

Molecular phylogeny of *Niphargus boskovic* (Crustacea: Amphipoda) reveals a new species from epikarst

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Abstract

We describe a new species of an amphipod *Niphargus zagorae* sp. n. and redescribe its nearest relative and morphologically similar species *N. boskovic* S. Karaman, 1952. We present the geographic distributions of both species, morphological diagnoses and infer their phylogenetic position within the genus based on COI, 28S and H3 markers.

Key words: *Niphargus*, taxonomy, cryptic species, epikarst

Introduction

The amphipod genus *Niphargus* is the most species-rich genus of hypogean amphipods in the world. It consists of over 300 species, which mostly occur in central and southeastern Europe (Väinölä *et al.* 2008). *Niphargus* species have been found in various aquatic habitats related to groundwater, including different types of springs, sinking rivers, cave lakes and interstitial water (Sket 1999). Many species are narrow range endemics—a pattern that reflects the fragmented nature of karstic systems and poor migratory abilities of cave amphipods (Fišer 2012; Trontelj *et al.* 2009).

The highest species diversity of *Niphargus* has been found in the Apennine Peninsula and Dinaric Karst (Zagmajster *et al.* 2014). The Dinaric Karst is a 650 km long limestone massif in the Western Balkans stretching from northwest to southeast along the Adriatic coast (Mihevc *et al.* 2010). Cave fauna of the area has been studied for more than a century and the region itself can be considered as one of the most thoroughly explored areas for subterranean fauna in the world. Despite this, new species from many taxonomic groups are still being discovered (e.g., G. S. Karaman 2014a; Bilandžija *et al.* 2013).

The taxonomy of the genus *Niphargus* is incomplete. Some species are rare, or may live in inaccessible parts of karstic systems; their collection requires additional sampling effort (Fišer & Zagmajster 2009). Some of the species have been simply overlooked, especially if not subject to detailed comparative analyses. Small sample sizes and limited knowledge of intra- and interspecific variation makes taxonomy even more difficult. Moreover, several molecular studies in the past years showed that many nominal species comprise a number of morphologically similar, so called cryptic species (Lefébure *et al.* 2006, 2007; Trontelj *et al.* 2009; Meleg *et al.* 2013; McInnery *et al.* 2014). Species experiencing a similar selective regime may resemble each other to a great extent and may differ only in few minute details (Fišer & Zagmajster 2009). Detection and taxonomic evaluation of such minute differences is demanding and hardly possible without a molecular taxonomic framework, especially if sample sizes are small (see Lim *et al.* 2012 for the problem of sample size in taxonomy). It is becoming more and more obvious that future species discovery within *Niphargus* strongly relies on the use of molecular techniques and that the most accurate morphological diagnosis can be defined *a posteriori*, once species are delineated within a molecular framework (e.g., Schlick-Steiner *et al.* 2010). As taxonomy progresses, new characters are discovered and old descriptions inherently become incomplete. Descriptions of new species therefore often require updated redescriptions of already established species for comparative purposes (Fišer *et al.* 2009).

In this paper we describe a new *Niphargus* species that had been identified as *Niphargus boskovicci* S. Karaman, 1952 on a morphological basis. *Niphargus boskovicci* was described from Vjetrenica Cave in southern Herzegovina (Bosnia and Herzegovina) by S. Karaman (1952), based on a sample of few females; the description of males followed in 1953 (S. Karaman 1953). Subsequent explorations led to the discovery of two subspecies *N. b. borkanicus* S. Karaman, 1960 from Boračko Lake in Bosnia and Herzegovina (S. Karaman 1960; G. S. Karaman 2014b) and *N. b. alatus* G. Karaman 1973 from Veruša spring near Kolašin in Montenegro (G. Karaman 1973) (Fig. 1). Recent cave explorations in southern Croatia resulted in the collection of individuals, morphologically resembling the nominate species *N. b. boskovicci*. However, molecular methods showed they should be treated as a new species. The aim of this paper is to 1) show the molecular evidence for species status of newly discovered individuals, 2) to morphologically characterize and describe the new species and 3) to analyze the morphology of the nominal *N. boskovicci* from the locality close to its type locality for comparative purposes. In addition, we revised our collection and present new localities of *N. boskovicci*.

Material and methods

Data. Specimens belonging to the newly described species, *N. zagorae* sp. n., were collected at three localities in Croatia (Golubinka pod Barišinovcem, Kevina jama and Tomina jama). In order to morphologically diagnose the newly discovered species, we analyzed also *N. boskovicci* collected in the Cave Bjelušica in Bosnia and Herzegovina. Bjelušica Cave is a part of the same system as the species' type locality, Vjetrenica Cave; the entrances to the caves are about 200 m apart. Both, *N. zagorae* sp. n. and *N. boskovicci* were collected in ponds of percolated water, suggesting that these species live in rock crevices, possibly at the soil-rock boundary called epikarst (Culver & Pipan 2014).

In order to determine the phylogenetic position of the newly described species, we compiled a dataset with 94 ingroup *Niphargus* species and one outgroup species (*Symurella ambulans* Müller, 1846). The ingroup sampling largely covers the geographic and phylogenetic diversity of the genus (Fišer *et al.* 2008a). Sequence data were retrieved from GenBank: 4 sequences are published for the first time (2 sequences 28S and 2 sequences COI; GenBank accession numbers KR827043-KR827046). We attempted to assemble as large a dataset as possible but also tried to keep missing data to a minimum. The concatenated dataset used in analyses contained about 14% missing data. The data for species, sampling sites and accession numbers can be accessed in the appendix. All samples used for morphological analyses, including the type series, are stored in the collection of the Department of Biology, Biotechnical Faculty, University of Ljubljana, Slovenia.

