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Rapid spread of a new alien and potentially invasive species, *Clathrocaspia knipowitschii* (Makarov, 1938) (Gastropoda: Hydrobiidae), in the Danube River

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Abstract: We examined the spread and distribution in the Danube River of a new alien gastropod species, *Clathrocaspia knipowitschii* (Makarov, 1938) (Gastropoda: Hydrobiidae: Caspiinae). First findings of this species for Hungary, Slovakia, Romania, Bulgaria and Serbia are presented. *Clathrocaspia knipowitschii* was initially found in 2013 in the Iron Gate stretch of the Danube River at the border between Romania and Serbia. In 2019 and 2020, the species was found at several sites in the lower Danube in Romania, Serbia and Bulgaria, and also upstream in the middle Hungarian Danube in high population densities. The species appears to have spread along more than 800 km in six years. This finding together with the available abundance data indicates that *C. knipowitschii* is potentially an invasive species, but further observations are needed.

Keywords: freshwater snails; non-indigenous species; DNA metabarcoding; Danube

INTRODUCTION

The Danube is the second longest river in Europe. Originating in Germany (Black Forest), it flows southeast for about 2,850 km (1,770 mi), passing through ten countries and many settlements and industrialized areas [1]. It has been an important waterway for centuries, and together with the rivers Rhine and Main and the Rhine-Main-Danube Canal, it connects the Atlantic coast with the Black Sea. As a key navigation and water transport route, the Danube is exposed to significant hydromorphological degradation [2] and biological invasions [3,4]. Examples of alien species include *Potamopyrgus antipodarum*, *Theodoxus fluviatilis*, *Corbicula fluminea*, *Sinanodonta woodiana*, *Dreissena polymorpha*, *Dreissena bugensis* and *Physella acuta* in the past decades [5-12].

In this paper, the finding of a new alien hydrobiid species, *Clathrocaspia knipowitschii* (Makarov, 1938) (Gastropoda: Hydrobiidae: Caspiinae), in the Hungarian, Serbian, Romanian and Bulgarian stretch

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of the Danube is reported. Representatives of the family Hydrobiidae, including the subfamily Caspiinae, show high morphological variability [13,14], and identification based on traditional taxonomic characters is complex and often unreliable. Molecular analyses were used to confirm the species presence via DNA barcoding, a method of species identification using a short section of DNA from a specific gene or genes, metabarcoding, the term used when a sample containing DNA from more than one organism is barcoded, and environmental DNA metabarcoding.

MATERIALS AND METHODS

Material collection and sample types

Macroinvertebrate samples were collected in 2013, 2018, 2019 and 2020. In 2013 and 2019 sampling was performed at 68 and 51 sites, respectively, covering a 2,581-km-long stretch of the Danube, as well as 15 major tributaries and Danube's side arms (Supplementary Fig. S1). The investigations were organized within the Joint Danube Survey 3 (JDS3 in 2013 [2]) and the Joint Danube Survey 4 (JDS4 in 2019; http://www.danubesurvey.org/jds4/; [15]), coordinated by the International Commission for the Protection of the Danube River (ICPDR), supported by the European Commission and Danubian Countries and with participation of more than 140 laboratories. In 2018, two samplings (June and September) were performed at the Iron Gate (Đerdap) stretch (Kladovo settlement, river km (rkm) 933) in habitats where the presence of *C. knipowitschii* was expected, based on the 2013 survey. Additionally, to better understand the habitat preference of this species, more detailed investigations were performed in the second part of 2019 and during 2020 along the stretch upstream of Budapest (Göd section, subsites 1,671.5-1,668 rkm and Zebegény site, rkm 1,704), where an abundant population of *C. knipowitschii* was detected in 2019.

Four methods were used for collection: (i) dredging with a triangle-shaped dredge in deeper zones (Supplementary Fig. S2; methodology described in [16,17]; (ii) multihabitat sampling [18]; (iii) kick-and-sweep sampling [19]; (iv) detailed visual search and hand collection. Using such a detailed sampling procedure assured the best possible samples to assess the distribution of *C. knipowitschii* and its habitat preferences and to achieve confident data comparability since changing water levels and discharge greatly influence the sampling efficiency, especially in large rivers [19,20].

The position of sampling sites was recorded with a GPS device, while water depth was measured by hydro-acoustic equipment. The dominant substrate type was assessed visually. Immediately after sampling and the removal of larger debris, the samples were preserved in undenatured 96% ethanol at a final concentration of >90% [21]. Samples for DNA metabarcoding analyses were collected in the littoral zone of the river following the multihabitat sampling technique [18].

