Molecular markers and SEM imaging reveal pseudocryptic diversity within the Ponto-Caspian low-profile amphipod invader Dikerogammarus bispinosus

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Abstract
The Ponto-Caspian amphipod Dikerogammarus bispinosus was originally described from the Black Sea basin. Its recent discovery in the Caspian Sea basin was puzzling because it was unknown whether it was an invasive or an overlooked native species in this area. Here, we examined specimens collected from both the Black and Caspian Sea basins by means of molecular species delimitation based on nuclear (28S) and mitochondrial (COI) DNA sequences, as well as scanning electron microscopy (SEM). Our analyses reveal that D. bispinosus comprises three evolutionary independent lineages that are molecularly and morphologically distinct. One lineage occurs throughout rivers in the Black Sea basin, while the other two inhabit the Caspian Sea and were found in sympatry, further reinforcing that they are distinct species. Our time-calibrated phylogeny indicates that these lineages split during the Late Miocene-Pliocene, a period corresponding with the separation of the Black and Caspian basins via the Caucasus mountain uplift. SEM imaging revealed morphological differences with respect to setal patterns on the gnathopod propodi among all three lineages. Therefore, our results clearly indicate not only that D. bispinosus is native in the Caspian region, but that it has been overlooked for a long time. Additional populations covering the entire range of this species complex need to be further studied in order to gain a more complete picture of its evolutionary history and resolve its taxonomy.

Keywords: Amphipoda, Ponto-Caspian, invasion, phylogeography, morphology

Introduction
The Ponto-Caspian region has a complex geological history due to its gradual separation, with recurrent re-connections, from the World Ocean and frequent sea-level fluctuations. These regression/transgression phases lead to recurrent episodes of connection and isolation between different basins within the Ponto-Caspian region. The current Black, Azov, Caspian and Aral Seas, formed in the Pleistocene and early Holocene, are products of this complex geological process (Zenkevich 1956; Jones & Simmons 1997; Cristescu et al. 2003, Figure 1; Popov et al. 2006; Hou & Li 2018, Figure 2). This dynamic history caused numerous environmental changes (salinity fluctuations) that led to biotic turnover and numerous evolutionary radiations, especially in crustaceans (Cristescu et al. 2003; Audzijonyte et al. 2006; Hou et al. 2014), mollusks (Sands et al. 2019a, 2019b; Neibera et al. 2021), and fish (Neilson & Stepien 2009).

Amphipods have greatly diversified in the Ponto-Caspian region (96 endemics), particularly in the
The Caspian Sea (71 endemic species) (Derzhavin 1948; Mordukhai-Boltovskoj 1960; Pjatakova & Tarasov 1996; Copilaș-Ciocianu & Sidorov 2021). The latter is even called the “Crustacean Sea” due to a high diversity of endemic amphipods, mysids and cumaceans (Zenkevich 1956; Cristescu & Hebert 2005). Ponto-Caspian amphipods are mostly euryhaline species with a high invasive potential, many of which have extended their ranges in the last 60 years (Bij de Vaate et al. 2002; Cristescu et al. 2004;...
Copilaş-Ciocianu et al. 2021) and are studied quite actively (e.g., Cristescu et al. 2004; Grabowski et al. 2007; Arbačiauskas et al. 2013; Rewicz et al. 2015; Copilaş-Ciocianu & Sidorov 2021).

Dikerogammarus bispinosus Martynov, 1925 described from the Dnieper River has been considered as native in the lower stretches of rivers that drain to the Black Sea (Martynov, 1925; Cărăuşu et al. 1955; Jazdewski & Konopacka 1988). Its non-native range stretches westwards along the middle and upper sectors of these rivers, reaching into the Rhine in western Europe (Labat et al. 2011). It is also widespread in the upper Danube River, particularly in Germany (Eggers & Martens 2001), Austria (Müller & Schramm 2001, Borza et al. 2015), and in the middle Danube in Hungary and Slovakia (Borza et al. 2015). More recently, it has started to expand northeast; it was reported as an invasive species from the Saratov reservoir (Volga River, Russia, Caspian Sea basin) between 2002 and 2006 (Voronin & Yermokin 2004; Filinova & Sonina 2012) and from the lower Don (Russia, Black Sea basin) in 2003 (Sayapin 2003). It is believed that this species could have dispersed naturally via the Volga-Don canal to the Caspian region or was passively introduced through shipping activity (Copilaş-Ciocianu & Arbačiauskas 2018). Yet, evidence of its presence in the Caspian basin before these reports were conflicting as D. bispinosus was considered for a long time a subspecies or synonym of D. villosus (Sowinsky, 1894), and sometimes even D. villosus was erroneously considered as a synonym of D. haemobaphes (Eichwald, 1841) (Pjatakova & Tarasov 1996). Mordukhai-Boltovskoj (1960) indicated that D. bispinosus does not occur in the Caspian Sea. Clear morphological features to distinguish these three species as well as molecular comparison were provided quite recently (Müller & Schramm 2001; Müller et al. 2002; Mamos et al. 2021), so D. bispinosus may have been overlooked in the Caspian basin for decades (Tarasov 1995; Copilaş-Ciocianu & Arbačiauskas 2018). Recently, this species was also reported in the Ural River (Kazakhstan), which drains into the Caspian Sea (Copilaş-Ciocianu & Arbačiauskas 2018). Nevertheless, given the currently available data, it has not been possible to determine whether D. bispinosus is an overlooked native or an invader in the Caspian basin (Copilaş-Ciocianu & Arbačiauskas 2018).