Morphological analyses. Selected specimens were treated in a 10% hot solution of KOH, briefly rinsed with diluted HCl and washed with distilled water. Cleared exoskeletons were stained with chlorazol black, partly dissected in glycerol and mounted on slides in a glycerol-gelatine medium. Morphology was studied under a stereomicroscope Olympus SZX9 (magnifications 3.14–114 ×) and a Zeiss microscope (magnifications 100–400 ×). Digital drawings (digital inking) were made in Adobe Illustrator CS3, using photographs of the appendages, Bamboo digital drawing board and digital pen (Coleman 2003, 2009).

The number of analyzed specimens was too small to conduct a proper statistical analysis; however, differences in proportions of appendages between the two species that may be important diagnostically were visualized on plots using PASW Statistics18.

Terminological note: true spines, i.e. extrusions of cuticle are not known in *Niphargus*. Species from this genus have appendages armed with flexible thin setae, flexible plumose setae and stout spiniform setae. To simplify descriptions, we refer to the thin flexible setae as 'setae' and stout spiniform setae as 'spines'.

Molecular methods. One of the pereopods of each specimen was used for DNA isolation, while the rest of the specimen was stored for morphological studies. Genomic DNA was isolated using GeneElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich) according to the Mammalian Tissue Preparation protocol. We amplified nuclear fragments of nuclear markers 28S rRNA and histone H3, and mitochondrial COI. Gene fragments were amplified in a polymerase chain reaction (PCR) using primer pairs 28S lev2 (Verovnik *et al.* 2005) and 28S des2 (Zakšek *et al.* 2007) for 28S, H3aF2 and H3aR2 (Colgan *et al.* 1998) for H3 and LCO 1490 and HCO 2198 (Folmer *et al.* 1994) for COI.

Amplification of 28S fragments followed the protocol from Zakšek *et al.* (2007). Histone H3 was amplified in 35 cycles of 94°C for 45 sec, 46°C for 1 min, 72°C for 1 min, followed by a final extension at 72°C for 3 min.

Mitochondrial COI was amplified in the following protocol: 40 cycles of 95°C for 1 min, 45°C for 1 min, 72°C for 2 min and 30 sec, followed by a final extension at 72°C for 7 min.

PCR products were purified using Exonuclease I and FastAP Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific INC., US) and commercially sequenced bidirectionally using amplification primers by Macrogen Europe (Amsterdam, the Netherlands). Chromatograms were assembled, manually checked and edited in Geneious 6.0.5. (Biomatters Ltd, New Zealand) with gaps coded as (–) and missing data as (?). Edited sequences were aligned in MAFFT v7 (Katoh & Standley 2013). Concatenated alignment was generated in Geneious.

Optimal models of molecular evolution for each partition were selected using PartitionFinder 1.1.1 (Lanfear *et al.* 2012); partition specific models for Bayesian analyses are shown in Table 1.

TABLE 1. Models of nucleotide evolution used in Bayesian analyses with reference to position in the concatenated alignment.

Gene fragment	Best Model	Site positions
28S rRNA	GTR+I+G	1-1078
Histone H3	GTR+G	1079-1409
COI codon1	GTR+I+G	1410-2070\3
COI codon2	GTR+I+G	1411-2070\3
COI codon3	SYM+I+G	1412-270\3

Phylogenetic relationships were reconstructed with Bayesian inference (BA) in MrBayes v3.2 (Ronquist & Huelsenbeck 2003) and Maximum likelihood (ML) in RAxML v7.8.4 (Stamatakis 2006). MrBayes ran for 20 million generations, sampling every 200 generation and producing two independent Monte Carlo Markov Chain (MCMC) with four cold chains each. After reaching the stationary phase, the first 25% trees were discarded as a burn-in while the remaining trees were used to assemble a majority-rule consensus tree. MrBayes phylogenetic analyses were run on the CIPRES Science Gateway, www.phylo.org (Miller *et al.* 2012).

Additionally, we used RAxML (Stamatakis 2006) to infer ML phylogenetic relationships via user-friendly graphical interface raxmlGUI v1.3 (Silvestro & Michalak 2012). Specific gene partitions were set to GTR+G+I model. To confirm robustness of the analysis we carried out 100 bootstrap runs and searched for the best ML tree.

To compare to which proportion the two sequences differ, uncorrected p-distance (*p*) was calculated in MEGA 6.06 (Tamura *et al.* 2013), by comparing *N. boskovic* and *N. zagorae* sp. n. COI sequences.

Results and discussion

Molecular phylogeny and species delimitation

Both BA and ML yielded phylogenetic trees with similar topologies. Although basal nodes are poorly supported, several monophyletic groups were highly supported (Fig. 1). The focal species belong to a highly supported monophyletic group distributed in southern Bosnia and Herzegovina, southern Croatia and Montenegro. This monophylum is ecologically extremely diverse and consists of two lineages. One lineage comprises of large-bodied species thriving in cave lakes (cave lake, lake giant and daddy longlegs ecomorphs, for terminology see Trontelj *et al.* 2012). Newly described species, *N. zagorae*, sp. n. is nested within the second lineage together with *N. boskovic*, *N. hvarensis* and *N. miljeticus*. These species live either in epikarst (*N. zagorae* sp. n., *N. boskovic*; both small pore ecomorph) or in springs, sometimes even in slightly brackish water (*N. hvarensis*, *N. miljeticus*; both cave stream ecomorph) (Fig. 1). Distributional data (Fig. 1) suggest that one lineage (*N. zagorae* sp. n. + *N. boskovic* + *N. hvarensis* + *N. miljeticus*) evolved through allopatric fragmentation, as ranges of these species do not overlap. The evolution of this lineage includes at least one shift in ecology of ancestral species, either from cave fissures to cave streams or *vice versa* (Fig. 1).

