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## The first records of *Niphargus hrabei* and *N. potamophilus* in Ukraine and Bulgaria significantly enlarge the ranges of these species

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### Abstract

*Niphargus* comprises hundreds of narrowly-endemic West Palaearctic subterranean taxa. However, a few exceptional species inhabit surface waters and have remarkably large ranges. Herein, based on morphological and molecular analyses, we provide important new records for two of these species. *N. potamophilus*, previously known from the eastern Azov Sea lowlands, is reported for the first time from Ukraine and Bulgaria from localities adjacent to the Black Sea. These findings expand its range westward by more than 1000 km along the coastline. From Bulgaria, we also report for the first time *N. hrabei*, a species previously known to occur along the middle and lower Danube lowlands and in isolated populations at the foothills of the Northern Caucasus. Our new record thus extends its range southwards by more than 150 km. Both species contained unique haplotypes at all of the sampled localities. These were, nevertheless, not very divergent from more distant populations, emphasizing their good dispersal ability. Ecologically, the sampling localities were generally characterized by stagnant to low running water, dense vegetation, and muddy substrate. Overall, our results bring important insights, shedding more light on the biogeography and ecology of *Niphargus*.

**Keywords:** *Amphipoda*, *epigean*, *Black Sea*, *biogeography*

### Introduction

The genus *Niphargus* Schiödte, 1849 is characterized by high species diversity (>400) which is distributed across Europe and West Asia (Horton et al. 2022). Although most species have small ranges (<200 km), some taxa show a wide distribution (Copilaş-Ciocianu et al. 2017). However, many of the large ranged species turned out to be complexes of cryptic species with smaller ranges (Lefébure et al. 2006, 2007; Trontelj et al. 2009). Most *Niphargus* live in hypogean (subterranean) waters (Fišer 2012), but few inhabit the ecotone zone between the hypogean and epigean (surface) realms (Straškraba 1972; Copilaş-Ciocianu et al. 2014; Copilaş-Ciocianu et al. 2017).

Remarkably, a few species of *Niphargus* are known almost exclusively from epigean habitats, and they also have the widest known ranges of any other niphargid, despite being blind and depigmented (Copilaş-Ciocianu et al. 2017). The best known are *Niphargus valachicus* Dobreaanu & Manolache, 1933 and *N. hrabei* S. Karaman, 1932, both inhabiting the middle and lower Danube lowlands as well as being occasionally reported around the Black Sea lowlands at the foothills of the Caucasus or the Anatolian Peninsula (Cărauşu et al. 1955; Nesemann et al. 1995; Copilaş-Ciocianu et al. 2014; Copilaş-Ciocianu et al. 2017; Palatov & Marin 2021). Although both are similar from a morphological, ecological and biogeographic

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perspective, they are not closely related (Copilaş-Ciocianu et al. 2018; Palatov & Marin 2021).

*Niphargus potamophilus* (Birstein 1954) is another lowland species that inhabits similar habitats and has a similar morphology to *N. hrabei* and *N. valachicus*, being closely related to the latter (Birstein 1954; Palatov & Marin 2021). These species have trapezoidal gnathopods, acutely produced infero-posterior corners of epimeres 2 and 3, additional spines on pereopod 3–4 dactily, non-sexually dimorphic 3rd uropod and a spoon-shaped appendix on the distal end of the uropod 1 peduncle. *Niphargus potamophilus* was originally described based on the specimens collected from the Don River delta, from a floodplain reservoir nearby Rostov-on-Don City, and from fish farms in Atkhyr, Kuban River delta, ca. 300 km southwest from the Don delta (Birstein 1954). The recognized range of this species is much smaller than that of *N. hrabei* and *N. valachicus*, for now being reported along the eastern Azov Sea shore (Palatov & Marin 2021), but it was suggested that its distribution might be much wider, covering southern Ukrainian rivers (Birstein 1954).

*Niphargus hrabei*, *N. potamophilus* and *N. valachicus* are epigeal species, rarely, if at all, being reported from groundwater (Karaman 1950; Dedju 1967; Sket 1981; Meijering et al. 1995; Nesemann et al. 1995; Copilaş-Ciocianu et al. 2014; Copilaş-Ciocianu et al. 2017; Palatov & Marin 2021). Only *N. hrabei* is known to have a handful of stable subterranean populations (Dudich 1941; Meijering et al. 1995; Pérez-Moreno et al. 2017). All three species inhabit the stagnant/slow-flowing, densely vegetated, eutrophic waters with a muddy substrate of lowland springs, streams, canals, rivers, ponds, lakes, and temporary water bodies (Nesemann et al. 1995; Copilaş-Ciocianu et al. 2014; Copilaş-Ciocianu et al. 2017; Palatov & Marin 2021).