Sampling equipment was cleaned before each sampling with 1% Virkon™ S solution by exposing it for a minimum of 30 min to the decontamination liquid. The decontaminated equipment was rinsed with clean water to remove any remains of Virkon™ S prior to use in the field.

Traditional taxonomical identification

Identification of the individuals to the genus and species level was performed as described [13,14,23]. Specimens were photographed using a Nikon-SMZ 800N stereomicroscope with a Nikon-DS-Fi2 camera.

DNA-based taxon identification

DNA-based taxon identification involved three procedures: (i) DNA barcoding by mitochondrial cytochrome oxidase subunit 1 (COI) analysis of selected individuals previously identified using morphological characters; (ii) DNA metabarcoding analysis of homogenized bulk macroinvertebrate sample; (iii) environmental DNA (eDNA) metabarcoding from the preservation liquid used for macroinvertebrate sample preservation.

To confirm the identity of the Danube populations, the standard barcoding DNA marker, a fragment of the COI gene of two Danube populations, was compared with that of verified *C. knipowitschii* from the Lower Dnieper River in Ukraine. DNA was isolated and the COI marker was amplified and sequenced according

to different protocols established by different work groups of the team of authors. Sequences Cakn5359 and Cakn5361, CasZ-1 to CasZ-4 according to Turóci [24] and CasK-1 and CasK-2 according to Weiss and Leese [25]. The sequences are deposited in GenBank.

For DNA metabarcoding of bulk macroinvertebrate samples, up to 1,000 specimens were randomly subsampled and homogenized to a fine powder before extracting DNA using a magnetic bead-based extraction protocol. In the process of subsampling specimens, mollusks were sorted from other invertebrates for separate processing. Two-step PCR was carried out using the primer pair BF3/BR2 [26]. For mollusks, a modified version of the BF3/BR2 primer pair with higher primer degeneracy was used to minimize the potential of false negative results caused by primer mismatch. Two extraction replicates per sample were used, separately amplified and sequenced. After sequencing, bioinformatics analyses were performed, involving quality filtering of the retained sequencing reads and clustering into molecular operational taxonomic units (MOTUs) of 97% sequence similarity, as well as the initial taxonomic assignment of MOTUs using BOLDigger [27] and the barcode of life reference sequence database (BOLD). Species names were assigned to MOTUs in cases where at least one published barcode sequence with a similarity of ≥97% was present in the database.

The third method involved macroinvertebrate preservation liquid environmental DNA metabarcoding of macroinvertebrate samples preserved with undenatured 96% ethanol. A volume of 250 mL of preservation liquid was filtered using a 0.45-µm cellulose nitrate membrane filter. DNA captured on the cellulose filter was extracted using a modified salt precipitation protocol and amplified in a two-step PCR protocol using the degenerate PCR primer pair fwh2n and EPTDr2n, used to minimize the amplification of non-invertebrate taxa [28]. Every sample was amplified in two PCR replicates in the first step and pooled prior to the second PCR reaction.

The protein-coding mitochondrial COI sequences were aligned using the program ClustalW with the parameters provided in the software package MEGA [29], ver. 7.0. The maximum likelihood (ML) tree was obtained using MEGA 7 software. Comparison of the obtained sequences with sequences in GenBank was performed using the basic local alignment tool (BLAST), available at http://www.ncbi.nlm.nih.gov. The robustness of the tree was assessed using a bootstrap analysis with 1,000 replicates.

Data processing

Relative abundance was assessed based on the number of individuals per sample. This assessment method was previously used to estimate the relative abundance of freshwater snails of the genus *Theodoxus* [22]. The relative scale was used to provide comparability of abundances between sites, since different methods of material collection have been applied.