The newly described species is morphologically similar and phylogenetically related to *N. boskovic*. Both, mitochondrial and nuclear markers suggest that *N. boskovic* and *N. zagorae* sp. n. have been evolving independently from each other and comprise two paraphyletic groups. The genetic distance between *N. zagorae* sp.

n. and *N. boskovici* is 14.2 % (uncorrected *p*) in COI, which is near to the threshold value at which interbreeding experiments detected a reproductive barrier (Cothran *et al.* 2013; Lagrue *et al.* 2014). Based on molecular data, we identified four morphological characters that may support separate species status of *N. zagorae* sp. **n.** and *N. boskovici*: i) unlike *N. boskovici*, *N. zagorae* sp. **n.** has a spine on urosomite III; ii) the propodus of gnathopod II is slightly larger and with a more inclined palm in *N. zagorae* sp. **n.** than in *N. boskovici* (Fig. 2), iii) the base of the nails of dactyls of pereopods III–IV have two setae in *N. zagorae* sp. **n.**, whereas in this position, *N. boskovici* has only one seta and one spine; and iv) *N. zagorae* sp. **n.** has a pereopod V that is proportionally longer than pereopods VI and VII compared to *N. boskovici* (Fig. 2).

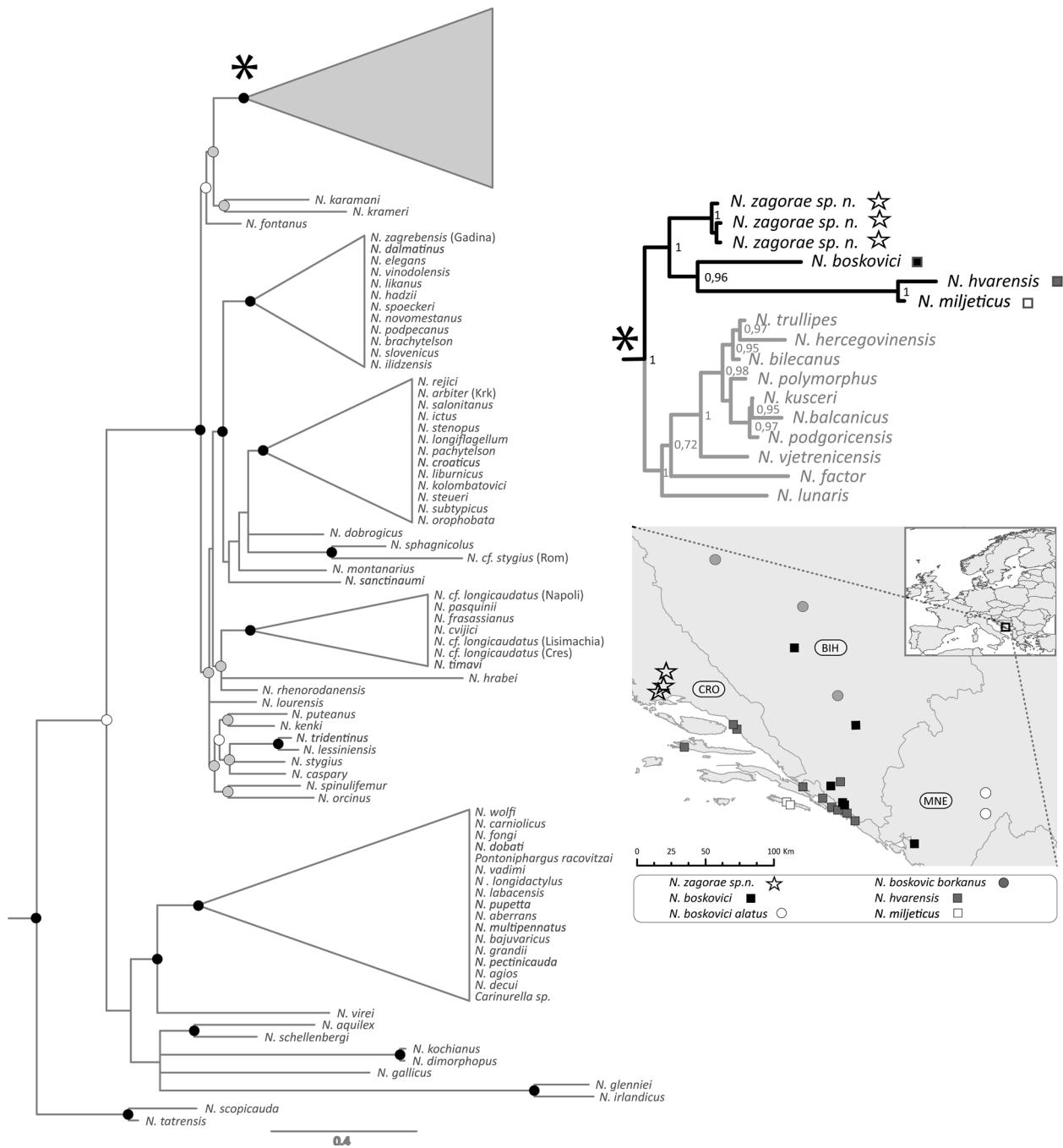


FIGURE 1. Phylogenetic position and distribution of the studied species. Left side: Bayesian phylogenetic tree of 94 *Niphargus* species. Support for the nodes is labeled with color (posterior probability 1—black, posterior probability 0.99—grey, posterior probability >0.95—white). Detailed phylogenetic structure of the shaded clade containing focal species is presented on the right side. Geographic distribution of the *N. zagorae*, *N. boskovici*, *N. hvarensis* and *N. miljeticus* (black-colored lineage in clade upper right) is presented on a bottom right map. Distribution of newly described species is presented with asterisk. Abbreviations—CRO—Croatia, BIH—Bosnia and Herzegovina, MNE—Montenegro.