In the present study, we report *N. potamophilus* and *N. hrabei* from new territories and river basins in

Bulgaria and Ukraine. Our combined molecular and morphological approach confirmed that these species are more widespread than previously known.

## Material and methods

### Sampling

Specimens were collected with a hand net during sampling campaigns in Ukraine in 2018 and in Bulgaria in 2014–2018 (for details see Table I). Animals were fixed in 96% ethanol. The material is stored in the collection of invertebrates at the Department of Invertebrate Zoology and Hydrobiology (University of Lodz, Poland) and Nature Research Center (Lithuania).

### DNA extraction, amplification, and sequencing

Samples were processed either at the University of Lodz (Poland) or at the Nature Research Centre (Lithuania) (see Supplemental Table SI for details). For samples analyzed at the University of Lodz, total DNA was extracted from the leg of each specimen using the Chelex procedure (see Casquet et al. 2012, Morhun et al. 2022 for details). For samples analyzed at the Nature Research Center DNA was extracted from the dorsal side using the Quick-DNA Miniprep Plus Kit (Zymo Research) (see Copilaş-Ciocianu et al. 2022 for details). The standard animal DNA barcode gene region (COI) (Hebert et al. 2003) was amplified using the primers LCO1490, 5'-GGTCAACAAATCATAAAGATAT TGG-3' and HCO2198, 5'-TAAACTTCAGGGT GACCAAAAATCA-3' (Folmer et al. 1994) with the PCR protocol described by Hou et al. (2007) or with LCO1490-JJ, 5'-CHACWAAYCATAAAGAT ATYGG-3' and HCO2198-JJ, 5'-AWACTTTCVGG RTGVCCAAARAATCA-3' (Astrin & Stüben 2008) with the PCR protocol described by Copilaş-Ciocianu

Table I. New localities and GenBank accession numbers of specimens were used for the current study.

Species	Code	Locality	N	Country	Lat., Long.	Date	Acc. no. COI
<i>Niphargus hrabei</i>	BS16	Tankovo	4	Bulgaria	42.701, 27.649	25.05.2018	ON134484, ON134488 - ON134490
<i>Niphargus potamophilus</i>	BG26	Tankovo	5	Bulgaria	42.701, 27.656	20.09.2014	ON134482, ON134483, ON134486, ON134491, ON134492
<i>Niphargus potamophilus</i>	BS12	Ezerets Lake	2	Bulgaria	43.587, 28.559	24.05.2018	ON134480, ON134487
<i>Niphargus potamophilus</i>	A08b	Dnipro delta, Stara Zburivka, spring	1	Ukraine	46.462, 32.357	01.05.2018	ON134481
<i>Niphargus potamophilus</i>	0038c	Mayaki Village, floodplains	1	Ukraine	46.412, 30.263	21.04.2018	ON134485

et al. (2022). PCR products (5 µl) were cleaned up with Exonuclease I (20 U/µl; EURx, Poland) and alkaline phosphatase Fast Polar-BAP (1 U/µl, EURx, Poland) treatment, according to the manufacturer's guidelines and then sequenced in Macrogen Europe (Netherlands) or BaseClear (Netherlands).

#### Dataset assembly and phylogeographic analyses

For phylogeographic analysis we included published nucleotide sequences from previous studies (Copilaş-Ciocianu et al. 2017; Copilaş-Ciocianu et al. 2018; Palatov & Marin 2021) in addition to our newly generated sequences. DNA sequences were deposited in the Barcode of Life Data Systems (BOLD - <http://v4.boldsystems.org>) (Ratnasingham & Hebert 2007), in order to obtain the Barcode Index Numbers (BIN) which group DNA sequences based on genetic distance (Ratnasingham & Hebert 2013). Relevant voucher information is accessible through the public dataset DS-PCNIPH (DOI: <http://dx.doi.org/10.5883/DS-PCNIPH>) in BOLD as well as in Supplementary Table SI. Sequence alignment was performed with MUSCLE (Edgar 2004) implemented in MEGA 6 (Tamura et al. 2013).

