

A new freshwater amphipod (Amphipoda, Gammaridae), *Gammarus tumaf* sp. nov. from the Gökgöl Cave, Türkiye

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Abstract

A new amphipod species belonging to the genus *Gammarus* is described from the Gökgöl Cave, Zonguldak Province, Türkiye. The newly-identified species is relatively small (13 mm) and is a member of the *Gammarus pulex*-group by the presence of numerous long setae along the posterior margins of pereopods 3 and 4. The specimens were sampled from a shallow pond located in the dark zone (about 1 km inside the entrance) of the cave. Minute eyes, setose (both peduncle and flagellar segments) second antenna, slightly swollen flagellar segments of the second antenna, setose pereopods 3 and 4 and relatively short endopod/exopod ratio of the third uropod are the character combination of the newly-identified species in addition to lacking body pigmentation. The molecular phylogeny, based on the concatenated dataset (28S+COI, 1495 bp) indicated that the new species was resolved from the other *Gammarus* species by high bootstrap (NJ: 100, ML: 100). In addition to *Gammarus tumaf* sp. nov., mtDNA COI and nuclear DNA 28S gene data of *Gammarus baysali* Özbek et al., 2013 were recorded for the first time. The newly-identified species was well-differentiated from the genetically closest species, *G. baysali*, with genetic distance of 12.22% and 0.55% for the COI and 28S genes, respectively. Detailed descriptions and drawings of the extremities of the holotype male were given and the morphology of the newly-identified species is compared with its relatives.

Key Words

benthos, cave, identification key, invertebrate, molecular identification, new species

Introduction

Gammarus Fabricius, 1775 is one of the richest genera of the Gammaridae Leach, 1814 family with more than 225 species worldwide (Vainola et al. 2008). The members of the genus are widely distributed in the Palearctic and Holarctic Regions. They inhabit both epigeal (seas, lakes and streams) and hypogean (caves, wells and groundwater) habitats (Karaman and Pinkster 1977).

The first study on the *Gammarus* genus in Turkish inland waters started with the identification of *Gammarus argaeus* Vávra, 1905 from Erciyes Mountain by Vávra (1905). In the last two decades, many studies have been reported by both foreign and native researchers, increasing compared to previous years. As a general

result of these studies, knowledge about the distribution of the *Gammarus* species in Turkish inland waters has increased. In addition, new *Gammarus* species, most of which are endemic, have been identified from both ground and surface waters. To date, 51 *Gammarus* taxa have been recorded from the inland waters of Türkiye and 30 of them are endemic to the country (İpek and Özbek 2022).

DNA barcoding, one of the molecular techniques developed in recent years, has brought an integrative approach by contributing to species identification, based on traditional taxonomy (Dayrat 2005). DNA barcoding studies, first introduced by Arnot et al. (1993) and later gained popularity with the work of Hebert et al. (2003), have allowed speedy, reliable and cost-effective species identification, based on specific nucleotide sequences on the genome.

Some mitochondrial (such as COI, Cytb, 16S, 12S) and nuclear DNA (such as 18S, 28S, ITS, EF1- α) genes are powerful markers for deducing evolutionary relationships at the species, genera, family and higher levels (Johns and Avise 1998; Englisch and Koenemann 2001; Cristescu and Hebert 2005; Kartavtsev and Lee 2006; Witt et al. 2006; Hou et al. 2007, 2011; Costa et al. 2009; Hupało et al. 2020; Morhun et al. 2022). Studies to date have confirmed that molecular techniques are a powerful tool for the discovery of cryptic species, as well as revealing speciation and population diversity within the genus *Gammarus* (Meyran et al. 1997; Meyran and Taberlet 1998; Müller 2000; Hou et al. 2007; Lagrue et al. 2014; Mamos et al. 2014; Weiss et al. 2014; Wysocka et al. 2014; Copilaş-Ciocianu et al. 2018; Copilaş-Ciocianu et al. 2019).

This study aims to examine the individuals collected from Gököl Cave, Zonguldak Province, Türkiye in terms of morphological and molecular features. Additionally, the molecular analysis of *Gammarus baysali* Özbek et al., 2013, which was reported from another cave (Cumayanı Cave) located in a geographically close location, was also carried out. Detailed descriptions and drawings of the extremities of the holotype male are given and the morphology of the newly-identified species is compared with its relatives.

Materials and methods

Gököl Cave is located on the road around Üzülmüş Region at the 4th km of Ankara highway, to the southeast of the city, in Ercek Village, NW Anatolia, Türkiye and is an active cave with a length of 3,350 m (Fig. 1) (Yamaç et al. 2021). The deepest point from the cave entrance is -5 m and the highest point is +35 m. From the entrance to the 830th metre of the cave, it is open to touristic activities. The main branch of the Cave lies down in the east-west direction and the Cave has four lateral branches extending in the north-south direction. Two of them extend in the north direction and the other ones in the south direction. The waters coming from the siphon, which is located at the end of the main branch, continue to flow from the first branch that develops in the south direction (Fig. 1a, b). Water flow is present throughout the year in the Cave. Additionally, a water inflow in the form of leakage from the cracks in the ceiling and walls of the Cave is observed.

Alive amphipod specimens were photographed and sampled with the help of a hand aspirator for the taxonomical investigation (Fig. 1c).

To measure the body length, individuals were straightened with forceps under a stereomicroscope and the distance between the rostrum and the base of the telson was measured.

Permanent slides of the holotype individual were prepared using the high-viscosity mount, CMCP-10. Photographs of the extremities were taken with a digital camera connected to a microscope (Olympus CX41). Photos were processed with image processing programmes and a standard pen. A digitiser board (Wacom PTH-451) and stan-

dard pen connected to a PC were used for detailed drawings of the extremities. Scaled drawings of the extremities were made on the photographs (Coleman 2003). Some of the collected samples are kept in Eskişehir Technical University Zoology Museum and some others in the Museum of the Faculty of Fisheries, Ege University (ESFM).

DNA extraction, PCR amplification and sequencing

Genomic DNA of *Gammarus* specimens was extracted on the Qiacube Automated DNA Isolation Device (Qiagen, Valencia, CA) according to the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) protocol. Mitochondrial cytochrome c oxidase subunit I gene (COI) and the nuclear large subunit ribosomal RNA gene (28S) were amplified from the extracted DNA. Amplification of the COI marker was performed with the primers UCOIF (5'- TAWACTTCDGGRTGRCCRAAAAYCA-3') and UCOIR (5'- ACWAAYCAYAAAGAYATYGG-3') according to the PCR protocol of Costa et al. (2009). Amplification of the 28S marker was performed with the primers 28F (5'- TTAGTAGGGGCGACCGAACAGGGAT-3') and 28R (5'- GTCTTCGCCCCATGCCCAACTGA-3') according to the PCR protocol of Hou et al. (2007).

PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and one-directional sequencing of PCR products was performed with an ABI PRISM 3730×1 Genetic Analyser using a BigDye Terminator 3.1 cycle sequencing ready reaction kit (Applied Biosystem) at MacroGen Europe according to the Sanger method.

Molecular data analyses

In the present study, a total of one individual of *Gammarus tumaf* sp. nov. and one of *Gammarus baysali* Özbek et al., 2013 were sequenced. In addition, previously-published sequences of 12 species as in-groups and one species as an outgroup from the GenBank (<http://www.ncbi.nih.gov>) containing both the COI and 28S sequences were downloaded for use in molecular analyses. GenBank accession numbers, locations and reference information of the sequences used in molecular analyses are given in Table 1.

The raw COI and 28S sequences generated in the present study were initially edited by checking their chromatograms in the *Bioedit* 7.2.5 programme (Hall 1999). To perform the molecular analyses, the COI and 28S sequences, both newly generated and downloaded from GenBank, were added end-to-end to obtain a concatenated dataset (28S+COI). All sequences were then aligned with the Clustal W method (Thompson et al. 1994), trimmed at the ends and converted to a FASTA format file.

The inter-specific pairwise genetic distances for both markers were calculated separately, according to the uncorrected *p*-distance in MEGA X software (Kumar et al. 2018). Phylogenetic relationships amongst the

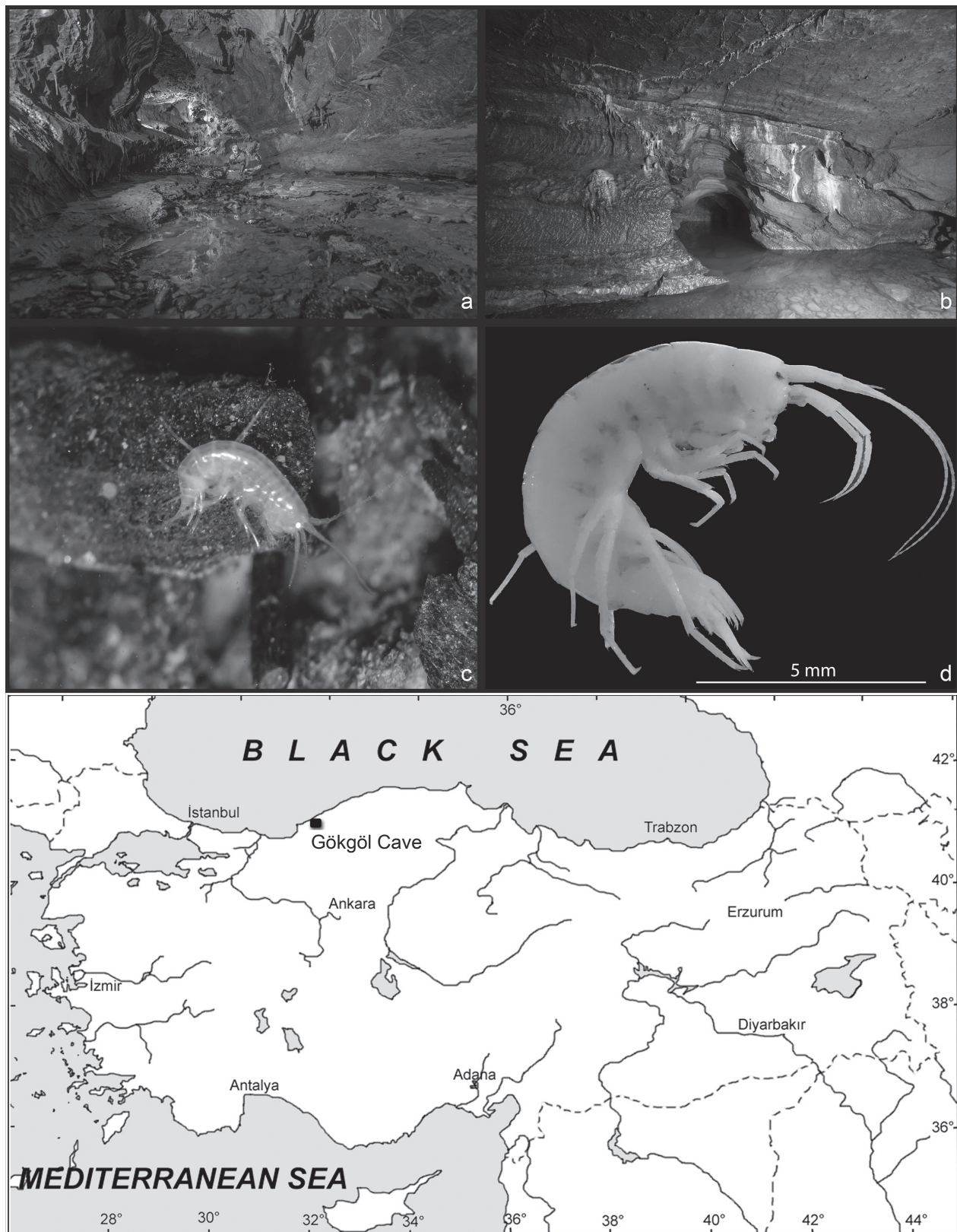


Figure 1. Inside the Gökgöl Cave (a); type locality of *Gammarus tumaf* sp. nov. (b); photo of an alive specimen (c); habitus of the holotype male (d) and the geographical location of the Gökgöl Cave (Cave photos: M. Elverici).

Gammarus species were estimated by using Neighbour-Joining (NJ) and Maximum Likelihood (ML) methods in *MEGA X* software. The NJ tree was generated according to the *p*-distance model. The ML tree was generated according to the GTR+G+I model (Tavaré 1986) and the

best-fit substitution model was selected with the lowest Akaike Information Criterion (AIC) score in jModelTest 0.1.1 (Posada 2008). Confidences of the NJ and ML analyses were estimated by the bootstrap test (Felsenstein 1985) using 1000 replicates.

Results

Gammarus tumaf sp. nov.

<https://zoobank.org/654377E5-2984-4189-B1EF-7D7346A414EF>

Figs 1–5

Holotype. Male, 12.6 mm (ESFM-MALI/20-10), Zonguldak Province, Türkiye (41°26'26.42"N, 31°49'57.48"E), 03.ix.2020; collected by M. Elverici.

Paratypes. 3 males and 3 females, (ESFM-MALI/20-11), same data as holotype.

Diagnosis. A medium-large species with a smooth body, lacking body pigmentation, minute eyes, setose (both peduncle and flagellar segments) second antenna, slightly swollen flagellar segments (second antenna), setose pereopods 3 and 4 and relatively short endopod/exopod ratio of the third uropod.

Description of holotype male. Head: Rostrum absent, inferior antennal sinus deep, rounded. Eyes small, ovoid; shorter than the diameter of the first peduncular segment of antenna 1 (Fig. 1d).