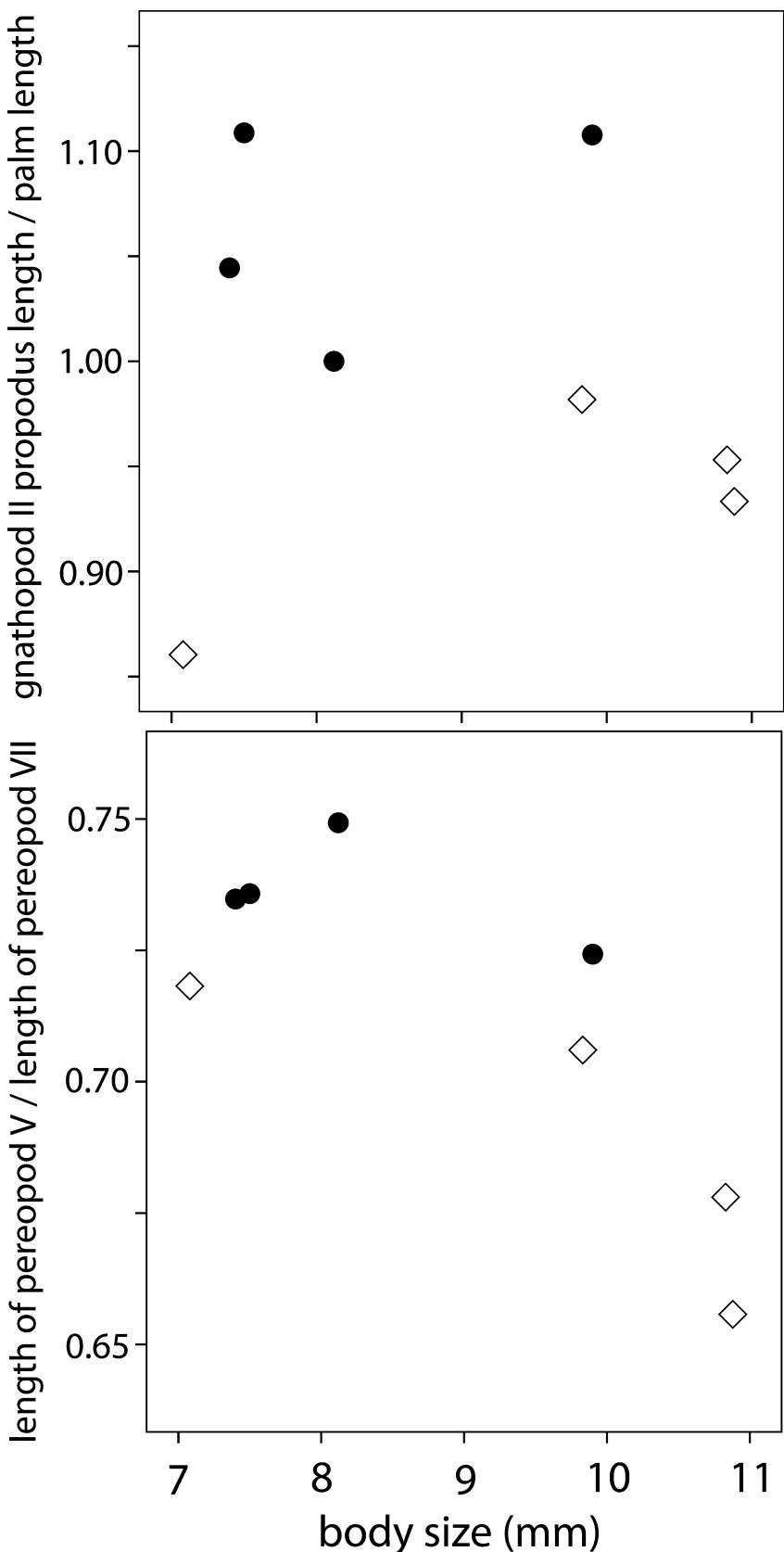


FIGURE 2. Differences in proportions between *N. zagorae* sp. n. (circles) and *N. boskoviczi* (diamonds). Upper: The ratio propodus length:palm length of gnathopod II plotted against the body size. Lower: The ratio pereopod V length:pereopod VII length plotted against body size. We plotted body proportions against body size as some appendages may grow allometrically (Fišer *et al.* 2008b). Note that values of ratio do not overlap between the two species.

In light of evidence presented here, we name *N. zagorae* sp. n. as a new species, described in systematic part of the article.

Systematics

Niphargidae Bousfield, 1977

Niphargus zagorae sp. n.

(Figs 1, 2, 3a–b, 4a–b, 5 a, c, e, g, 6a, d, 7a–b, 8a–c, 9a–c, 10a, c)

Type material. 5 individuals from type locality (slides #1, 2, 5 juvenile or immature males, slides #3, 4 ovigerous females, voucher No. NB943–NB947), holotype ovigerous female, slide #3, all collected in Golubinka pod Barišinovcem, Croatia, coll. Tonči Rađa, 2007. The type series is deposited in collection of Department of Biology, Biotechnical Faculty, University of Ljubljana.

Type locality. Golubinka pod Barišinovcem, Čvrljevo, Šibenik, Croatia. Coordinates, WGS-84: 43° 696558°N, 16° 305139°E

Etymology. The species name is derived from the Latinized Croatian name of the geographical area (Croatian: Zagora) where the new species was collected.

Distribution. Golubinka pod Barišinovcem, near Čvrljevo, vicinity of Šibenik; Kevina jama near Radošić, vicinity of Split; Tomina jama, near Labin dalmatinski, vicinity of Split (Fig. 1).

Description. [Based on holotype; variation among individuals given in square brackets].

Body (Fig. 3a, 10c). Body elongated, 9.9 [7.4–9.9] mm long. Head from 0.11 [0.09–0.12] of body length, without rostrum. Pereonite VII with 1 [1–2] postero-ventral setae, pleon segments I–III with 8 [5–10] dorso-posterior setae. Epimeral plates II–III rectangular, with posterior and distal margins convex. Epimeral plates II–III with 4–5 [3–5] setae posteriorly; subventrally with 1 [1–2] and 2 spines respectively. Urosomites I–III with 1, 2, and 1 dorsolateral spines, respectively, 1 each side of body. Base of uropod with 1 strong seta.