Sequence divergences for the COI-5P barcode region (mean and maximum intraspecific variation and minimum genetic distance to the nearest-neighbor species) were calculated using the analytical tools of the BOLD workbench ("Barcode Gap Analysis"), employing the Kimura 2-parameter model (K2P; Kimura 1980). We estimated the haplotype number, using the DnaSP v5 software (Librado & Rozas 2009). The genetic structure within both species was visualized through a Median-joining network using PopART (Leigh & Bryant 2015). Phylogenetic analyses were performed in MEGA 6 (Tamura et al. 2013) using the Neighbor-Joining algorithm with Kimura 2-parameter distances, and 1000 bootstrap replicates to visualize relationships among haplotypes.

## Results

### Field research

In the Dniester basin (Ukraine), *N. potamophilus* was collected in 2018 from the flooded meadow area beside the river (near Mayaki), in shallow, well-warmed floodplain puddles (both temporary and permanent ones) with almost no water flow (Figure 1(a,b)). Amphipods co-occurred alongside *A. aquaticus* and diverse fauna of freshwater mollusks and leeches. It should be noted that this

floodplain meadow has recently been greatly reduced in size due to ongoing urbanization, and the natural floodplain puddles (Figure 1(b)) are rare now.

*Niphargus potamophilus* in the delta of the Dniro River (Ukraine) was found in 2018 in a shallow stream (near Stara Zburivka) with a lot of decaying vegetation, that flows over an alder forest, in a place where the spring starts its outflow (Figure 1(c)). The water in this place was colder than in the surroundings and was flowing slowly. The amphipods co-occurred alongside *A. aquaticus* and *Haemopsis sanguisuga* (Linnaeus, 1758). This stream is part of the channel that connects the estuarine bay to a system of fish breeding ponds. The amphipods were found in the least anthropogenically transformed part of a former floodplain channel.

In Bulgaria in the Tankovo area, both *Niphargus hrabei* and *N. potamophilus* were sampled four years apart. *Niphargus hrabei* was collected in 2018 from a ditch with apparently temporary water (Figure 1(d)). The substrate was muddy with a lot of decaying vegetation. The riparian vegetation was very abundant. The density of the animals was rather high as several tens of individuals were sampled, including 21 females (a few ovigerous), 52 males, and 16 juveniles. The water louse *Asellus aquaticus* (Linnaeus, 1758) was also highly abundant. This sampling point represents the southernmost record of this species, being situated ca. 150 km south of the previously known locality (Hagieni, Romania; Flot et al. 2014). *Niphargus potamophilus* was sampled in 2014 from Hadjiyska stream, a few tens of meters eastward from where *N. hrabei* was collected (Figure 1(e)). It occurred among reeds on the shallow and muddy shores. Few adult specimens were sampled including both males and females. Accompanying crustacean fauna consisted of *Gammarus* cf. *arduus* G. Karaman, 1975, *Pontogammarus robustoides* (Sars, 1894), and *A. aquaticus*. This sampling point is the furthest locality from the previously known records (ca. 1000 km) (Palatov & Marin 2021).

*Niphargus potamophilus* in Lake Ezerets (Bulgaria) was sampled in dense aquatic vegetation and muddy substrate along the lake shore in 2018 (Figure 1(f)). Only a few immature individuals were sampled that could be properly identified only by DNA barcoding. It was accompanied by typical Ponto-Caspian amphipod fauna such as *Pontogammarus robustoides*, *Dikerogammarus haemobaphes* (Eichwald, 1841), *Chaetogammarus ischnus* (Stebbing, 1899), *Trichogammarus trichiatus* (Martynov, 1932), *Shablogammarus shablensis* Carausu, 1943,