Antennae: Antenna 1 is longer than half of the body length; the length ratio of the peduncular segments is 1:0.7:0.5; peduncle segments bear a few groups of minute setae; the length of the setae is much shorter than the segment where they are implanted; the main flagellum with 30 segments; each segment bears a few short setae in distal side; aesthetasc absent; accessory flagellum 5 segmented (Fig. 3D). Antenna 2 is shorter than antenna 1 (ratio 1:0.56); the antennal gland cone is straight and short; setation is rich both on peduncular and flagellar segments; peduncular segments 4 and 5 bear many groups of setae; the setae on the ventral part of the peduncle segments are longer than the dorsal ones and can be up to 1.5 times longer than the diameter of the segment; flagellum consists of 13 segments; flagellar segments are setose and swollen; each segment bears many long setae on both dorsal and ventral sides; calceoli absent (Fig. 3A).

Mouthparts: Left mandible (Fig. 2H) with 5-toothed incisor, lacinia mobilis with 4 dentitions, molar tritritative. The first article of palp without setae, the second one bears 13 setae; the setae become shorter from distal to proximal. The third segment has 28 D-setae, 4–5 E-setae, one group of A- and one group of B-setae. C-setae absent.

Right mandible (Fig. 2I) has a 3-toothed incisor and bifurcate lacinia mobilis.

Right maxilla 1 (Fig. 2D) is asymmetric to the left, it has 20 plumose setae along the inner margin of the inner lobe. The outer lobe bears 11 distal stout serrate spines and some tiny setules on the inner margin. Palp of the outer lobe with no setae in the first segment and five stout spines and two simple setae on the distal part of the second segment, in addition to two marginal setae along the outer margin. The second article of left palp elongated and bears 8 spines and 5 simple setae on its distal part and no setae along the outer margin.

Lower lip (Fig. 2B) has no inner lobe and bears numerous small simple setae along the distal margins of both lobes.

Upper lip (Fig. 2A) with numerous minute setules in the distal part.

Maxilla 2 (Fig. 2F, G) has 20–25 simple setae in the distal part of the outer lobe and a few tiny hairs along the outer margin. The inner lobe also has 8–10 simple setae in the distal part in addition to 20 plumose setae located in a diagonal row along the inner margin. There are also a few tiny hairs in the proximal part of the inner margin of the lobe.

Maxilliped (Fig. 2E) inner plate has 3 tooth-like spines and a spine in the distal part and the distal corner, respectively. Additionally, there are 6 plumose setae along the inner margin of the lobe. Outer plate armed with 4–5 serrate stout setae in the distal part and 13 spines along its inner margin.

Coxal plates: Coxal plate 1 is rectangular, the distal part slightly widened, the ventral margin slightly convex and bears 4 antero-distal setae and one postero-distal seta in addition to some tiny setules along the ventral margin (Fig. 3B). Coxal plate 2 is in the shape of an elongated rectangle, distal part narrower than the proximal, the ventral margin is highly convex and setation is similar to that of coxal plate 1 (Fig. 3C). Coxal plate 3 is similar in shape and setation to coxal plate 2, with less narrowing in the distal part (Fig. 4A). The ventral edge of the fourth coxal plate is almost straight and bears 4 and 7 setae along the anteroventral and posterior margins, respectively (Fig. 4B). Coxal plates 5 and 6 bilobate, each one having one seta in the anterior and 4 setae in the posterior lobes (Fig. 4D, E). Coxal plate 7 with 3–4 setae on the posteroventral margin (Fig. 4C).

Gnathopods: Basal segment of gnathopod 1 bears many long setae along both margins, the length of the setae can be more than twice the diameter of the segment. Ischium bears a group of setae in posteroventral corner. Posterior margin of the merus with 4 groups of setae. Carpus triangular and bears two groups of setae along the anterior margin in addition to many setae groups on both ventral and posterior sides. Propodus pyriform, the length/width ratio is 1: 0.34, anterior margin with three groups of setae, medial palmar spine is present, posterodistal corner armed with a strong spine in addition to some small spines, posterior margin bears 4–5 groups of setae. Dactylus reaches the posterodistal corner and bears a simple seta along the outer margin in addition to a small setule around the distal part of the inner margin (Fig. 3B, B').

Basis and ischium of gnathopod 2 have a similar setation to that of gnathopod 1. Merus and carpus are more setose than those of gnathopod 1. Carpus triangular, densely setose along the posterior margin in addition to three groups of setae along the anterior margin. Propodus is densely setose and has a sub-rectangular shape, the length/width ratio is 1: 0.55, anterior margin bears 4 groups of setae, some setae have curled distal tips, posterior margin with many groups of setae, medial palmar spine is present, posterodistal corner armed with three strong spines in addition to some small spines. Dactylus reaches the posterodistal corner and bears a simple seta along the outer margin in addition to a small setule around the distal part of the inner margin (Fig. 3C, C').