Telson (Fig. 10c) length:width ratio as 1:1.1 [1:0.95–1.1], telson cleft 0.67 [0.65–0.75] of telson length. Telson with 3 [3–4] apical spines (per lobe), 0 [0–1] lateral spines (per lobe), 0–1 [0–1] mesial (per lobe) and 2 [0–2] dorsal spines (per lobe). Laterally two plumose setae on each lobe. Longest apical spines as long as 0.33 [0.30–0.38] of telson length.

Antennae I (Fig. 4a, b). Length 0.40 [0.35–0.40] of the body length. Peduncle segments 1–3 in ratio 1:82:38 [1:(0.73–0.82):(0.36–0.40)], flagellum of 26 [17–26] articles, each bearing 1 seta and 1 aesthetasc. Accessory flagellum biarticulated, distal article about 1/3 of the length of basal article.

Antennae II (Fig. 4b) 0.49 [0.49–0.55] of antenna I length. Lengths of peduncle articles 4:5 as 1:0.86 [1:(0.86–0.95)]. Flagellum II with 11 [8–11] articles, each bearing short seta and another chemosensory seta.

Mouthparts (Fig. 5a, c, e, g). Left mandible pars incisiva with 4 teeth and lacinia mobilis with four teeth, pars molaris triturative. Right mandible (Fig. 5a) with 4-dentate incisor blades, multidenticulated lacinia mobilis and 6 plumose setae between lacinia and triturative molar.

Mandibular palp tri-articulate, basal article without setae, middle article with 5–9 long setae along inner margin, distal article with group of 6 [1–6] A setae, 4 [3–4] groups of B setae, 24 [19–24] D setae and 4 [3–4] E setae. Palp articles 2 and 3 in ratio 1:1.24 [1:1.20–1.35].

Maxilla I (Fig. 5c) with 4 [2–4] setae on inner lobe, outer lobe with 7 spines; innermost spine pluridentate, others with up to 2 denticles. Palp biarticulated, with 7 [5–7] distal and subdistal seta.

Maxilla II (Fig. 5e) with sub-equal lobes, each with a group of long apical and subapical seta. Labium with inner lobes.

Maxilliped (Fig. 5g) inner lobe with 2 [2–3] strong flattened spines and 7 [6–8] strong plumose setae apically and subapically. Outer lobe with 10 [7–10] strong mesial flattened spines and 5 [5–6] plumose apical seta. Dactylus with setae at base of nail.

Gnathopods. Gnathopod I (Fig. 6a) coxa of rhomboid shape with 9 [6–9] setae ventrodistally. Article 2 length: width as 1:0.44 [1:(0.41–0.50)]. Article 3 with one row of 8 [5–9] posteroventral setae. Article 5 length 0.80 [0.78–0.86] length of article 6. Article 5 with proximal bulb; 1 group of setae distoanteriorly; setae also on bulb and along

posteromesial margin. Article 6 rectangular in shape, antero-distal corner slightly inclined. Anterior margin with 3 [2–3] groups of setae (in total 14 [9–14] setae) and antero-distal group of 11 [8–11] setae. Posterior margin with 6 [5–6] rows of setae. Palmar corner with long palmar spine, small smooth inner spine and 2 [2–3] outer denticulated spines. Outer surface proximal to palmar spine with group of 3 [2–3] long setae; inner surface with several groups of small setae. Dactylus with 6 [4–6] single setae along outer margin, inner margin with small setae; nail 0.25 [0.25–0.33] of dactylus length.