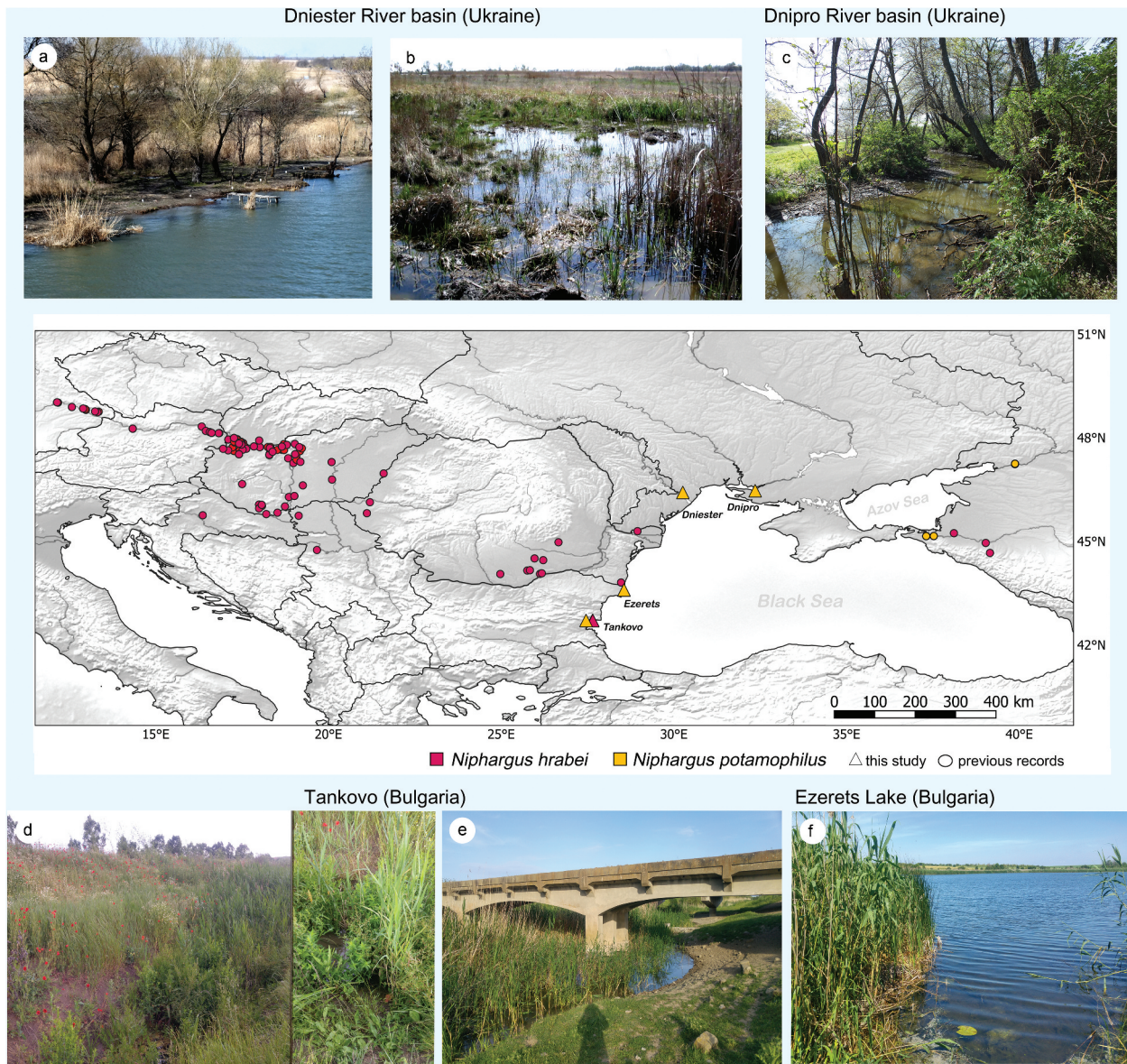


Figure 1. Sampling sites and biotopes where *Niphargus hrabei* and *N. potamophilus* were collected: A, B, Dniester River (Ukraine); C, Dnipro River (Ukraine); D, E, Tankovo (Bulgaria); F, Ezerets Lake (Bulgaria).

*Lanceogammarus andrussovi* (G.O. Sars, 1896), *Chelicorophium curvispinum* (G. O. Sars, 1895), *Ch. sowinskyi* (Martynov, 1924), and various unidentified Mysidae.

