004).

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The number of base differences per site (p-distances) among *Macrobrachium nipponense* sequences used for phylogenetic analyses was calculated using MEGA X (Kumar et al. 2018). All positions containing gaps were eliminated, resulting in a final dataset with a total of 579 positions.

Results

Based on morphological data, all four shrimp specimens were identified as the oriental river prawn, *Macrobrachium nipponense* (De Haan, 1849). The species diagnosis followed the descriptions provided by Cai and Ng (2002) and Zheng et al. (2019). The total body length of the captured specimens ranged from 78.14 to 94.11 mm, with an average of 86.79 mm. The thirth, fourth, and fifth pereiopods had simple dactylus. The second pereiopods were much longer than the other legs (Fig. 2A and D). A distinctive feature was the long second pereiopods, which exceeded the body length in larger adults and had claws bearing numerous setae (Fig. 2A and B). The rostrum was long and almost straight, with 12–14 dorsal teeth with feathery setae between them, including 3 teeth posterior to orbital margin and 2–4 ventral teeth (Fig. 2B and C). The carapace had a hepatic spine but lacked a branchiostegal spine. There were three pairs of antennae with the longest antennae exceeding the body length (Fig. 2A and D). The body of living specimens was almost translucent or light brownish.

Figure 2. Photographs of *M. nipponense:* A - general view, B- carapace, C - rostrum, D – lateral view.

The invasion of the species (size 8-10 cm) in the lower Danube (Bulgarian part) near Ruse, around 500 river km, was reported also on the social media (YouTube) in October 2023. Juveniles were caught in June 2024 near Pozharevo and Dunavetz, Tutrakan minusipality (personal communication with local fishermen).

No identical sequences were found in the GenBank database for the LD1 isolate (669 bp lenght). The sequence differed by only 1 bp from a strain from China (PP747057) and the two available sequences from Ukraine (MT881674 and MT881675), but these had shorter lenghts (629 and 632 bp). In the phylogenetic tree, *Macrobrachium nipponense* formed a monophyletic clade (ML 100%, BI 0.98) divided into two subclades (Figure 3). The *Macrobrachium nipponense* isolate LD1 from Bulgaria (Danube river) clustered with isolates of the species from various geographic locations, while the second subclade comprised specimens from Japan.

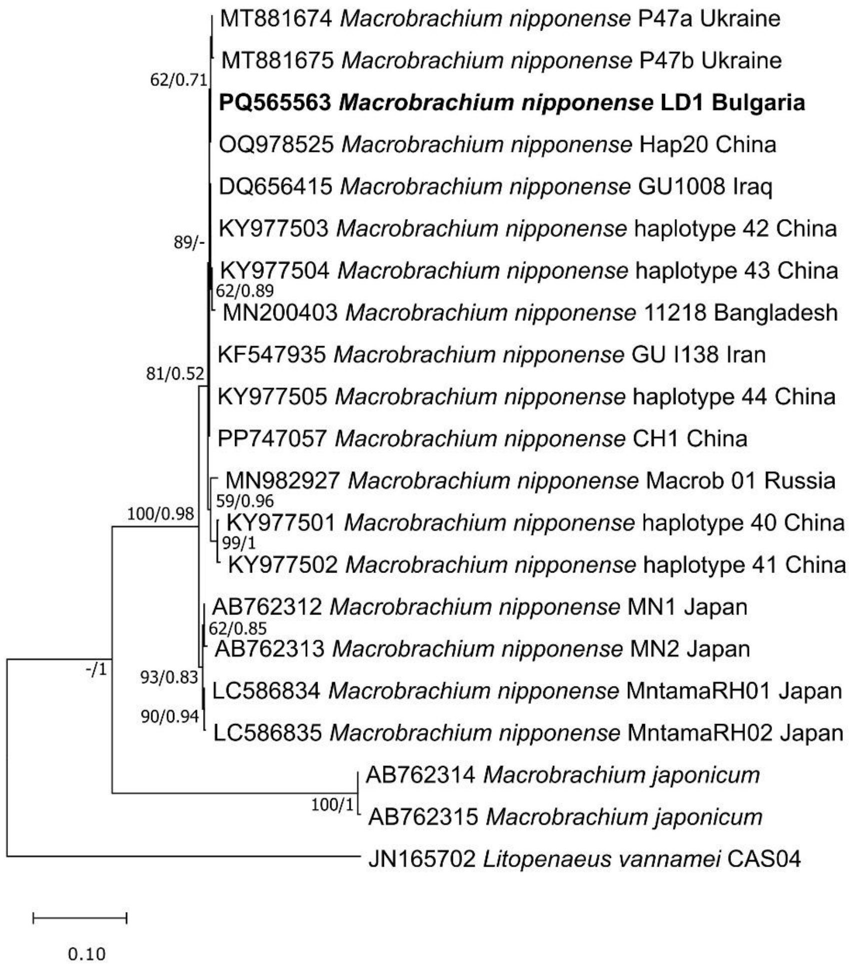

Figure 3. A Maximum Likelihood tree derived from a partial COI alignment including 18 sequences of *Macrobrachium nipponense*, and *Macrobrachium japonicum* and *Litopenaeus vannamei* serving as outgroups. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Node labels correspond to bootstrap values from the maximum likelihood method and posterior probabilities from the Bayesian inference analyses (ML/BI). The sequence from the current study is indicated in bold.