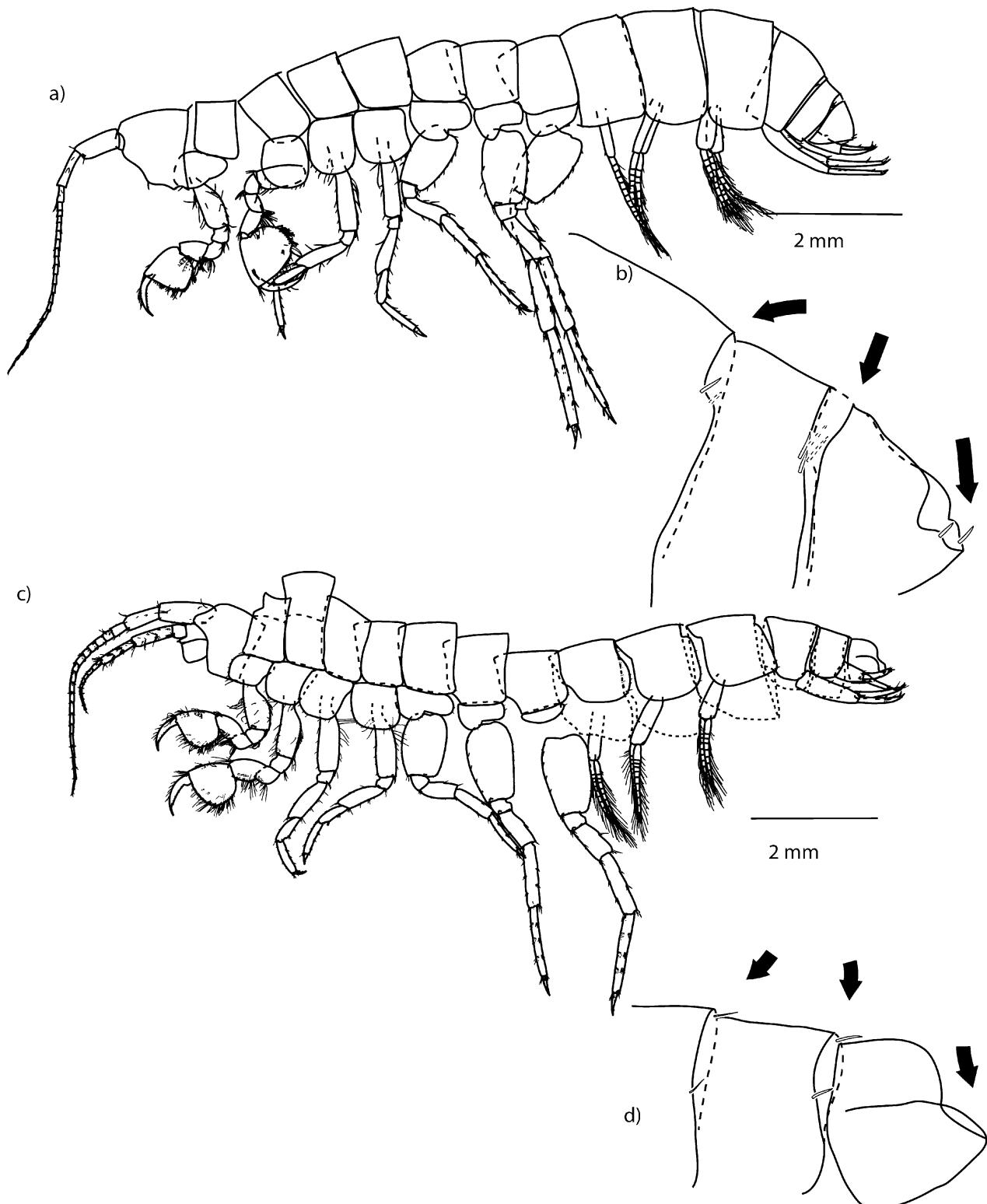


FIGURE 3. Habitus and diagnostic details (arrows) on urosoma in *N. zagorae* sp. n. (a, b) and *N. boskovicci* (c, d).

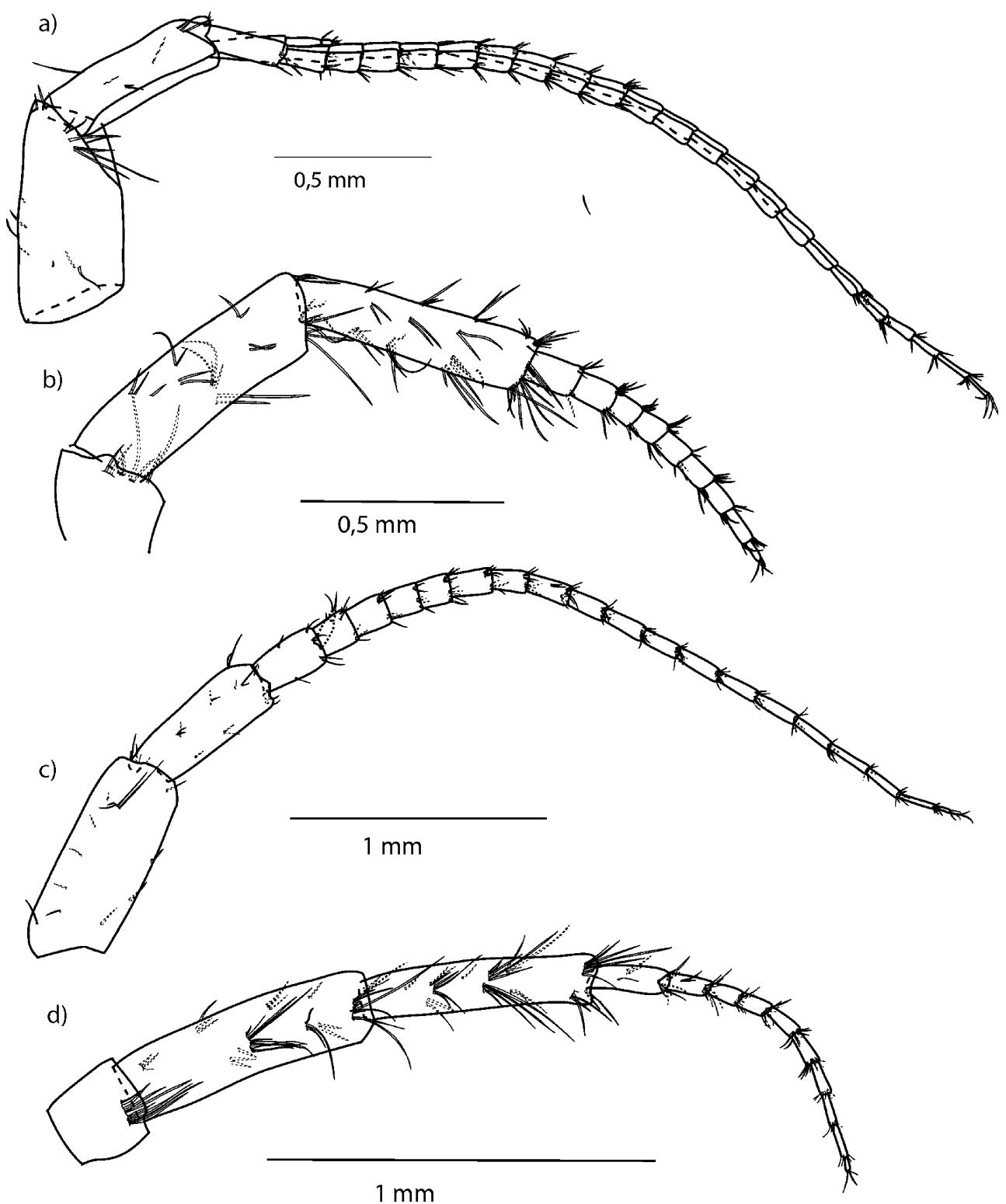


FIGURE 4. Antenna I and II of *N. zagorae* sp. n. (a, b) and *N. boskoviци* (c, d).