In this study, we aim to provide insight into the native versus invasive status of D. bispinosus in the Caspian Sea basin by using a phylogeographical approach based on mitochondrial and nuclear markers, combined with Scanning Electron Microscopy (SEM) imaging. We hypothesize that, given the long geological isolation of the Black and Caspian Seas, the local populations of D. bispinosus may belong to different lineages, as observed in several other crustacean species in this region (Cristescu et al. 2003; Cristescu & Hebert 2005; Audzijonyte et al. 2006; Nahavandi et al. 2013).

Material and methods

Sampling

Dikerogammarus bispinosus was collected with a hand net and by visual inspection of submerged substrates from three localities on the coast of the Caspian Sea: near Baku (Azerbaijan) and 43 km north of Aktau in Kazakhstan in August 2018, as well as in Yalama (Azerbaijan) in July 2019. Additionally, specimens of D. bispinosus, D. villosus, D haemobaphes and Pontogammarus robustoides (Sars, 1894) that originated from previous sampling campaigns in Austria, Croatia, Ukraine and Hungary, were used as a comparative material (for details see Table I). Animals were fixed in 96% ethanol. The specimens are stored in the collection of invertebrates at the Department of Invertebrate Zoology and Hydrobiology (University of Lodz, Poland).

Morphological identification and SEM imaging

Identity of the collected D. bispinosus was confirmed using the keys by Cărăuşu et al. (1955), Stock (1974), Karaman & Barnard (1979), Stock et al. (1998) and Özbek & Özkam (2011). The diagnostic morphological features for D. bispinosus were defined as follows: pillar-shaped protuberances on first and second urosomites, antenna 2 peduncular segments with tufts of setae longer than the underlying segment and a postero-distal lobe on the basis of the 7th pereopod.

Adult, male individuals used for SEM documentation were dehydrated in an ethanol series, air dried, and sputter-coated with gold (11 nm). Images were produced with a PHENOM PRO X SEM in the Department of Invertebrate Zoology and Hydrobiology, University of Lodz. Given that second antennae and second gnathopods are known to bear important taxonomic features in amphipods, we imaged these appendages for two male individuals per each lineage identified with molecular methods (see below).

DNA extraction and PCR

Samples were processed either in the Canadian Center for DNA Barcoding (CCDB), Guelph,
Table I. Localities and GenBank accession numbers of specimens used for the current study. N = 54.