The genetic distances between the Bulgarian isolate (LD1) and the analyzed sequences of *Macrobrachium nipponense* (a total of 18 sequences, 579 bp) ranged from 0% to 2.3% (Supplementary Table 2). The greatest difference was observed with the isolates for Japan.

Discussion

Macrobrachium nipponense is native and broadly distributed throughout East Asia (i.e., China, Japan, Korea, Vietnam, and Myanmar) (Cai and Ng 2002). The species has been introduced into Singapore, the Philippines, Uzbekistan, Iraq, Russia, Belarus, Moldova, and Iran (Chong et al. 1987; Alekhnovich and Kulesh 2001; Mirabdullaev and Niyazov 2005; Cai and Shokita 2006; De Grave and Ghane 2006; Salman et al. 2006).

Since 2019, *Macrobrachium nipponense* has been part of the benthic fauna of the Danube Delta (Zhmud et al. 2022, Bushuiev et al. 2023) and, until now, has been reported up to Galați in the Danube upstream (Surugiu 2022). Over the five-year period from 2019 to 2023, the species spread up to 427 rkm (Pozharevo Island) and 500 rkm (Ruse), demonstrating its plasticity and invasion potencial. This spread can be attributed to speies' high tolerance to environmental conditions, fairly high fecundity, and mobile reproduction strategy (Mashiko and Numachi 2000). The temperature optimum for *M. nipponense* is 25–28°C, and it begins to reproduce at water temperatures above 20°C (Kulesh 2013). However, *M. nipponense* can also withstand low winter temperatures (2–4°C) for extended periods (De Grave and Ghane 2006). Additionally, it is important to consider the adaptive abilities of this invasive species to extreme conditions at the edge of its distribution range (Nekrasova et al. 2024).

Our study, based on morphological characteristics and molecular data, confirmed the expansion of the East Asian river prawn (*Macrobrachium nipponense*) in the lower Danube, Bulgarian part. Multiple records of adult specimens and the presence of juveniles in the sampling area (Pozharevo Island) suggest that it has likely established a large population here. Given the biological characteristics of *M. nipponense*, it can be expected to soon become a common species in the freshwater ecosystems of the Danube River and its tributaries.

The lack of identical sequences for the Bulgarian isolate (partial COI gene, 669 bp) in the GenBank database suggests it is a unique haplotype. Nevertheless, the sequence was highly similar to haplotypes from Ukraine and China, presuming an invasion pathway of this species from Ukraine to the lower Danube (Bulgarian part) and supporting the Chinese origin of the Ukrainian population (Son et al., 2020). This is further confirmed by the distinct subclade of sequences from specimens collected in Japan.

The most probable vector for its introduction into the Danube River Basin is a deliberate or accidental introduction from the Dniester Basin into fish farms near Sarata town (Zhmud et al. 2022). Estimating the potential impact of the oriental river prawn on the Danube ecosystem is challenging. However, it is associated with a high risk for native species and ecosystems, enhanceing competitive pressure on the native narrow-clawed crayfish, *Pontastacus leptodactylus* (Eschscholtz 1823), as well as on the entire community. The introduction of this invasive shrimp species will potentially affect the structure of benthic macroinvertebrate communities, as *M. nipponense* is a predator that feeds on various aquatic organisms (Afanasyev at al. 2020). Additionally, numerous populations of edible shrimp could alter the trophic preferences of some native fish species (De Grave and Ghane 2006).

In the context of global change, and especially biological invasions, the spread of *M. nipponense* poses a high risk to native species and ecosystems, making it necessary to monitor existing populations in the Danube River to prevent further invasion.

Conclusions

The oriental river prawn, *Macrobrachium nipponense*, was found for the first time in the Bulgarian part of the Danube River. Its entry occurred upstream from the Danube Delta, where the species has established a permanent population over the last five years. Most likely, the species will be established in a much larger part of the Bulgarian Danube basin and continue its invasion to west along the Danube River. Future comprehensive research is required to assess the invasive potential and ecological impact of this species. Long-term monitoring is needed to effectively manage *M. nipponense* populations already present in the Bulgarian part of the Danube River to mitigate their negative effects on native species and ecosystems.

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Author contributions

Sampling: YK, EV, KY, Formal analysis: PI, ND. Methodology: PI, ND, VR. Software: ND. Visualization: PI, ND, VR. Writing - original draft: PI, ND, VR. Writing - review and editing: PI, ND, VR. All authors contributed to the final version of the manuscript and approved the submitted version.

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