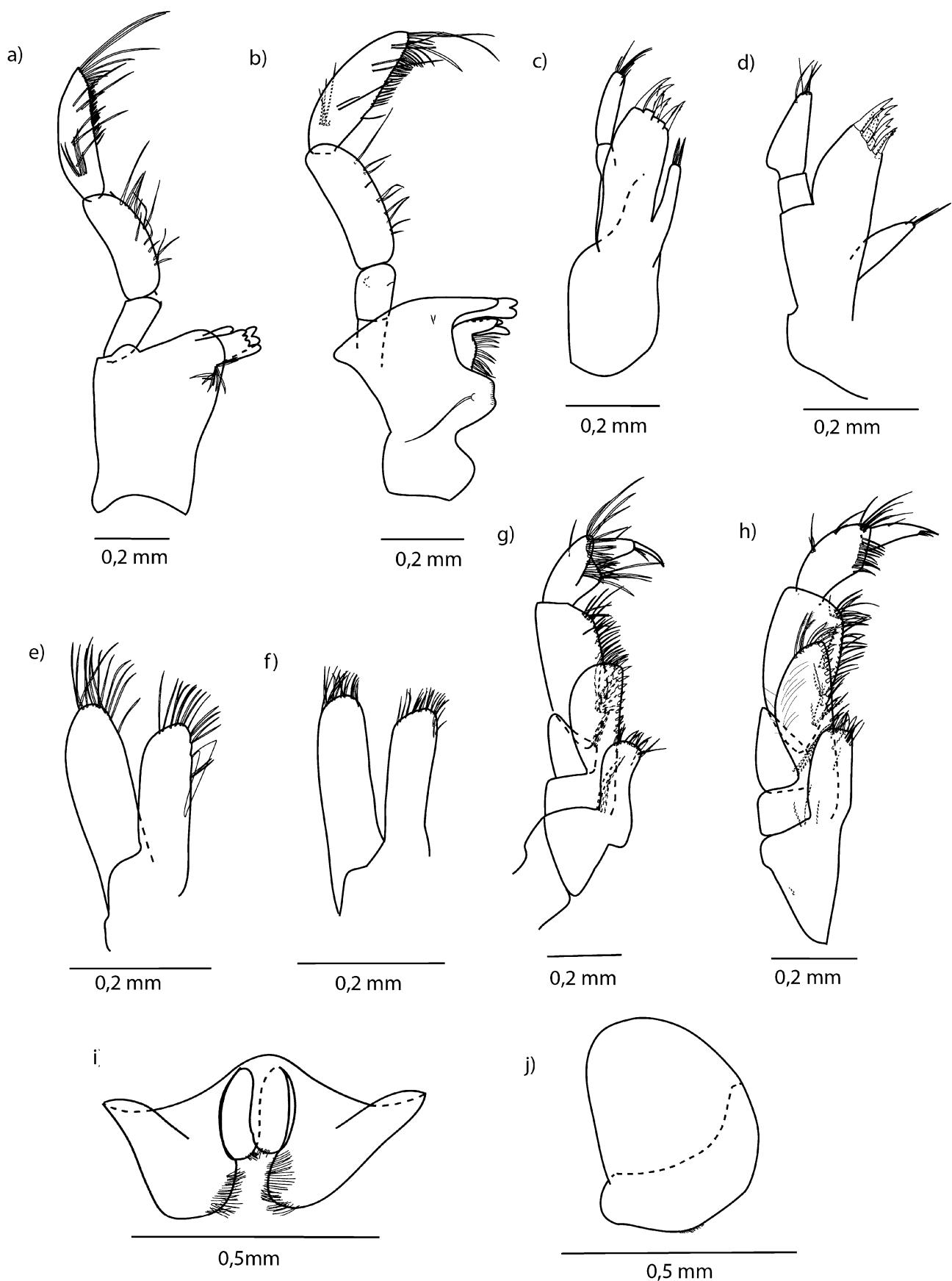


FIGURE 5. Mouthparts of *N. zagorae* sp. n. (a,c,e,g) and *N. boskoviczi* (b,d,f,h,i,j). Mandibula (a,b), maxilla I (c,d), maxilla II (e,f), maxilliped (g,h), labrum (i), labium (j).