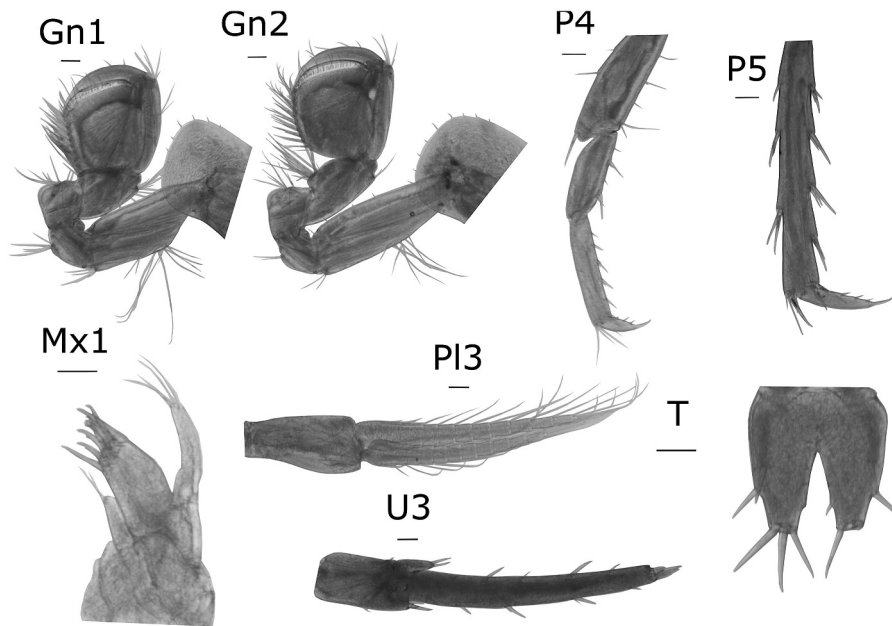
*Morphology of specimens*

All of the examined morphological characters of our material fell within the range indicated for *N. hrabei* and *N. potamophilus* species by Birstein (1954) and the recent redescription of the latter species by Palatov and Marin (2021). Both species are characterized by trapezoidal gnathopod propodi, acutely produced infero-

posterior corners of epimeres 2 and 3, additional spines on dactyly of pereopods 3 to 4, uropod 3 non-sexually dimorphic, and the presence of a spoon-shaped appendix on the basis of uropod 1 in males (Figures 2 and 3). The two species can be distinguished from one another by the lack of dorsal spines on telson lobes in *N. hrabei* (present in *N. potamophilus*), presence of lateral setae on peduncle of pleopod 3 in *N. hrabei* (absent in *N. potamophilus*), inner lobe of maxilla 1 with 3 setae in *N. hrabei* (one seta in *N. potamophilus*) (Palatov & Marin 2021) (Figures 2 and 3). The morphological identification is in full agreement with the molecular identification through the COI mtDNA barcode.

## *Niphargus potamophilus*

Tankovo (Bulgaria)



Dnipro River (Ukraine)

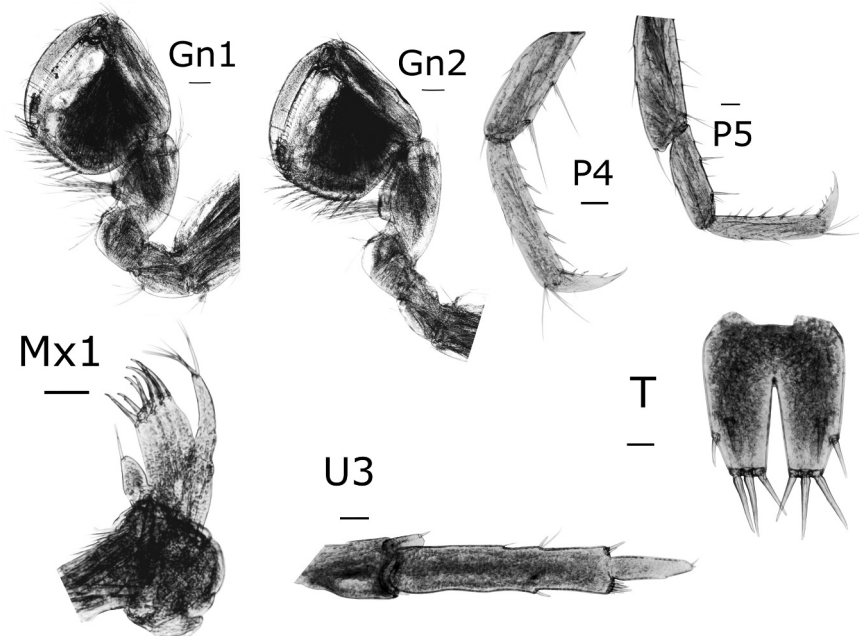


Figure 2. *Niphargus potamophilus* morphology (Gn1, first gnatopod; Gn2, second gnatopod; P4, fourth pereopod; P5, fifth pereopod; Mx1, first maxilla; PI3, third pleopod; U3, third uropod; T, telson).