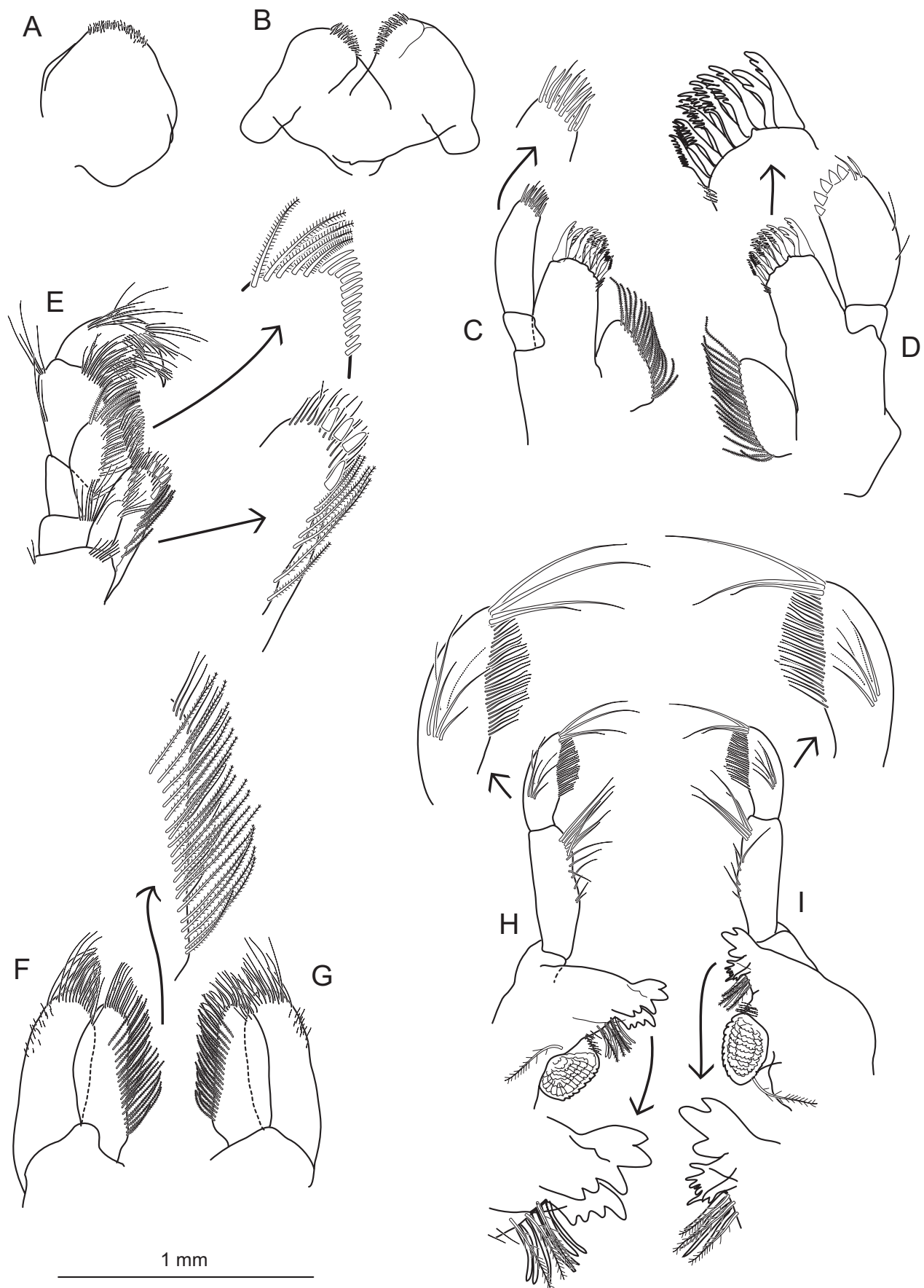


Figure 2. Mouth parts of *Gammarus tumaf* sp. nov., (holotype male). **A.** Upper lip; **B.** Lower lip; **C.** Left maxilla 1; **D.** Right maxilla 1; **E.** Maxilliped; **F.** Left maxilla 2; **G.** Right maxilla 2; **H.** Left mandible; **I.** Right mandible.

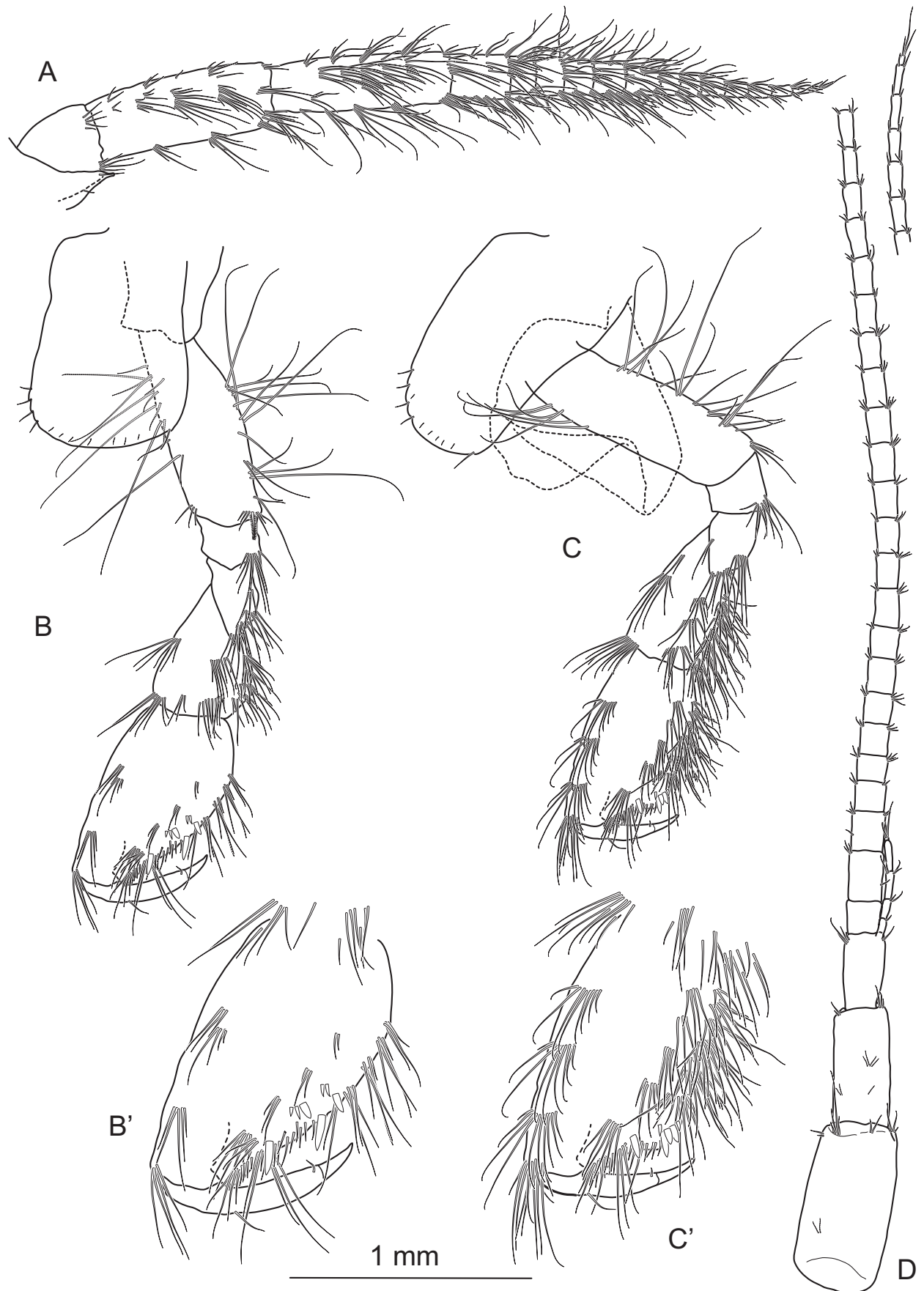


Figure 3. Extremities of *Gammarus tumaf* sp. nov., (holotype male). **A.** Antenna 2; **B.** Gnathopod 1; **B'.** Palm of gnathopod 1; **C.** Gnathopod 2; **C'.** Palm of gnathopod 2; **D.** Antenna 1.

Pereopods: Anterior and posterior margins of the pereopod 3 bear 4–6 groups of setae, the setae along the posterior margin are much longer than those in the anterior margin and posterior margins of the merus, carpus and propodus bear long and slightly curved setae, the setae can be three times longer than the diameter of the segment where they are implanted. Dactylus slim, a minute plumose seta occurs on the outer margin; the inner margin with two small setules (Fig. 4A).

The basal segment of pereopod 4 has a similar setation to that of pereopod 3. Ischium, merus, carpus and propodus have groups of setae along their posterior margins, but they are much shorter and less than those in pereopod

3, the length of the setae can be as long as (or slightly longer) than the diameter of the segment where they are implanted. Dactylus slim, a minute plumose seta occurs on the outer margin; the inner margin with two small setules (Fig. 4B).