| Code   | N  | Locality                       | Latitude | Longitude | Date          | Acc. no. COI                                      | Acc. no 28S                                      | Reference                          |
|--------|----|--------------------------------|----------|-----------|---------------|--------------------------------------------------|--------------------------------------------------|------------------------------------|
| 1Casp  | 5  | Kazakhstan, Caspian Sea        | 44.773   | 51.027    | 09.08.2018    | MZ261668, MZ261672, MZ261679, MZ261684, MZ261693 | MZ261645, MZ261647, MZ261652, MZ261656, MZ261660 | This study                         |
| CAU52  | 3  | Azerbaijan, Caspian Sea        | 41.795   | 48.661    | 03.07.2019    | MZ261678, MZ261686, MZ261688                      | MZ261651, MZ261657, MZ261658                  | This study                         |
| A03    | 3  | Azerbaijan, Caspian Sea        | 40.301   | 49.777    | 19.08.2018    | MZ261663, MZ261665, MZ261675                      | MZ261642, MZ261644, MZ261649                  | This study                         |
| HBUĐ   | 2  | Hungary, Danube                | 47.518   | 19.042    | 13.04.2012    | MZ261670, MZ261689                                |                                                  | This study                         |
| DHDN   | 1  | Hungary, Danube                | 47.785   | 18.960    | 12.03.2011    | -                                                  | MN343752                                      | Jadzewska et al. 2020             |
| HRB100 | 2  | Croatia, Danube                | 45.531   | 18.949    | 28.10.2016    | MZ261677, MZ261697                                | -                                                | This study                         |
| AUSTR1*| 2  | Austria, Danube                | 48.294   | 15.405    | 06.11.2010    | MZ261666, MZ261682                                | -                                                | This study                         |
| UKR_167| 3  | Ukraine, Dniester              | 48.792   | 25.286    | 13.07.2019    | MZ261669, MZ261673, MZ261695                      | MZ261646                                      | This study                         |
| UKR_173| 2  | Ukraine, Dniester              | 48.808   | 25.341    | 19.07.2019    | MZ261671, MZ261692                                | MZ261659                                      | This study                         |
| UKR_186| 3  | Ukraine, Dniester              | 48.725   | 25.611    | 09.07.2019    | MZ261676, MZ261681, MZ261694                      | MZ261650, MZ261653                              | This study                         |
| GB1    | 1  | United Kingdom,                | 52.292   | -0.32     | 03.09.2010    | MZ261683                                        | -                                                | Rewicz et al. 2015                |
| D08    | 1  | Netherlands, IJssel            | 52.239   | 6.160     | 10.06.2010    | MZ261680                                        | -                                                | Rewicz et al. 2015                |
| TR41   | 1  | Turkey, Durusu Lake            | 41.316   | 28.620    | 01.09.2007    | MZ261691                                        | -                                                | Rewicz et al. 2015                |
| PLWW   | 1  | Poland, Vistula                | 52.384   | 20.187    | 08.07.2008    | MZ261698                                        | -                                                | Rewicz et al. 2015                |
| UKR_040| 2  | Ukraine, Dniester Liman        | 46.198   | 30.343    | 29.04.2018    | MZ261667, MZ261696                                | MZ261661                                      | This study                         |
| UKR_042| 2  | Ukraine, Danube                | 45.339   | 28.809    | 01.05.2018    | MZ261664, MZ261674                                | MZ261643, MZ261648                              | This study                         |
| DUA    | 1  | Ukraine, Akkarzhanka           | 46.347   | 30.597    | 22.08.2009    | MN343183                                        | MN343750                                       | Jadzewska et al. 2020             |
| DUDZ   | 1  | Ukraine, Dnieper               | 47.7918  | 35.1255   | 11.08.2009    | MN343090                                        | MN343744                                       | Jadzewska et al. 2020             |
| DTDL   | 2  | Turkey, Durusu Lake            | 41.316   | 28.620    | 01.09.2007    | MN343268, MN343032                                | MN343753, MN343745                             | Jadzewska et al. 2020             |
| DRM    | 3  | Russia, Moskva                 | 55.5969  | 37.1223   | NA            | MN343066, MN343164, MN343203                      | MN343746, MN343749, MN343751                 | Jadzewska et al. 2020             |
| DUEM   | 2  | Ukraine, Malo Odina            | 46.4008  | 30.5929   | 22.08.2009    | MN342878, MN343114                                | MN34374, MN343748                               | Jadzewska et al. 2020             |
| UKR_P10| 1  | Ukraine, Desna                 | 51.449   | 32.4766   | 10.07.2018    | MZ261690                                        | -                                                | This study                         |
| UKR_030| 1  | Ukraine, Conca                 | 46.5382  | 32.5322   | 01.05.2017    | MZ261695                                        | -                                                | This study                         |
| DOP    | 1  | Poland, Oder                   | 52.4396  | 14.5779   | 09.05.2001    | MN343242                                        | -                                                | Jadzewska et al. 2020             |
| CAU_37 | 1  | Azerbaijan, Varvara Lake       | 40.681   | 47.09     | 02.07.2019    | MZ261687                                        | -                                                | This study                         |

(Continued)
| Code | N  | Locality                  | Latitude | Longitude | Date       | Acc. no. COI                  | Acc. no 28S         | Reference               |
|------|----|---------------------------|----------|-----------|------------|-------------------------------|---------------------|-------------------------|
| DUKP | 4  | Ukraine, North Crimean Canal | 46.1828 | 33.5432   | 11.07.2011 | MN342932, MN343079, MN343110, MN343287 | MN343747, MZ261662 | Jaźdżewska et al. 2020 |
| DUDL | 1  | Ukraine, Dniestrovskij Liman | 46.3309 | 30.0956   | 21.08.2009 | MN342950                      | -                   | Jaźdżewska et al. 2020 |
| DUDM | 2  | Ukraine, Dniester          | 46.4128 | 30.2585   | 22.08.2009 | MN343124, MN343134            | MZ261655            | Jaźdżewska et al. 2020 |
| HUNI | 1  | Hungary, Maros             | 46.196  | 20.4709   | 27.03.2019 | MN322614                      | MZ261654            | Csabai et al. 2020     |

*specimens proceeded in CCDB according to their protocols of extraction, amplification and sequencing*
Canada (two individuals, see Table I for details) or in the Department of Invertebrate Zoology and Hydrobiology, University of Lodz (all the remaining individuals).