*Niphargus hrabei*

Tankovo (Bulgaria)

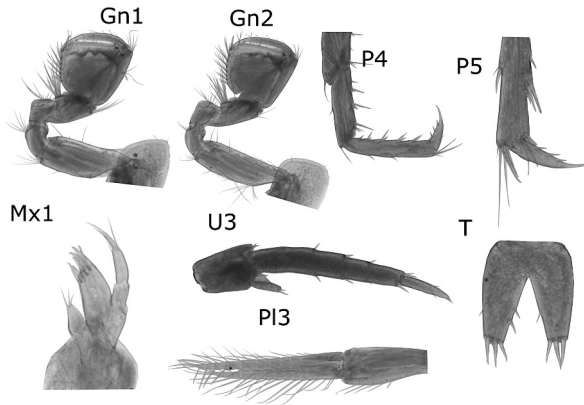


Figure 3. *Niphargus hrabei* morphology (Gn1, first gnathopod; Gn2, second gnathopod; P4, fourth pereopod; P5, fifth pereopod; Mx1, first maxilla; PI3, third pleopod; U3, third uropod; T, telson).

*Molecular studies*

We generated four new sequences for *Niphargus hrabei* (representing two new haplotypes), and nine new sequences for *Niphargus potamophilus* (representing six new haplotypes). Analyzing the whole assembled dataset, we identified 15 haplotypes within 48 sequences for *N. hrabei*, with a 0.41% mean, and 1.31% max intraspecific K2P distance; all sequences were assigned to a single BIN - BOLD:ACQ4737. For *N. potamophilus* we identified 10 haplotypes within 13 sequences, with a 1.15% mean, and 2.85% max intraspecific K2P distance; all sequences were assigned to a single BIN - BOLD:AEB5889. K2P distance between these two species was 17.25%, clearly indicated in the Neighbor-Joining tree (Figure 4). The Median-Joining network of *N. hrabei* shows that individuals from Russia and Bulgaria have only private haplotypes (Figure 5(a)). The network for *N. potamophilus* shows quite different private haplotypes from Russia, with a minimum distance of six substitutions, to the closest haplotypes from Bulgaria and Ukraine (Figure 5(b)).

**Discussion**

The epigeal *Niphargus* species from the Azov-Black Sea region, *N. hrabei*, *N. potamophilus*, *N. valachicus*, and *N. magnus* Birstein, 1940, have formerly been considered as members of the subgenus *Phaenogammarus* (Dudich 1941) based on their ecological and morphological similarity (Birstein 1954;

Straškraba 1972; Sket 1981; Palatov & Marin 2021). However, recent molecular research has shown that this similarity is due to convergence, thus rendering *Phaenogammarus* obsolete (Borko et al. 2021). Nevertheless, regarding our focal species, a recent analysis showed that *N. potamophilus*, *N. valachicus*, and *N. magnus* are closely related, forming a well supported clade that is phylogenetically distant from *N. hrabei* (Palatov & Marin 2021). *Niphargus hrabei* and *N. valachicus*, are widespread across the middle and lower Danube lowlands and the Black Sea coast (Copilaş-Ciocianu et al. 2018; Palatov & Marin 2021), while *N. magnus* and *N. potamophilus* are known from the eastern Black Sea and Azov-Black Sea basins, respectively (Palatov & Marin 2021). Our findings of *N. hrabei* and *N. potamophilus* across the west part of the Black Sea basin significantly increased the range of these species, especially for the latter, confirming its westward distribution suggested in the original description (Birstein 1954).

The previous and newly gathered ecological data indicates that *N. hrabei*, *N. potamophilus* and *N. valachicus* have similar environmental preferences, occupying a wide range of habitats ranging from springs to small and large streams, large deltaic lakes and channels, estuarine water bodies, and floodplains (Birstein 1954; Zorina-Sakharova 2017; Copilaş-Ciocianu et al. 2018; Palatov & Marin 2021; this study). Conversely, *N. magnus* is distributed both in subterranean and surface water. However, it seems to occupy a narrower spectrum of surface habitats, preferring well-shaded small forest ponds filled with fallen leaves (Birstein 1940, 1952; Palatov & Marin 2021). Such habitats are more isolated as evidenced by the pronounced genetic structuring within this species (Palatov & Marin 2021). This is probably the reason why it has a much smaller range than the other three species.