RESULTS

Clathrocaspia knipowitschii was recorded at seven sampling sites (Table 1) belonging to the Middle (Hungarian stretch) and Lower Danube (stretch shared by Serbia, Romania and Bulgaria). The abundance data, individuals per sample and relative abundance, bottom habitat characteristics of localities, as well as notes on the water level at the time of sampling are presented. The first record of this new snail species for the Danube River came from two sites in the Lower Danube stretch during the JDS3 in 2013 [2] – Kladovo (Vrbica-Simian cross section at 926 river km (Supplementary Fig. S3A), and the second one downstream of Kozloduy (686 rkm cross section). Based on the material from 2013, several live specimens of *C. knipowitschii* were identified using morphological characters. Although detailed sampling was performed along the shore region using multihabitat and kick-and-sweep methods, as well as visual survey, *C. knipowitschii* was detected in 2013 only in dredge samples at a depth ranging between 3.6 and 12 m (Table 1). At the time of sampling, the water level was high, following a rise of about one meter in the period before the sampling (72 h). Resampling of *C. knipowitschii* was performed in 2018 (June 5) at the site Kladovo (rkm 933), seven km upstream from the position where the snail was detected in 2013. This time, three specimens of *C. knipowitschii* were discovered in the shore region at a depth of 40 cm on the lower surface of stones (Supplementary Fig. S3B). At the same site (Kladovo), 28 snail individuals were collected in

Table 1. Location, date, geographic coordinates and individual collectors of *C. knipowitschii* along the Danube.

Site name	Date	Coordinates (y; x)	No. of Individuals	Ab	Depth (m)	Substrate	Note	Legator
Lower Dnieper, Kherson	05.05.2005	46.6441; 32.6539	3	2	3.5	U		TA
d/s Kladovo (Vrbica-Simian, 926 rkm)	10.09.2013	44.6008; 22.6981	86	7	4.2	A	recent change of the water level	BCS, MP, JSZ
d/s Kladovo (Vrbica-Simian, 926 rkm)	10.09.2013	44.6030; 22.7006	11	3	6.7	A	recent change of the water level	BCS, MP, JSZ
d/s Kladovo (Vrbica-Simian, 926 rkm)	10.09.2013	44.6053; 22.7022	13	3	12.0	A	recent change of the water level	BCS, MP, JSZ
d/s Kladovo (Vrbica-Simian, 926 rkm)	10.09.2013	44.6073; 22.7040	25	3	10.1	A	recent change of the water level	BCS, MP, JSZ
d/s Kozloduy (686 rkm)	13.09.2013	43.7482; 22.8795	1	1	3.6	B	recent change of the water level	BCS, MP, JSZ
Kladovo (rkm 933)	05.05.2018	44.6127; 22.6168	3	2	0.4	B	low and stable water level	BCS
Kladovo (rkm 933)	26.09.2018	44.6127; 22.6168	28	5	0.3	B	low and stable water level	BCS, MP
Medve/Medvedov*	01.07.2019	47.7895; 17.6598	N/A	N/A	1.2	A	recent change of the water level	MO
Gönyű*	01.07.2019	47.7426; 17.8440	N/A	N/A	1.2	A	recent change of the water level	MO
Gönyű (Slovakian side)	01.07.2019	47.7427; 17.8239	12	3	2.6	A	recent change of the water level	JSZ, BCS
Zebegény, 1704 rkm	29.08.2019	47.8031; 18.9058	5	2	3.7	A	recent change of the water level	BCS, JSZ
Zebegény, 1704 rkm	27.01.2020	47.8022; 18.9069	35	5	1.6	A	low and stable water level	BCS
Göd, Felsőgöd, Duna Csárda	04.04.2020	47.7111; 19.1288	14	3	0.70	A	stable water level	BCS
Göd, u/s Gödi Island	06.04.2020	47.6927; 19.1282	17	3	0.30	A	stable water level	BCS
Göd, between 2 groynes	29.11.2020	47.6888; 19.1260	82	7	0.20	A	stable water level	BCS
Göd, Alsógöd	29.11.2020	47.6805; 19.1259	1	1	0.30	B	stable water level	BCS
Göd, Alsógöd, 11th road	04.12.2020	47.6754; 19.1237	2	2	0.20	B	stable water level	BCS
Göd, between 2 groynes	09.12.2020	47.6888; 19.1263	7	3	0.20	B	stable water level	BCS
Göd, between 2 groynes	09.11.2020	47.6888; 19.1261	3	2	0.10	B	stable water level	BCS

Leg. names: TA – Tatiana Alexenko; BCS – Béla Csányi, MP – Momir Paunović; JSZ – József Szekeres; MO – Miroslav Očadlik; asterisks denote results of bulk sample DNA analyses; thus quantification was not possible; N/A – not applicable; Ab – relative abundance at site according to scale presented in Supplementary Table S3; substrates U – unknown; A – coarse sand, gravel, pebbles and small stones; B – coarse sand, gravel and pebbles.

Scale for relative abundance estimation.