Posterior margins of the basal segments of pereopods 5 to 7 are more or less convex and bear many short setae, anterior margins with 5–7 small spines and no setae present on the inner surfaces of the basal segments; there is a spine in the posteroventral corner of the basal segment of pereopod 7. Pereopods 6 and 7 bear no setae along the anterior margins of ischium, merus and carpus, while pereopod 5 has a few setae longer than the accompanying

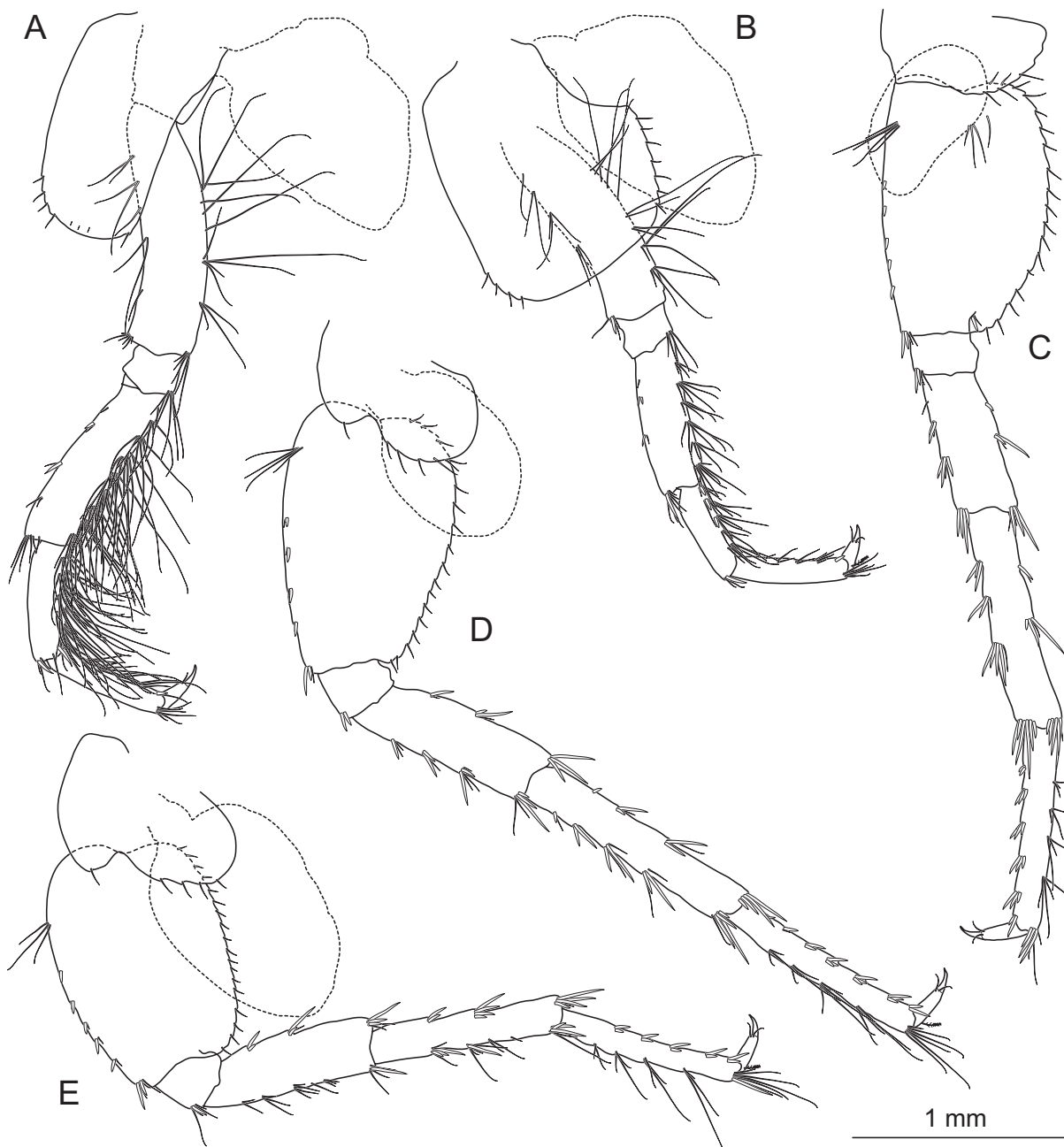


Figure 4. Extremities of *Gammarus tumaf* sp. nov., (holotype male). **A.** Pereopod 3; **B.** Pereopod 4; **C.** Pereopod 7; **D.** Pereopod 6; **E.** Pereopod 5.

spines along with the mentioned segments. Propodus of pereopod 5 to 7 with 4–5 groups of long setae groups along their outer margins in addition to 5–6 groups of small spines along their inner margins. Setae on the outer margins of the propodus of pereopod 7 are shorter than those in pereopod 5 and 6. Dactylus slim, a minute plumose seta occurs on the outer margin; the inner margin with two small setules (Fig. 4C–E).

Epimeral plates: They are neither curved nor sharply pointed. Epimeral plate 1 bears 5–6 long setae in addition to a few setules along the anterior margin and posterior margin with 6–7 tiny setae. The posteroventral corner is angular (Fig. 5E). Epimeral plate 2 bears 3–4 setae in the anteroventral corner, the ventral margin is armed with 3 spines and the posterior margin with 4–5 setules. The posteroventral corner is angular (Fig. 5F). Epimeral plate 3 is slightly pointed; the anteroventral corner bears 6–7 setae; the ventral margin is armed with 4 spines; the posterior margin bears 6–7 setules (Fig. 5G).

Urosomites: Not elevated. Each segment bears a median and two dorsolateral groups of armaments; each of them consists of 1–2 spines and 3–4 accompanying setae (Fig. 5H).

Uropods: Uropod 1 has a spine in the distoventral corner of the base; the peduncle is longer than rami; the length ratio is about 1:0.75. Peduncle with a spine in the outer margin of the proximal part in addition to 6 spines along the inner margin and 2 spines in the distal part. Both rami are of equal size and bear 4–5 spines along their inferior margins in addition to 4–5 distal spines (Fig. 5D).

Uropod 2 is smaller than the first one; the length ratio is about 1:0.7; the peduncle segment is longer than the rami and bears 4 spines along the inner margin and the outer margin is bare. The length and armaments of both rami are similar to each other, they bear 2–3 spines along their inner and outer margins in addition to 4–5 longer spines on their distal tips (Fig. 5C).

Uropod 3 is setose and bears simple and plumose setae. The peduncle segment is much shorter than the outer ramus and the length ratio is about 1:0.38. The outer ramus has two articulated and densely setose along both margins; the outer bears 4 groups of spines accompanied by groups of long simple setae; the inner margin with plumose setae; the second article is well developed and longer than the surrounding distal spines. The inner ramus is about 0.75× the length of the outer ramus. It bears two spines along the outer margin in addition to groups of simple and plumose setae; the inner margin bears both simple and plumose setae (Fig. 5B).