For samples analysed at the CCDB, a leg was removed from each specimen and transferred into 96 well plates for subsequent DNA extraction (protocol available: https://ccdb.ca/site/wp-content/uploads/2016/09/CCDB_DNA_Extraction.pdf). The standard animal DNA barcode gene region (COI) (Hebert et al. 2003) was amplified using the primers LCO1490, 5′-GGTCAAAATCTATAAGATATGG-3′ and HCO2198, 5′-TAAACTTCAGGGTGACCAAAAAA TCA-3′ (Folmer et al. 1994) (https://ccdb.ca/site/wp-content/uploads/2016/09/CCDB_Amplification.pdf) and bi-directionally Sanger sequenced at place in the CCDB (https://ccdb.ca/site/wp-content/uploads/2016/09/CCDB_PrimerSets.pdf).

For samples analysed at the Department of Invertebrate Zoology and Hydrobiology (University of Lodz), total DNA was extracted using either the Chelex procedure (Casquet et al. 2012), or the phenol-chloroform method (see Grabowski et al. 2012 for details). The standard animal DNA barcode gene region (COI) (Hebert et al. 2003) was amplified using the primers described above (Folmer et al. 1994). The amplification of a nuclear fragment of gene 28S rRNA was done with 28 F 5′-TTAGTGGGCCCACGACACGGG-3′ and 28 R 5′-GTCTTTCGCCCCTATGCGACAACCTGA-3′ primers published by Hou et al. (2007). The amplification of both gene regions was conducted under the same PCR protocol described by Hou et al. (2007). 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 (COI – forward direction only, 28S – bi-directional) using standard Sanger method by Macrogen Europe (Netherlands), using the same PCR primers.

**Dataset assembly**

BLAST search (blast.ncbi.nlm.nih.gov/Blast.cgi) was performed to check for the identity of amplified sequences. The resulting sequences were manually assembled in Geneious v. 10.2.6 (https://www.geneious.com, Kearse et al. 2012) and trimmed by removing the remaining parts of the primer regions (length COI: 490–695; 28S: 862–1264) and then submitted to GenBank (Accession: COI: MZ261663-MZ261 698; 28S: MZ261642-MZ261662) and simultaneously deposited in the Barcode of Life Data Systems (BOLD) System (Ratnasingham & Hebert 2007).

Additional COI and 28S sequences of *D. haemobaphes* and *D. villoides* (28 individuals) as well as the outgroup sequence (one individual) of *P. robustoides* were acquired either from the publicly available datasets deposited in the open databases (GenBank and BOLD) or from own data (see Table I for details).

Relevant voucher information is accessible through the public data set “DS-DIKS” (DOI: http://dx.doi.org/10.5883/DS-DIKS) in the BOLD (http://v4.boldsystems.org).

**Phylogenetic analyses**

The COI fragment was aligned with MUSCLE (Edgar 2004) in MEGA X (Kumar et al. 2018) and subsequently amino acid translated to check for stop codons that would indicate pseudogenes. The 28S fragment was aligned online with MAFFT 7 (Katoh & Standley 2013) (https://mafft.cbrc.jp/alignment/server/) using the G-INS-I method. Evolutionary models for each gene fragment (including the three codon positions in COI) were selected with PartitionFinder (Lanfear et al. 2012) using the greedy algorithm and the Bayesian information criterion.

Gene trees were calculated separately for the COI and 28S fragments. For this, we used the maximum likelihood (ML) method implemented on the W-IQ-TREE webserver (Trifinopoulos et al. 2016). Node support was assessed with 1000 ultra-fast bootstrap replicates (Hoang et al. 2018) and the Shimodaira-Hasegawa approximate likelihood ratio test (Shimodaira & Hasegawa 1999). For COI we used the GTR+G model further partitioned into codon positions, and for 28S the K80+I model. A concatenated analysis of both markers was also run in IQTREE using a partitioned edge-linked model and the GTR+I+G model.

In order to gain a more detailed insight into evolutionary patterns, we generated haplotype networks for each marker. For this purpose we used Haploviewer (Salzburger et al. 2011) with input ML phylogenetic trees calculated in MEGA X.

In addition, the number of base differences per site (p-distance) between COI and 28S sequences was calculated in MEGA X, including all codon positions. All positions containing gaps and missing data were eliminated.

**Molecular species delimitation and divergence time estimation**

We applied three unilocus and one multilocus species delimitation methods to test the hypothesis that *D. bispinosus* harbors putative cryptic species. The
unilocus methods were applied for the COI and 28S fragments separately while the multilocus method was applied to both markers simultaneously. In order to increase the accuracy of the results the dataset consisted of sequences of all the species, i.e., *D. haemobaphes*, *D. villosus* and *P. robustoides*.