Relative abundance	Description	Number of individuals per sample
1	very rare/individual findings	1
2	low abundance	2-5
3	moderate abundance	6-30
5	high abundance	31-60
7	very high abundance	61-100
9	mass occurrence	>100

September 2018, also in the shore region, noted by visual observation from a depth of 30 cm. The water level in both periods of sampling in 2018 was low and stable for a few weeks before sampling (with minor oscillations of several cm). In June 2019, the species was found by dredging in the Middle Hungarian Danube, at site Zebegény (5 individuals) at a depth of 5 m. One month later, in July 2019, the snail was found again at Gönyű during the JDS4 survey [15], JDS4 site 18 in the Upper Hungarian (Slovakian-Hungarian) Danube

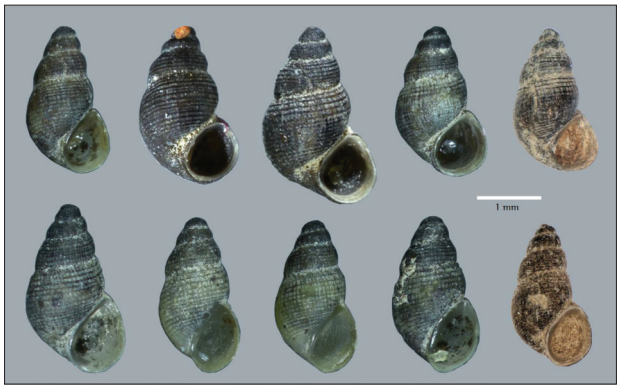

Fig. 1. Two individuals (right) from Kladovo, June 2018 (Photo by Arne J. Beermann) and eight individuals of *C. knipowitschii* from Zebegény, January 2020. Scale bar: 1 mm (Photo by Zoltán Fehér).

section by dredging at a depth of 1.2 m. In subsequent studies, *C. knipowitschii* was detected at several locations along a 5-km-long stretch of the Danube near the settlement Göd (upstream of Budapest) in January, April, November and December 2020, often in high individual abundance (Table 1). The snail was found at different depths, mostly along shore areas up to a depth of 70 cm. The relative abundance of *C. knipowitschii* at sites in the Danube ranged from several individuals to 86 individuals in the sample, or from 1 individual finding to 7 (very high abundance) according to the scale of relative abundance (Table 1), with moderate abundance (3) being the most common (according to the scale for relative abundance estimation (Table 1).

Based on our observations from different sampling locations, we concluded that the species prefers riverbed habitats characterized by coarse sand, gravel or pebbles. The species occurs at highest density at riverbed habitats paved with small stones, and the species was not detected at locations characterized by mud or fine sand (Supplementary Fig. S4). The collected specimens varied with respect to shell morphology within a population (Fig. 1), rendering the commonly used height-width ratio [23] an uninformative character for identification of this species.

Identification of specimens by DNA barcoding

To obtain reliable identification, individual snails collected at sites Kladovo (in 2018) and Zebegény (in 2019) were subjected to COI analysis. The eight new sequences could be separated into three haplotypes. Kladovo (CasK-1 and CasK-2), two Kherson (Cakn5359 and Cakn5361) and two Zebegény (CasZ-3 and CasZ-4) specimens bear the same haplotype, whereas the other two Zebegény specimens (CasZ-1 and CasZ-2) differed by one base pair each. The ML tree for the 14 specimens shows the position of the three haplotypes of *C. knipowitschii* in relation to other hydrobiid species (Table 2, Fig. 2).

Following a recently published review of the Caspiinae [14], the shells from Kherson represent typical *C. knipowitschii*. Therefore, the COI haplotype that characterizes Cakn5361 and Cakn5359 isolates can be considered as the barcode of this species. The occurrence of the species was also confirmed by DNA bulk sample analyses at an upstream station near Medve/Medvedov during the JDS4 2019 sampling program – the analyses resulted in identical sequences of 418 bp. The species was not detected by eDNA metabarcoding of the preservation liquid used for sample preservation (2019, JDS4 survey).

DISCUSSION

The first detection of *C. knipowitschii* in the Danube was reported in 2013 within the frame of the Joint Danube Survey 3 international expedition [2]. The species was confirmed at five additional sites, including those located 800 km upstream of the site of first occurrence (2019/2020 – Middle Danube, Hungarian and Slovak stretch). Having in mind the intensive investigations along the Danube prior to the first finding [2,4,30,31], the species was either not present before 2013, or its abundance was low and thus it escaped detection.