Telson: Telson lobes cleft, each lobe bears 2 spines and 2–3 simple setae in their distal parts. The setae are longer than the spines. There are some setae on the dorsal surface of the lobes in addition to two plumose setules. The length/width ratio of each is about 1:0.5 (Fig. 5A).

Etymology. The species epithet (*tumaf*) is the abbreviation of the Turkish Caving Federation.

Description of females. Smaller than males. Except for the sexual dimorphism indicated for the genus

Gammarus, females do not show obvious differences from males. Setation and armaments of the extremities are more or less similar to those of males.

Variability. In some individuals, the size of the eyes is slightly smaller than in the holotype. The number of flagellar segments in Antenna 1 and Antenna 2 can be variable. The number of flagellar segments of Antenna 1 in paratype individuals ranged from 32 to 37. Similarly, the number of flagellar segments of Antenna 2 varied between 11 and 14.

Results of molecular data analyses

We produced the partial sequences of the COI and 28S genes of *Gammarus tumaf* sp. nov. and *Gammarus baysali* Özbek et al., 2013 (from Cumayanı Cave) and performed molecular analyses, based on concatenated data. A concatenated dataset with a total length of 1495 bp (for the COI fragment 573 bp and for the 28S fragment 922 bp including gaps) were sequenced. While no stop codon, insertion, deletion and a gap were detected in the protein-coding mtDNA COI gene, there are insertions and deletions in the nuclear 28S gene. Additionally, newly-generated sequences are deposited in GenBank accession numbers, for COI [ON749780–ON749781](#) and 28S [ON751931–ON751932](#).

We performed phylogenetic and genetic distance analyses with the topotype sample sequences of the nominal taxa that we could find especially in GenBank. Otherwise, sequences considered representative of the species were preferred (Table 1).

For the COI gene, the genetic distance amongst the species ranged from a minimum of 12.22% (*Gammarus tumaf* sp. nov. – *G. baysali*) to a maximum of 16.00% (*G. kesslerianus* – *G. plaitisi*). The next minimum genetic distance value is 16.06% (*G. uludagi* – *G. plaitisi*).

For the 28S gene, it ranged from a minimum of 0.55% (*Gammarus tumaf* sp. nov. – *G. baysali*) to a maximum of 7.38% (*G. roeselii* – *G. balcanicus* Kolasin Montenegro). The next minimum genetic distance value is 0.66% (*G. pulex* – *G. plaitisi*) (Table 2).

According to phylogenetic results, NJ and ML methods provided similar topologies for the *Gammarus* species. Many species lineages were supported by high bootstrap values. *Gammarus tumaf* sp. nov. is closely related to *G. baysali* in phylogenetic trees, but differs from it (Fig. 6).

Discussion

Gammarus tumaf sp. nov. belongs to the *Gammarus pulex*-group because of the presence of long setae along the posterior margins of the merus and carpus of pereopods 3 and 4 (Fig. 4A, B). The newly-identified species shows some common characteristics seen in many other members of the group. *Gammarus tumaf* sp. nov. is very similar to *Gammarus komareki* Schäferna

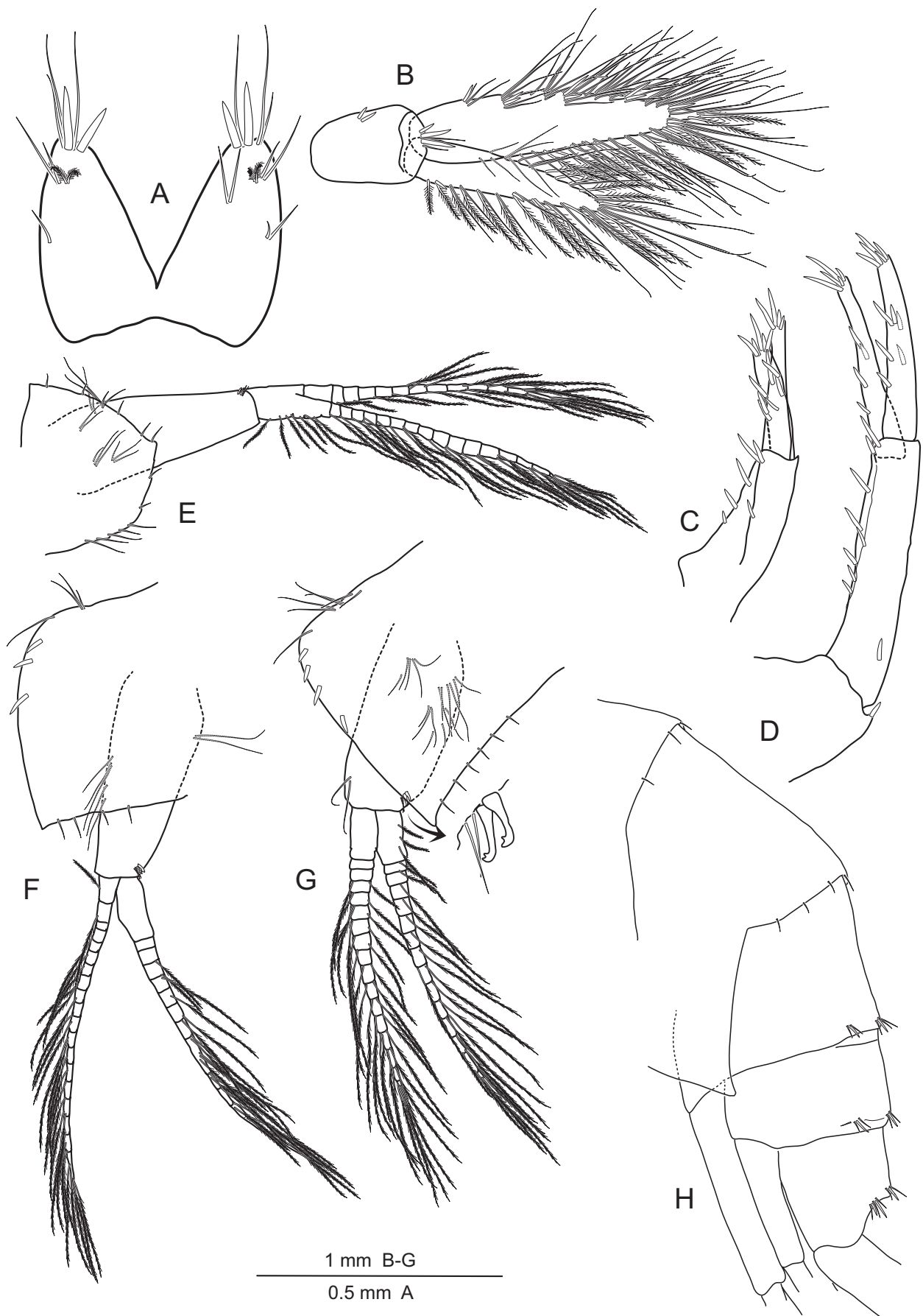


Figure 5. Extremities of *Gammarus tumaf* sp. nov., (holotype male). **A.** Telson; **B.** Uropod 3; **C.** Uropod 2; **D.** Uropod 1; **E.** Epimeral plate 1; **F.** Epimeral plate 2; **G.** Epimeral plate 3; **H.** Urosomites.