This increase in reports of epigeal *Niphargus* species in the last decade around the Black Sea basin is noteworthy, and could either be a result of more active monitoring, or recent range expansion. The latter is unlikely given the changes in the last century in the condition of large river ecosystems, associated with the simultaneous effects of climate change and anthropogenic flow regulation, which affected the abundance of Ponto-Caspian species and communities (Gogaladze et al. 2021), and promoted the spread of non-native species. Furthermore, our molecular analysis showed that Ukrainian and Bulgarian populations of both species are characterized by unique haplotypes, not present anywhere else in the Pannonian or Black Sea basins.

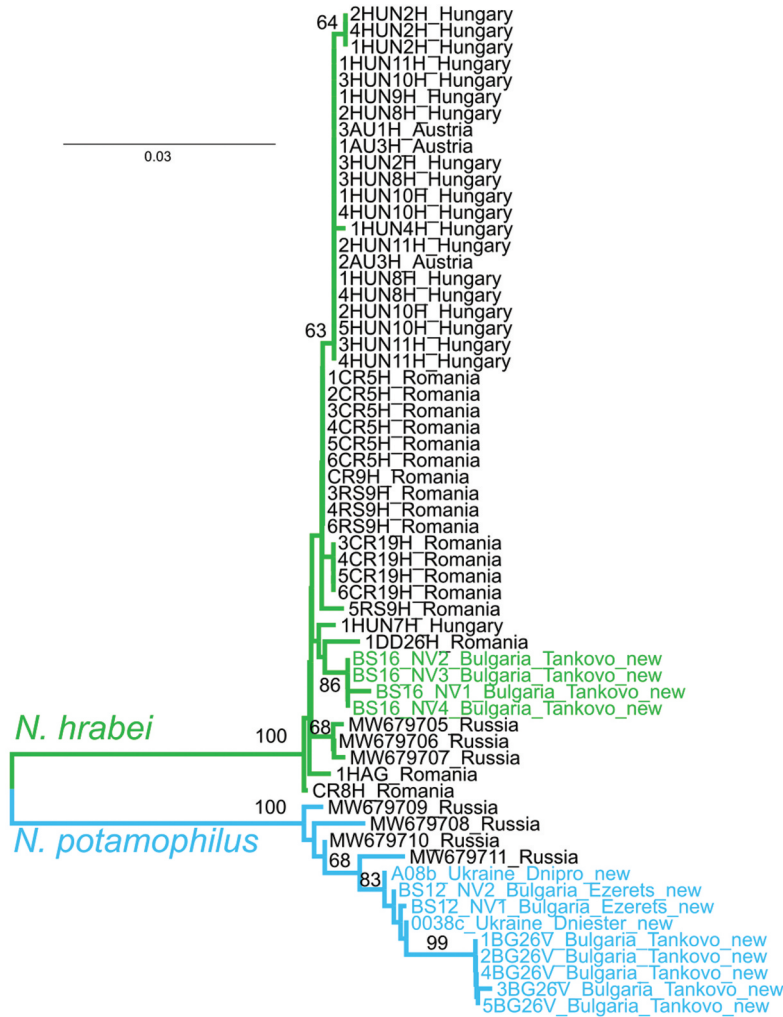


Figure 4. Neighbor-Joining tree depicting the relationship between our newly generated sequences (shown with colored text) and those from previous studies. Numbers at nodes are bootstrap support values (not shown if <60%).

As such, it is clear that *N. hrabei* and *N. potamophilus* are native species in these areas.

It is, therefore, more likely that these species might have been overlooked previously in the Azov-Black Sea basin, and their dispersal across the marine/brackish waters of the Black Sea might have occurred a long time ago, at the Last Glacial Maximum (30–25 thousand years ago), when this sea was a freshwater lake, isolated from the world ocean (Ryan et al. 1997; Bahr et al. 2006; Georgievski & Stanev 2006). At this time, the Pre-Danube, Pre-Don and Pre-Dnipro paleo-basins were merged into one common alluvial valley (Chepalyga 2007; Yanko-Hombach et al. 2017) and lakes in the Syvash Gulf were desalinated (Olenkovsky 2012). This alluvial valley lasted until the latest Mediterranean transgression 16–9 thousand years ago (Yanko-Hombach & Yanina 2019; Figure 9). This palaeogeographic context most likely