Little is known about the autecological characteristics of species belonging to the subfamily Caspiinae [14]. *Clathrocaspia knipowitschii* has been previously considered as a 'highly endangered' brackish water species [32] endemic to the Dnieper River [14]. Based on our data, the species prefers riverbed substrates consisting of coarse sand, gravel, pebbles and stones. Most probably, stones provide shelter from hydrological stress (drifting) and predators. *Clathrocaspia knipowitschii* has been found at different depths, ranging from several cm up to 12 m, but it occurs at highest

Table 2. Hydrobiid specimens included in the DNA sequence analyses, locality information, GenBank accession numbers and references for published sequences

No.	Taxon / Code	Locality	GenBank Accession # COI
	Subfam: Caspiinae		
1	*Clathrocaspia knipowitschii* (Makarov, 1938) / Cakn5359	Lower Dnieper, Kherson, Ukraine	MW385256 (Present study)
2	*Clathrocaspia knipowitschii* (Makarov, 1938) / Cakn5361	Lower Dnieper, Kherson, Ukraine	MW385257 (Present study)
3	*Clathrocaspia knipowitschii* (Makarov, 1938) / CasK-1	Danube, d/s Kladovo, Serbia	MW385254 (Present study)
4	*Clathrocaspia knipowitschii* (Makarov, 1938) / CasK-2	Danube, d/s Kladovo, Serbia	MW385255 (Present study)
5	*Clathrocaspia knipowitschii* (Makarov, 1938) / CasZ-1	Danube, Zebegény, Hungary	MW385250 (Present study)
6	*Clathrocaspia knipowitschii* (Makarov, 1938) / CasZ-2	Danube, Zebegény, Hungary	MW385251 (Present study)
7	*Clathrocaspia knipowitschii* (Makarov, 1938) / CasZ-3	Danube, Zebegény, Hungary	MW385252 (Present study)
8	*Clathrocaspia knipowitschii* (Makarov, 1938) / CasZ-4	Danube, Zebegény, Hungary	MW385253 (Present study)
	Subfam: Belgrandiinae		
9	*Belgrandiella kusceri* (Wagner, 1914)	Spring of Rakek, Slovenia	JX970610 [37]
	Subfam: Islamiinae		
10	*Islamia pallida* Arconada & Ramos, 2006	Spring in Patones de Abajo, Madrid, Spain	MH350230 [38]
11	*Fissuria boui* Boeters, 1981	Spring near La Prouveresse, Alpes, France	AF367654 [39]
	Subfam: Hydrobiinae		
12	*Hydrobia acuta* (Draparnaud, 1805)	Laguna di Orbetello, Toscana, Italy	AF213344 [40]
	Subfam: Pyrgulinae		
13	*Laevicaspia* sp.*	Lower Dnieper, Kherson, Ukraine	EF379295 [41]
14	*Pyrgula annulata* (Linnaeus, 1767)	Lake Garda, Brescia, Italy	AY341258 [41]

*[36]

abundance in the zone just below the annual minimum water level. Therefore, sampling success strongly depends on the selection of suitable methods, the hydrologic conditions and the optimal depth of sampling.

In the DNA metabarcoding analyses of bulk samples, *C. knipowitschii* was detected at two out of 46 sites only (Medve/Medvedov and Gönyű). In DNA metabarcoding of homogenized bulk samples there is a negative bias towards species of low biomass [33], which most likely contributed to the species not being detected by eDNA metabarcoding from the fixative used for sample preservation (2019, JDS4 survey). Further, for DNA-based analysis, only 1,000 specimens per site were used, and thus rare or small taxa might have been missed. These potential issues that reduce confidence of the analyses can be addressed by the selection of an optimal sampling procedure (taking samples from shallow as well as deep habitats), separating small from large organisms prior to DNA metabarcoding analyses and by processing all and not only a subset of the sample.

The lack of *C. knipowitschii* eDNA metabarcoding records can be additionally attributed to the primers' abilities