The two unilocus methods consisted of two fundamentally different approaches. First, we implemented two distance-based approaches: (1) automatic classification of sequences into putative species clusters based on a barcode gap using the online version of the ASAP software (https://bioinfo.mnhn.fr/abi/public/asap/) (Puillandre et al. 2021), distances were computed using the K2p model; (2) Barcode Index Number (BIN), which is a method implemented in BOLD, where newly submitted and already available sequences (COI 5’ only) are indexed based on the genetic distance method, and clustered in unique BINS (Ratnasingham & Hebert 2013). Second, we applied a tree-based approach which models speciation along the branches of a given phylogenetic tree using the online version of the mPTP software (https://mcmc-mptp.h-its.org/mcmc/) (Kapli et al. 2017). As an input we used the ML trees calculated with IQTREE (see above).

For the multilocus species delimitation, we implemented the Bayes factors species delimitation approach (Grummer et al. 2014). For this we used a Bayesian implementation of the multispecies coalescent in BEAST 1.8.2 (Drummond et al. 2012) with the *BEAST* package (Heled & Drummond 2010). The species hypotheses proposed by ASAP and mPTP were tested against each other as well as against the null hypothesis of a single species. The marginal log likelihood of each species hypothesis was calculated using path and stepping stone sampling (Baele et al. 2012, 2013). The marginal likelihood estimator was run for one million generations, 10 paths steps and alpha set to the default 0.3. Model fit was evaluated by calculating Bayes factors (Kass & Raftery 1995). A value of at least 10 was considered as strong support against the competing model.

Within this Bayesian multilocus species tree approach, we also estimated divergence times and phylogenetic relationships. The evolutionary models used were TrNeF+G, HKY and GTR for the 1st, 2nd and 3rd COI codon positions, respectively, and the K80 + I model for 28S (as determined with PartitionFinder). The molecular clock was calibrated using amphipod-specific substitution rates for both markers (0.01773 ± 0.004 substitutions/site/Ma for COI, and 0.00161 ± 0.0003 substitutions/site/Ma for 28S) estimated for the freshwater families Crangonyctidae and Pseudocrangonyctidae (Copilaș-Ciocianu et al. 2019, 2020). A lognormal relaxed clock was used for the fast-evolving COI fragment and a strict clock for the slow-evolving 28S. Speciation was modelled using the Birth-Death process. Two independent runs were conducted for 30 million generations, a thinning of 1000, and the first 1000 trees were discarded as burn-in after visual examination in TRACER 1.6 (Rambaut et al. 2014). The two runs converged on identical results.

Results

Phylogenetic analyses

The inferred phylogenetic relationships among populations and species are shown in Figures 1 and Figures 2. Three well supported divergent mitochondrial lineages were observed within *D. bispinosus* and presence of the two previously known lineages of *D. haemobaphes* was confirmed. These mitochondrial lineages were also distinct at the nuclear level, albeit with lower support (Figure 1). Although the *D. bispinosus* lineages are well supported, the exact phylogenetic relationships between them are not very clear at the individual gene level. The concatenated ML analysis also recovered a similar pattern with poorly supported deep nodes. However, the multilocus species tree was well resolved, with each node receiving maximum support (Figure 2).

MOTU delimitation

The distance-based ASAP method gave identical results for both the COI (ASAP-score 2, p = 0.00001, threshold distance 0.05) and 28S (ASAP-score 2, p = 0.0185, threshold distance 0.003) fragments by delimiting the same seven MOTUs (Molecular Operational Taxonomic Units): three within *D. bispinosus*, two within *D. haemobaphes* and one for *D. villosus* (Figure 1). The tree-based mPTP method recovered the same seven MOTUs for COI (probability ≥ 0.95), and only three MOTUs for 28S, lumping together *D. bispinosus* MOTU 1 and 2, as well as both MOTUs of *D. haemobaphes* with *D. villosus* (Figure 1). However, the three 28S MOTUs had a low probability, ranging from 0.63 to 0.78.

The BIN delimitation method revealed seven BINS, fully congruent with the MOTUs delimited with ASAP and mPTP based on COI. Sequences of *D. bispinosus* grouped under three BINS: ADM9911 (known already from Europe), ADY3531 (Kazakhstan and Azerbaijan – new BIN), and AEC3943 (Kazakhstan, also new BIN). *Dikerogammarus haemobaphes* grouped in two BINS (AAX9262, ADB9467), recently presented and discussed in Jażdżewska et al. (2020), and *D. villosus* grouped
under one BIN (AAI9938) which is up to now the only known BIN for this species in BOLD.