Table 1. List of samples used in molecular analysis.

Species	Locality	28S	COI	References
<i>Gammarus tumaf</i> sp. nov. (T)	Gököl Cave, Türkiye	ON751931	ON749780	This study
<i>G. baysali</i> (T)	Cumayani Cave, Türkiye	ON751932	ON749781	This study
<i>G. balcanicus</i> (T)	Kolašin, Montenegro	JF965640	JF965834	Hou et al. (2011)
<i>G. balcanicus</i>	Virpazar, Montenegro	JF965654	JF965848	Hou et al. (2011)
<i>G. fossarum</i> (T)	Regensburg, Germany	JF965696	JF965886	Hou et al. (2011)
<i>G. kesslerianus</i> (T)	Simferopol, Crimea, Ukraine	JF965721	JF965909	Hou et al. (2011)
<i>G. kischineffensis</i> (T)	Targu Bujor, Romania	MG987529	MG987571	Copilaş-Ciocianu et al. (2018)
<i>G. komareki</i>	Mazandaran, Iran	JF965723	JF965911	Hou et al. (2011)
<i>G. komareki</i> (T)	ca 200km SE Sofia, Bulgaria	JF965725	JF965913	Hou et al. (2011)
<i>G. lacustris</i>	Bled, Slovenia	JF965728	JF965915	Hou et al. (2011)
<i>G. plaitisi</i>	Tinos, Komi, Greece	MT999102	MT999049	Hupalo et al. (2020)
<i>G. pulex</i> (T)	Slovenia	JF965767	JF965943	Hou et al. (2011)
<i>G. roeselii</i>	Netherlands	JF965771	JF965947	Hou et al. (2011)
<i>G. uludagi</i>	Evia, Greece	JF965817	JF965986	Hou et al. (2011)
<i>Pontogammarus robustoides</i>	Delta Volgi, Russia	JF965822	JF965990	Hou et al. (2011)

Note: (T) Topotype samples of nominal taxa.

1922, which is distributed over a large geographical area including the Balkans, Black Sea coasts, northern parts of Türkiye and Iran (Karaman and Pinkster 1977; Zamanpoore et al. 2011; İpek and Özbek 2022). The characteristic densely setose peduncular and flagellar segments of antenna 2 and many other morphological features of the newly-identified species suggest that it can be closely related to *G. komareki*. Molecular analysis results also support this assumption. On the other hand, it differs from *G. komareki* by the following features:

- having fewer setae on the first segment of the mandible palp,
- having fewer D-setae on the mandible palp.
- absence of long setae along the anterior margins of pereopods 5–7,
- having a shorter inner lobe of uropod 3.

Karaman and Pinkster (1977) state that the setation of the anterior margins of pereopods 5 to 7 is a variable feature, even in some populations there are no setae along the anterior margins of pereopods 5–7 in some populations.

We included two *G. komareki* reference sequences in molecular analyses. The two most distant individuals constituting the geographical distribution line of *G. komareki* species are Sofia, Bulgaria (topotype sample) and Mazandaran, Iran samples. The newly-identified species was resolved in the phylogenetic tree with high support values (NJ: 95–100, ML: 95–100) from these two *G. komareki* samples. *Gammarus tumaf* sp. nov. differs from both samples with pairwise genetic distance values of 18.15% and 22.34% for the COI gene and 1.75% and 1.75% for the 28S gene, respectively.

Gammarus tumaf sp. nov. also shows morphological similarities with *Gammarus baysali* which was reported from another geographically close cave. However, *G. baysali* has 4 setae along the outer margin of the palp of the right maxilla in addition to 6 blunt distal teeth, whereas the present species has 2 setae and 5 distal teeth (Özbek et al. 2013).

In this study, in addition to *Gammarus tumaf* sp. nov., mtDNA COI and nuclear DNA 28S gene data of *Gammarus baysali* Özbek et al., 2013 were recorded for the first time. It is important to create genetic records from type specimens to avoid confusion that may occur in species identification. The molecular phylogeny, based on the concatenated dataset (28S+COI, 1495 bp) indicated that the new species was resolved from *Gammarus baysali* species by high bootstrap (NJ: 100, ML: 100). Additionally, the newly-identified species are well differentiated from the genetically closest species, *G. baysali*, with a genetic distance of 12.22% and 0.55% for the COI and 28S genes, respectively.

Gammarus tumaf sp. nov. is similar to *G. kesslerianus* by having setose antenna 2, but differs from it by having a shorter inner lobe of uropod 3.

Gammarus tumaf sp. nov. and *G. kesslerianus* resolved in the phylogenetic tree with relatively strong bootstrap values (NJ:76, ML:65). The genetic distance between the two species is 17.45% and 1.42% for the COI and 28S genes, respectively. Phylogenetic analysis placed *G. kesslerianus* as a sister branch to *G. tumaf* and *G. baysali* species (Fig. 6).

Gammarus tumaf sp. nov. is similar to *Gammarus microps* Pinkster & Goedmakers, 1975 by having minute eyes, but differs from it by having shorter extremities, fewer flagellar segments of antennae 1 and 2, less setose pereopod 4 and more setose carpus of pereopods 5 to 7 (Karaman and Pinkster 1977). Genetic comparison could not be made because molecular data were not available.

The newly-identified species differs from *Gammarus pulex pulex* (L., 1758) by having more setae on the peduncle segments of antenna 2 and from *Gammarus pulex polonensis* Karaman & Pinkster, 1977 by having minute eyes. *Gammarus tumaf* sp. nov., morphologically described in the *G. pulex*-group, differs from the topotype sample of *G. pulex* with its high genetic distance (for COI: 26.18% and for 28S: 4.51%) and moderately-supported bootstrap values (NJ:74, ML:80).