facilitated the dispersal of these freshwater amphipods around the Azov and Black Sea coastline. The phylogeography of both *N. hrabei* and *N. valachicus* is consistent with dispersal along the Black Sea coast during this time frame (Copilaş-Ciocianu et al. 2018). The subsequent flooding of the Black Sea with saline Mediterranean waters divided the common valley into separate estuaries of the Dnieper, Dniestr, and Danube River basins (Yanko-Hombach et al. 2017; Figure 16.22), forming the modern Azov and Black Sea (7–9 thousand years ago) (Federov 1971; Ryan et al. 1997; Badertscher et al. 2011; Yanko-Hombach & Yanina 2019; Figure 9). Since then, all the major river basins have maintained their isolation. As such, *N. hrabei*, *N. potamophilus* and *N. valachicus* could be present across the entire north Black Sea basin for at least 9 thousand years.



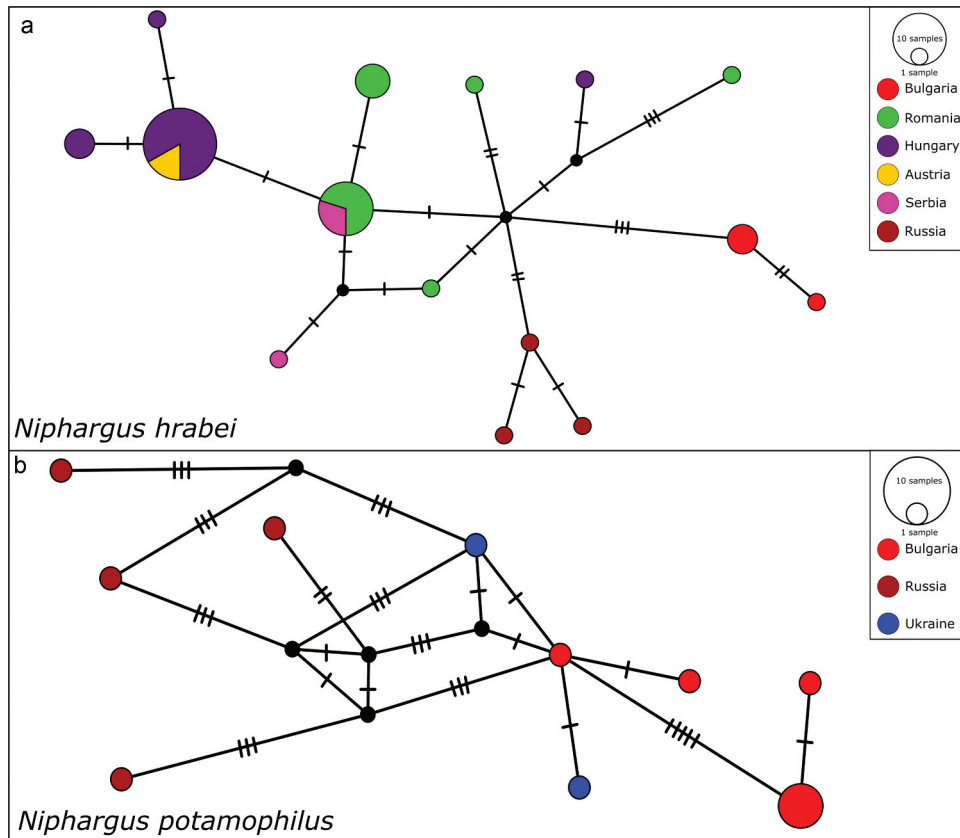


Figure 5. Median-joining network showing the relationships among the haplotypes: a, *Niphargus hrabei*; b, *Niphargus potamophilus*. Colors indicate individuals representing different countries of origin. Each bar represents one substitution, whereas small black dots indicate undetected/extinct intermediate haplotype states. The sizes of the circles are proportional to frequencies of haplotypes (see open circles with numbers).

In conclusion, we provide the first morphological and molecular evidence of the presence of *N. hrabei* in Bulgaria, and *N. potamophilus* in Bulgaria and Ukraine. Our findings expand the known range of the former ca. 150 km southwards and for the latter species ca. 1000 km westwards. Molecular population structure shows that only Pannonian populations of *N. hrabei* shared haplotypes between sites and countries, and populations of both species along the Black Sea coast are genetically isolated.

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### Disclosure statement

No potential conflict of interest was reported by the author(s).

### Supplementary material

Supplemental data for this article can be accessed online at <https://doi.org/10.1080/24750263.2022.2126534>

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