For the multilocus MOTU delimitation approach we compared three MOTU hypotheses regarding the number of putative cryptic MOTUs within *D. bispinosus*: three MOTUs (as suggested by ASAP based on COI and 28S, and mPTP based on COI), two MOTUs (as suggested by ASAP based on 28S), and the null hypothesis of one MOTU. The model that had the highest fit to the data was the three-MOTU hypothesis (ln = −4988.44), followed by the two MOTU (ln = −5002.78) and one MOTU (ln = −5016.96) hypotheses (Table II). The three-MOTU model had a decisive support (>10) versus the two MOTU model (2ln Bayes factors = 28.66) or one MOTU (2ln Bayes factors = 57.04) (Table II).

Thus, based on the above results, we define three MOTUs within *D. bispinosus*: *D. bispinosus* 1, comprising populations from the Black Sea drainages (Hungary, Austria, Croatia and Ukraine), followed by *D. bispinosus* 2 and *D. bispinosus* 3, both apparently restricted to the Caspian basin (Figure 3). Detailed MOTU distribution is shown on Figure 3.

There is a high genetic divergence between the population of *D. bispinosus* from Europe (Hungary, Austria, Croatia and Ukraine) and the populations from Caspian regions (Kazakhstan and Azerbaijan). The p-distance between *D. bispinosus* 1 (corresponding to European MOTU) and *D. bispinosus* 2 (from Kazakhstan and Azerbaijan) and *D. bispinosus* 3 (Kazakhstan) are 10 and 8% respectively for COI, and 0.6% and 3% for 28S; p-distance between *D. bispinosus* 2 and *D. bispinosus* 3 = 12% for COI and 3% for 28S (Table III).

### Divergence times

The time-calibrated multilocus species tree indicated a crown age for the *Dikerogammarus* species of ca. 9.2 Ma (HPD = 13–6 Ma). The species tree was well resolved, each node receiving a maximum support (Figure 3). The *D. bispinosus* complex probably started diversifying at ca. 6.5 Ma (HPD = 9.7–3.8 Ma), when MOTU 3 branched off. MOTU 1 and 2 diverged around 2.5 Ma ago (HPD = 4.9–1.7 Ma). *D. bispinosus* appears to be in a sister relationship to a clade containing both lineages of *D. haemobaphes* and *D. villosus*. These latter two species split probably ca. 6 Ma ago. The two *D. haemobaphes* lineages split around 3.8 Ma ago (Figure 2).

### SEM imaging

The SEM imaging indicated noticeable differences among *D. bispinosus* MOTUs regarding the

![Figure 3. Distribution of *D. bispinosus* MOTUs: *D. bispinosus* 1 – red; *D. bispinosus* 2 – yellow; *D. bispinosus* 3 – blue.](image-url)
setation patterns of the inner side of the second gnathopod propodus (Figure 4). Specifically, *D. bispinosus* 1 and 2 had tufts of dense, long and somewhat curled setae along the palmar edge and the posterior side of the propodus, while *D. bispinosus* 3 had much shorter, sparser and straight setae. *D. bispinosus* 1 and 2 could be further differentiated by the presence of long setae along the anterior margin of the propodus in the former, while the latter had two to three times shorter setae. Additionally, *D. bispinosus* 3 had eight setal clusters along the anterior margin of the propodus versus four clusters in *D. bispinosus* 1 and 2.

Table III. Estimates of evolutionary divergence between groups (mean p-distances) based on COI gene region (below diagonal) and 28S (above diagonal).

|                | [1]   | [2]   | [3]   | [4]   | [5]   | [6]   |
|----------------|-------|-------|-------|-------|-------|-------|
| *D. bispinosus* 3 | 0.030 | 0.031 | 0.031 | 0.030 | 0.031 |       |
| *D. bispinosus* 2 | 0.016 | 0.006 | 0.012 | 0.013 | 0.011 |       |
| *D. bispinosus* 1 | 0.100 | 0.081 | 0.012 | 0.012 | 0.011 |       |
| *D. villosus*    | 0.159 | 0.165 | 0.156 | 0.010 | 0.013 |       |
| *D. haemobaphes* | 0.176 | 0.168 | 0.166 | 0.169 | 0.010 |       |

![Figure 4](image-url) SEM pictures of antenna 2 and gnathopod 2 from *D. bispinosus* 1 (A, B), *D. bispinosus* 2 (C, D) and *D. bispinosus* 3 (E, F) respectively.

With respect to the second antenna the differences were not so pronounced (Figure 4). *D. bispinosus* 1 and 2 appear to have a larger number of setae (>10) than *D. bispinosus* 3 (max. 5–6) within the setal clusters located on the peduncular segments. Overall, with respect to the studied appendages it appears that *D. bispinosus* 1 and 2 are more similar to each other than to *D. bispinosus* 3.

**Discussion**

Our study reveals that *D. bispinosus* comprises three evolutionary lineages that are distinct at the molecular, morphological and, partially, at the geographical level. These findings have important implications regarding the taxonomy as well as the alien vs. native status of this species within various geographic areas.