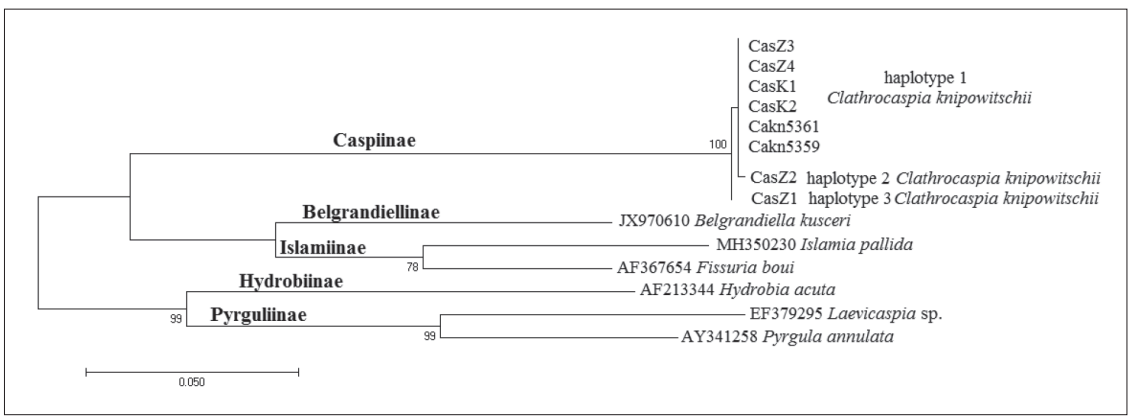

Fig. 2. Phylogenetic trees based on mitochondrial COI gene, obtained using the Maximum Likelihood (ML) method; bootstrap values are indicated below the branches; scale bar indicates the number of substitutions per site.

(the fixative primers have a negative bias towards underrepresented taxa), but also the characteristics of the species of concern, thus species with an external hard skeleton, such as freshwater snails, were previously found to be underrepresented [34].

Caspia milae Boeters, Glöer & Georgiev, 2015 from Vardim Island (Bulgaria) has been described [23] based in part on minor morphological differences as well as the large geographical distance and presumed isolation from its congeners. No fundamental differences from *C. knipowitschii* in terms of shape, size and sculpture were found [36], and therefore their identity is uncertain; *C. milae* as the synonym of *C. knipowitschii* with a question mark is noted [14].

We show that already in 2013, the geographic range of *C. knipowitschii* extended far beyond the Middle Bulgarian Danube section. Although not confirmed by DNA barcoding thus far, the lack of any geographical isolation is another strong argument that the Bulgarian population at the Vardim Island is conspecific with *C. knipowitschii*.

Molecular taxonomic methods (DNA barcoding) have been used extensively to complement morphological approaches for species identification and for establishing phylogenetic relationships. An integrative method of classification that includes morphological, molecular, and distributional data is an essential prerequisite for understanding the cryptic and small freshwater snails [35]. Our study demonstrates that a combination of different DNA barcoding methods provides more reliable results, concerning species identification and distribution ranges.

CONCLUSIONS

The JDS4 revealed that this snail species, detected in 2013 (JDS3) in the Lower Danube, populates a large section along the Middle Danube as well. This is another example demonstrating that our knowledge about the nature of biological invasions is limited, as a taxon that was considered a 'highly endangered' brackish water species less than a decade ago has rapidly spread outside its native range. Further, our study demonstrates that a combination of different sampling techniques and detection methods is the most reliable approach to study the distribution of a macroinvertebrate taxa group in large rivers, specifically freshwater mollusk species that have a small size and specific distribution within aquatic habitats. The combination of traditional identification based on morphological characters and molecular methods allows for confident recognition of taxa and consequently contributes to resolving taxonomical issues. The results of all JDS missions confirmed that the Danube harbors several non-indigenous species and is a suitable recipient area for bioinvasion.

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Conflict of interest disclosure: The authors declare no conflict of interest.

Data availability: Data underlying the reported findings are available at: GenBase https://www.ncbi.nlm.nih.gov/genbank/ as follows: Ukraine, Lower Dnieper, Kherson, Isolates Cakn5359 and Cakn5361, COI Gene Bank numbers MW385256 and MW385257; Serbia, Danube, d/s Kladovo, CasK-1 – MW385254 and CasK-2 – MW385255; Hungary, Danube, Zebegény, CasZ-1 – MW385250, CasZ-2 – MW385251, CasZ-3 – MW385252, CasZ-4 – MW385253. Details on the JDS4 investigation, including sampling sites data and data on DNA barcoding results, are available at: http://www.danubesurvey.org/jds4/, and at: https://jds4.icpdr.org/portal/ (on request from the ICPDR Secretariat, Vienna International Centre, Room D0412, Wagramer Strasse 5, A-1220 Vienna, Austria, e-mail: secretariat@icpdr.org; tel.: +431 260 60 5738).

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Supplementary material

The Supplementary Material is available at: https://www.serbiosoc.org.rs/NewUploads/Uploads/Paunovic%20et%20al_7492-Supplementary%20Material.pdf