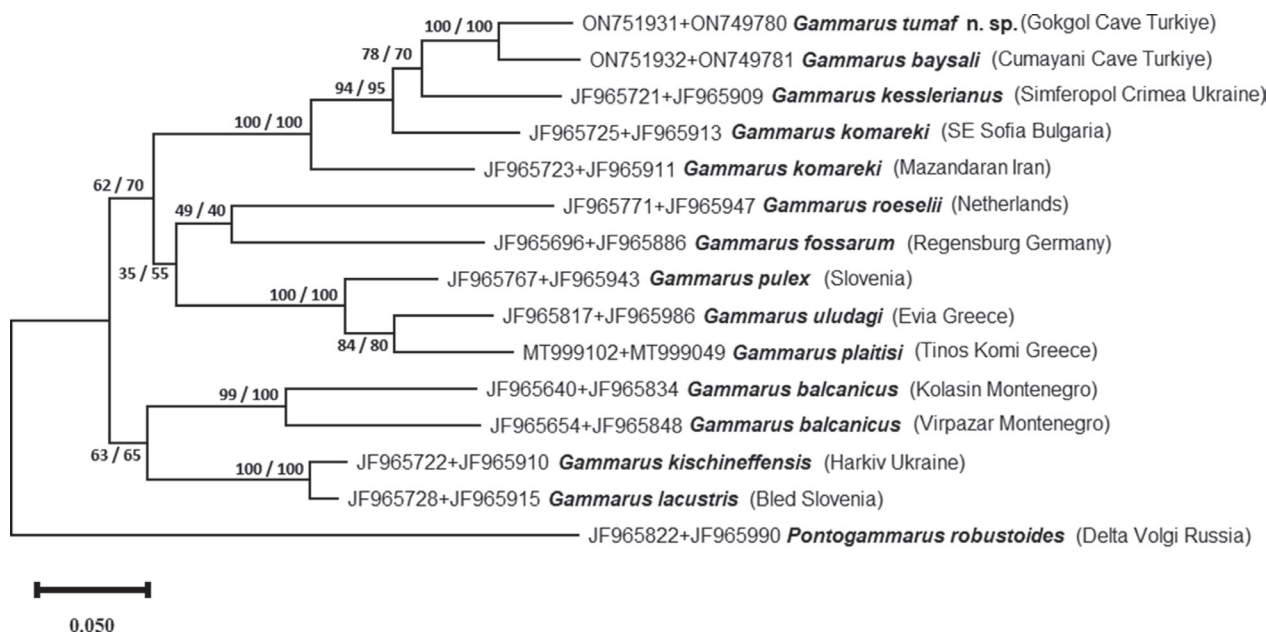


Figure 6. Maximum Likelihood (ML) phylogenetic tree generated, based on the concatenated dataset (28S+COI). ML and NJ methods yielded the same topologies and, therefore, only the ML tree is shown. The bootstrap values of NJ and ML are shown on nodes (NJ/ML).

Table 2. The pairwise genetic distance values amongst the *Gammarus* species, based on the COI dataset (below the diagonal) and 28S dataset (above the diagonal).

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1 <i>Gammarus tumaf</i> sp. nov.		0.0055	0.0142	0.0175	0.0175	0.0551	0.0464	0.0451	0.0441	0.0495	0.0670	0.0664	0.0638	0.0540
2 <i>G. baysali</i>	0.1222		0.0154	0.0165	0.0187	0.0530	0.0454	0.0442	0.0431	0.0463	0.0650	0.0622	0.0618	0.0531
3 <i>G. kesslerianus</i>	0.1745	0.1902		0.0142	0.0098	0.0518	0.0408	0.0418	0.0407	0.0440	0.0615	0.0586	0.0583	0.0474
4 <i>G. komareki</i> (SE Sofia Bulgaria)	0.1815	0.1728	0.1850		0.0197	0.0529	0.0442	0.0396	0.0386	0.0440	0.0593	0.0620	0.0540	0.0453
5 <i>G. komareki</i> (Mazandaran Iran)	0.2234	0.2094	0.2216	0.2112		0.0485	0.0353	0.0341	0.0330	0.0385	0.0582	0.0586	0.0550	0.0496
6 <i>G. roeselii</i>	0.2705	0.2810	0.2705	0.2688	0.2740		0.0475	0.0540	0.0518	0.0551	0.0738	0.0721	0.0673	0.0586
7 <i>G. fossarum</i>	0.2496	0.2653	0.2426	0.2321	0.2618	0.2391		0.0364	0.0353	0.0397	0.0695	0.0633	0.0631	0.0643
8 <i>G. pulex</i>	0.2618	0.2723	0.2792	0.2513	0.2583	0.2356	0.2304		0.0066	0.0099	0.0649	0.0653	0.0595	0.0552
9 <i>G. plaitisi</i>	0.2653	0.2653	0.2862	0.2653	0.2688	0.2618	0.2461	0.1745		0.0088	0.0639	0.0654	0.0585	0.0542
10 <i>G. uludagi</i>	0.2670	0.2757	0.2775	0.2513	0.2600	0.2391	0.2461	0.1640	0.1606		0.0681	0.0686	0.0628	0.0585
11 <i>G. balcanicus</i> (Kolasin Montenegro)	0.2635	0.2548	0.2513	0.2548	0.2461	0.2443	0.2443	0.2164	0.2513	0.2304		0.0365	0.0220	0.0441
12 <i>G. balcanicus</i> (Virpazar Montenegro)	0.2548	0.2565	0.2653	0.2548	0.2757	0.2408	0.2461	0.2112	0.2304	0.2356	0.2042		0.0365	0.0576
13 <i>G. kischineffensis</i>	0.2531	0.2478	0.2408	0.2391	0.2426	0.2461	0.2147	0.2059	0.2321	0.2234	0.1815	0.1955		0.0364
14 <i>G. lacustris</i>	0.2356	0.2269	0.2513	0.2304	0.2443	0.2234	0.2182	0.2094	0.2129	0.2164	0.2234	0.2007	0.1745	

Gammarus tumaf sp. nov. differs from *Gammarus uludagi* Karaman, 1975 by the shape and armaments of telson in addition to the absence of long and curled setae on the palm of gnathopod 2. Similarly, the newly-identified species differs from *Gammarus obruki* Özbek, 2012 by having smaller eyes, shorter antenna 1 and more setose peduncular segments of antenna 2 (Özbek 2012). No molecular data were available for the *G. obruki* species. *Gammarus tumaf* sp. nov. is located far from the *G. uludagi* in the phylogenetic tree. They were resolved with moderately-supported bootstrap values (NJ:74, ML:80) from the common ancestor from which they originated. The genetic distance between the two taxa is also quite high (for COI: 26.70% and for 28S: 4.95%).

Although the phylogeny of *Gammarus* is still not fully resolved, the species on the common branch from which

the new species originated were well resolved with high bootstrap values and indicating that the new species is an independent branch. The newly-identified species is well supported by molecular data. In this study, morphological and molecular data which we handled with an integrative approach, strongly supported the taxonomic status of *Gammarus tumaf* as a new species.

All of the studies on the taxonomy of amphipods that inhabited the inland waters of Türkiye so far have been based on the examination of morphological features only. Although these studies have contributed to species identification or new species identification, the phylogenetic relationships between species are still not fully understood. This is the first study in which both morphological and molecular analyses have been used to define a new amphipod species from the freshwaters of Türkiye.

Taxonomic studies supported by molecular and DNA analyses help to understand the relationships of species. On the other hand, making detailed morphological definitions also help other studies (ecological, taxonomic, population dynamics etc.), especially in accurate species determination. The authors agree that taxonomic studies taking into account both molecular analyses and detailed morphological features would be more beneficial.

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