The status (native or invasive) of *D. bispinosus* in the Caspian basin was highly uncertain due to conflicting reports and fluctuating taxonomy (Copilaş-Ciocianu & Arbačiauskas 2018). The most probable invasion pathway is considered from the Black Sea basin, via the Volga-Don canal, to the Caspian basin (Sayapin 2003; Voronin & Yermokhin 2004; Filinova & Sonina 2012). On the other hand, the
taxonomy of the invasive *Dikerogammarus* species was also uncertain until recent molecular studies (Müller et al. 2002; Mamos et al. 2021) and the possibility was raised that *D. bispinosus* might have been overlooked for a long time in the Caspian basin (Copilaş-Ciocianu & Arbačiauskas 2018). Our study finally settles the issue by indicating that the Caspian and Black Sea basins harbour two and one, respectively, evolutionary independent lineages that have diverged before the Pleistocene. This clearly indicates that *D. bispinosus* is native to both the Caspian and the Black Sea basins. However, a detailed molecular study of specimens from the Don and Volga rivers is required to confirm the origin of these particular populations.

**Geological history and phylogenetic pattern**

We consider that the uncovered phylogenetic patterns and associated divergence times coincide with the geological history of the Ponto-Caspian region. In the late Miocene the Paratethys Sea was divided into more or less isolated parts (western part became more brackish, eastern – marine) by the mountain uplift in the Alpine belt (Popov et al. 2004; Palcu et al. 2021). Based on our molecular clock results, it appears that the divergence of *D. bispinosus* from the clade encompassing *D. haemobaphes* and *D. villosus* took place in this period (Figure 2). This is also consistent with the timing of several extant Ponto-Caspian radiations (8–12 Mya) observed not only in amphipods (Hou & Sket 2016), but also myid crustaceans and gobiiid fishes (Auzijonyte et al. 2006; Neilson & Stepken 2009). Then, at the end of the Miocene, the uplift of the Caucasus started dividing the Caspian Sea basin from the Azov-Black Sea basin (Zenkevich 1963; Cristescu et al. 2003, Figure 1(c);Grigorovich et al. 2003, Figure 1; Popov et al. 2006, Figure 2). This event could be the reason why *D. bispinosus* 3 separated from *D. bispinosus* 2 and *D. bispinosus* 1 (possibly as a result of allopatry). Later, in the early Pliocene, the Caspian Sea was completely separated from the Azov-Black Sea and, since then, the fauna of these two basins developed in reciprocal isolation (Zenkevich 1956). However, in the late Pliocene, the recurrent transgressions of the Caspian Sea led to reconnections of the two basins via the Kumomanič Strait (Popov 1955; Mordukhai-Boltovskoj 1960, Table 13; Grigorovich et al. 2003, Table 1; Doluhkanov et al. 2010; Krijgsman et al. 2019, Figure 3(b)). These reconnections (2–3.4 Mya, Grigorovich et al. 2003, Table 1) could be the reason for the splitting of the MOTUs *D. bispinosus* 1 and *D. bispinosus* 2 (Figure 2), the latter emerging possibly as consequence of re-colonisation of the Caspian Sea. However, the aforementioned scenario should necessarily be considered with caution because it is also possible that *D. bispinosus* 3 and the ancestor of clades 1 and 2 evolved in sympathy in the Caspian Sea, and then *D. bispinosus* 1 evolved as a result of colonization of the Pontic basin. It is not possible to confidently reject either of these scenarios based on the available data. More localities have to be examined in order to gain a more complete picture of the evolutionary history of *D. bispinosus*.

**Remarks on distribution and ecology of *D. bispinosus***

*Dikerogammarus bispinosus* was originally described as a subspecies of *D. villosus* (*D. villosus bispinosus*), occurring in areas with clean and cold water near deep “pits” (12–22 meters) of the Dnieper River (Martynov 1925), which are presently flooded by the Kakhovka reservoir. *Dikerogammarus bispinosus* samples from the Dnieper River preceded by Martynov (1925) were collected by Dr. Beling and Dr. Markovsky. In their later works Beling (1930, 1939) reported a freshwater *D. villosus* population distributed upstream the Dnieper River (above cities Nikopol, Dniepr, Kremenchuk), while Markovsky (1954) described the distribution of two subspecies for the lower Dnieper, confirming *D. v. bispinosus* in the area of the modern Kakhovsk reservoir and the nominative *D. villosus* from the delta and estuary. The latter author reported also an intentional introduction of the nominative *D. villosus* to the upper Dnieper area (Kyiv). Thus, the distribution of *D. bispinosus* in the Dnieper River is restricted only to the region it was described from. Yet, this species was also reported from the Dniester and Danube basins.

In the Dniester Basin, *D. v. bispinosus* was reported to occur upstream from Galich (upper river basin) (Dedyu 1967). Our *D. bispinosus* samples from the Dniester River were also collected in this region.

In the Danube Basin, Cărăuşu (1943) reported *D. v. bispinosus* from the lower Danube (near Oltenița). There are also records of *D. bispinosus* from the middle Danube (Germany, Austria, Hungary, Slovakia, Croatia, Serbia, Romania) (Dudich 1947; Borza et al. 2015, 2017, 2021; Brtek 2001; Copilaş-Ciocianu & Arbačiauskas 2018, Figure 3) where it prefers riverine habitats in the middle stretches of large rivers (Borza et al. 2017). To sum up, almost all the historical records of *D. bispinosus* from the Black Sea basin are of riverine freshwater origin. However, it also occurs along the rocky coast of the Black Sea near Odessa,
but that zone is desalinated by coastal runoff (Son et al. 2010).

In the Azov region, *D. bispinosus* is considered as an invasive species with reliable reports known from the lower Don (Sayapin 2003). In the Caspian basin it is reported also as an invasive from the middle Volga (Voronin & Yermokhin 2004; Filinova & Sonina 2012) and from the lower Ural (Copilaș-Ciocianu & Arbačiauskas 2018). Some ecological differences between the studied MOTUs can be observed, in particular biotopes where they were retrieved from. While our records of *D. bispinosus* 1 from riverine freshwater (the Dniester and Danube Rivers) are congruent with literature data, both *D. bispinosus* 2 and *D. bispinosus* 3 came from the littoral of Caspian Sea, and are therefore ecologically distinct. This distribution pattern may also be explained by (1) different historical past, (2) the effects of different salinity compositions in the neighboring seas (Beklemishev 1922; Mudie et al. 2017), especially the high level of sulfates and the low level of chlorides in the Caspian water which controls the distribution of biota through “chloridophobia” (Mordukhai-Boltovskoj 1960), and (3) the impact of a stronger competing fauna (in particular, the Mediterranean one), which may displace local species.

The putative ecological differences observed among MOTUs raise the interest to reveal the intra-population affinity of the populations reported from the Don, Volga, and Ural Rivers (Voronin & Yermokhin 2004; Filinova & Sonina 2012; Copilaș-Ciocianu & Arbačiauskas 2018), as well as those occurring in the coastal waters of the Black Sea (Son et al. 2010). So far, it appears that *D. bispinosus* 1 is the only invasive lineage. Additional molecular data from the Volga, Don and Ural rivers would show whether the remaining two lineages extend beyond their native range or not.

**Morphological differences of lineages**

Until recently there were some issues regarding the taxonomic status of *D. bispinosus* with respect to *D. villosus* or *D. haemobaphes*. Some authors considered it a subspecies of *D. villosus* (e.g. Cărăuşu et al. 1955), while others even went as far as considering that *D. villosus* (together with *D. bispinosus*) is a synonym of *D. haemobaphes* (Piatakova & Tarasov 1996). However, the integrative taxonomic study made by Müller et al. (2002), and based on mitochondrial genomes by Mamos et al. (2021) revealed genetic distinctions among these three species and provided clear morphological features for identification: they differ in number of spines on 1st and 2nd urosomites, length of setation on peduncle and flagellum of 2nd antenna, and length of propodus setation of gnathopods. Within the framework of our research we did not observe clear differences among *D. bispinosus* MOTUs regarding the second antenna, but there were significant differences regarding the setal patterns on the inner side of the propodus of the second gnathopod (Figure 4). MOTUs *D. bispinosus* 1 and 2 seem more similar to each other by having longer and denser setae, which reflects well their phylogenetic relationship (Figures 1, 2). The setal patterns of gnathopod two in *D. bispinosus* specimens from the lower Ural (Kazakhstan) published by Copilaș-Ciocianu & Arbačiauskas (2018; Figure 2) are similar to these two.

This suggests that the MOTUs may in fact be distinct species, yet more material from the Black, Azov, and Caspian seas, as well as additional molecular markers and morphometry are needed to fully clarify the taxonomic status of the *D. bispinosus* lineages identified in this study.

**Conclusions**

Our study revealed that *D. bispinosus* comprises three independently evolving lineages which are distinct at the molecular (nuclear and mitochondrial), and morphological level and, partially, geographically. One of these lineages, native to the lower reaches of Pontic rivers, is gradually spreading throughout southwestern and Central European rivers, while the two others are apparently restricted to the Caspian Sea. We also clarify that *D. bispinosus* is not a recent invader in the Caspian Sea, but a native species. Our findings show that even relatively simple pilot molecular research may bring surprising and valuable results also in the case of long-studied invasive species.

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