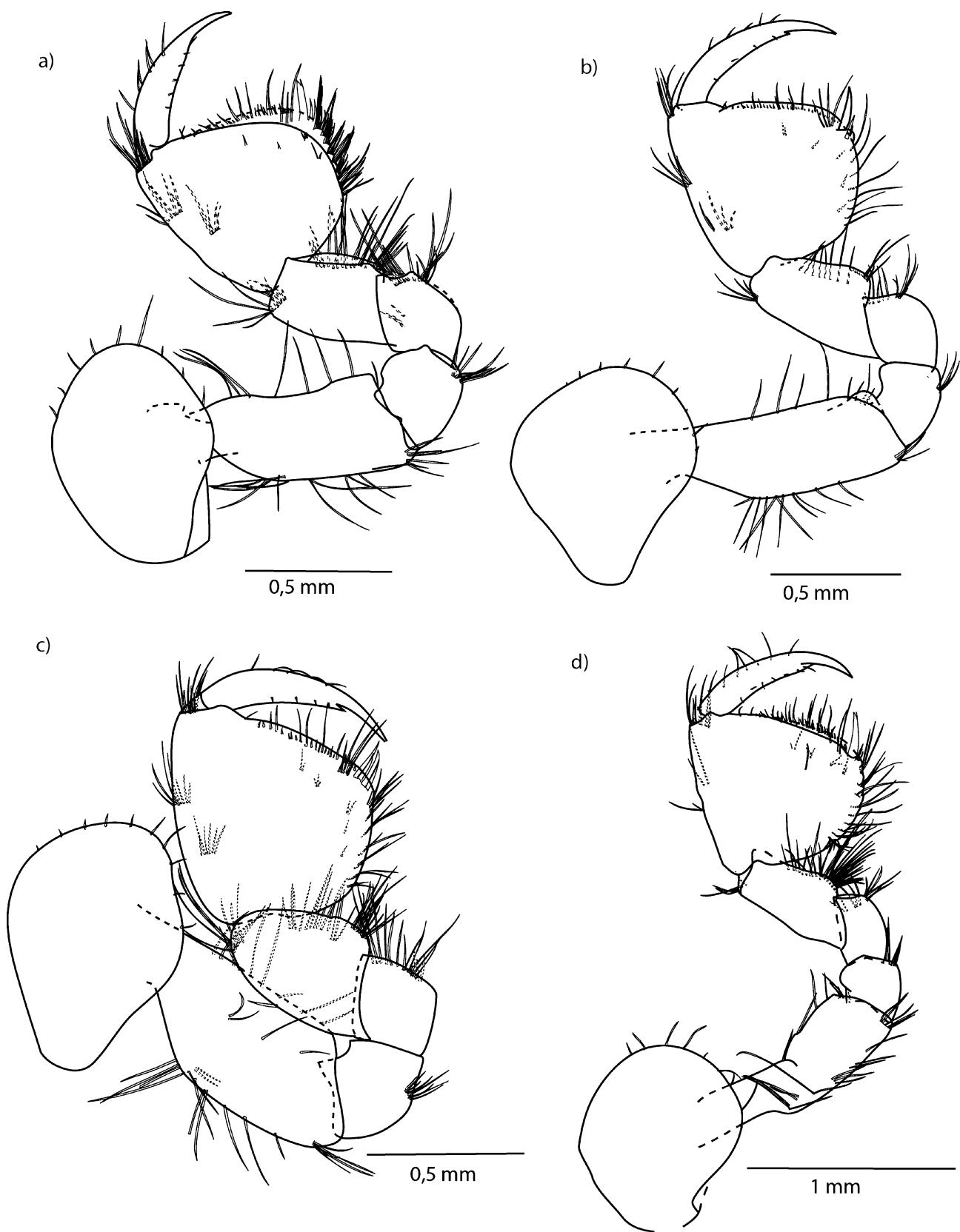


FIGURE 6. Gnathopods I and II of *N. zagorae* sp. n.(a,d) and *N. boskovići* (b,c).

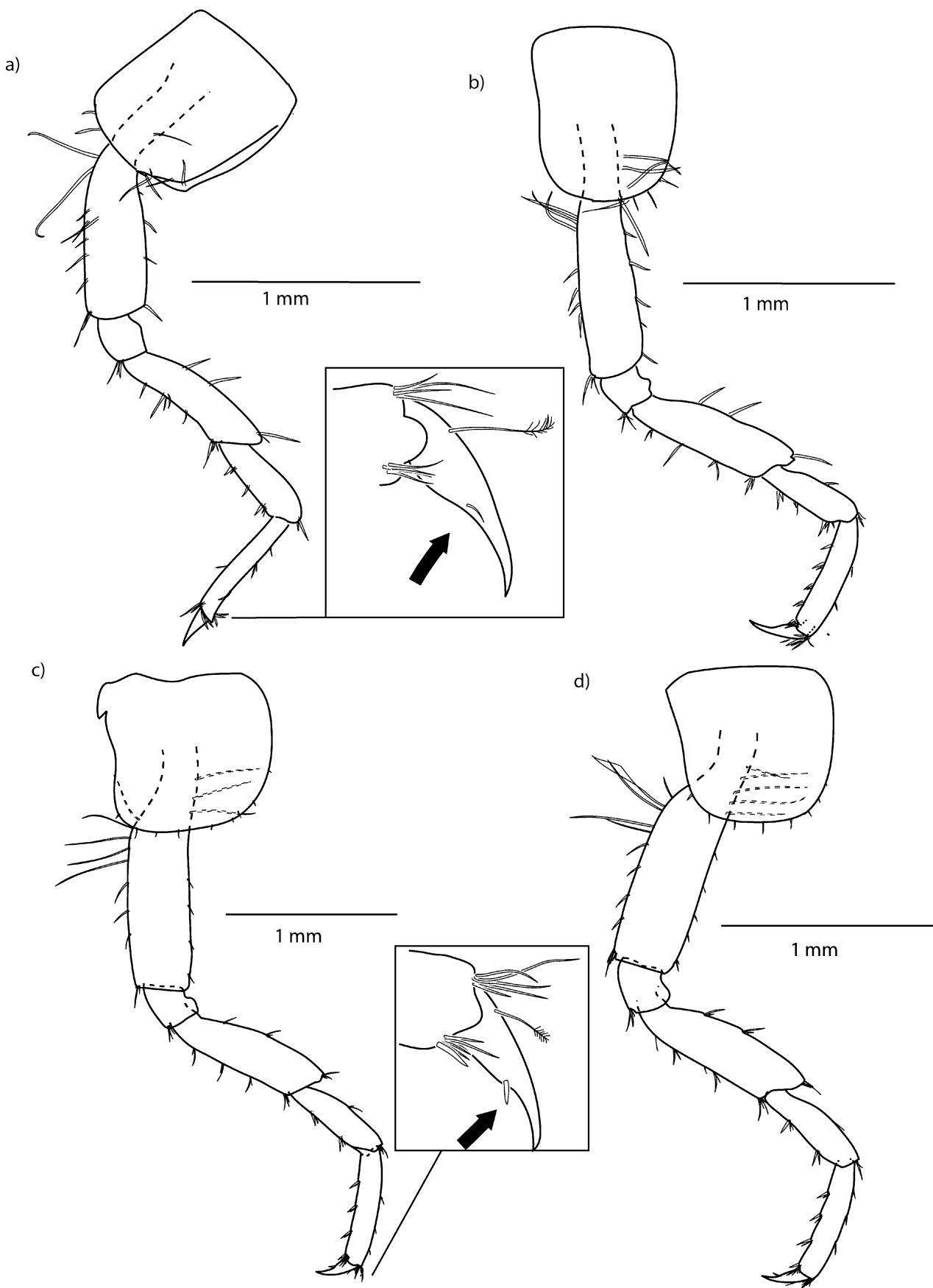


FIGURE 7. Pereopods III and IV of *N. zagorae* sp. n. (a, b) and *N. boskovici* (c, d) with more precise drawing of dactylus of the third pereopods. Arrows indicate diagnostic difference.

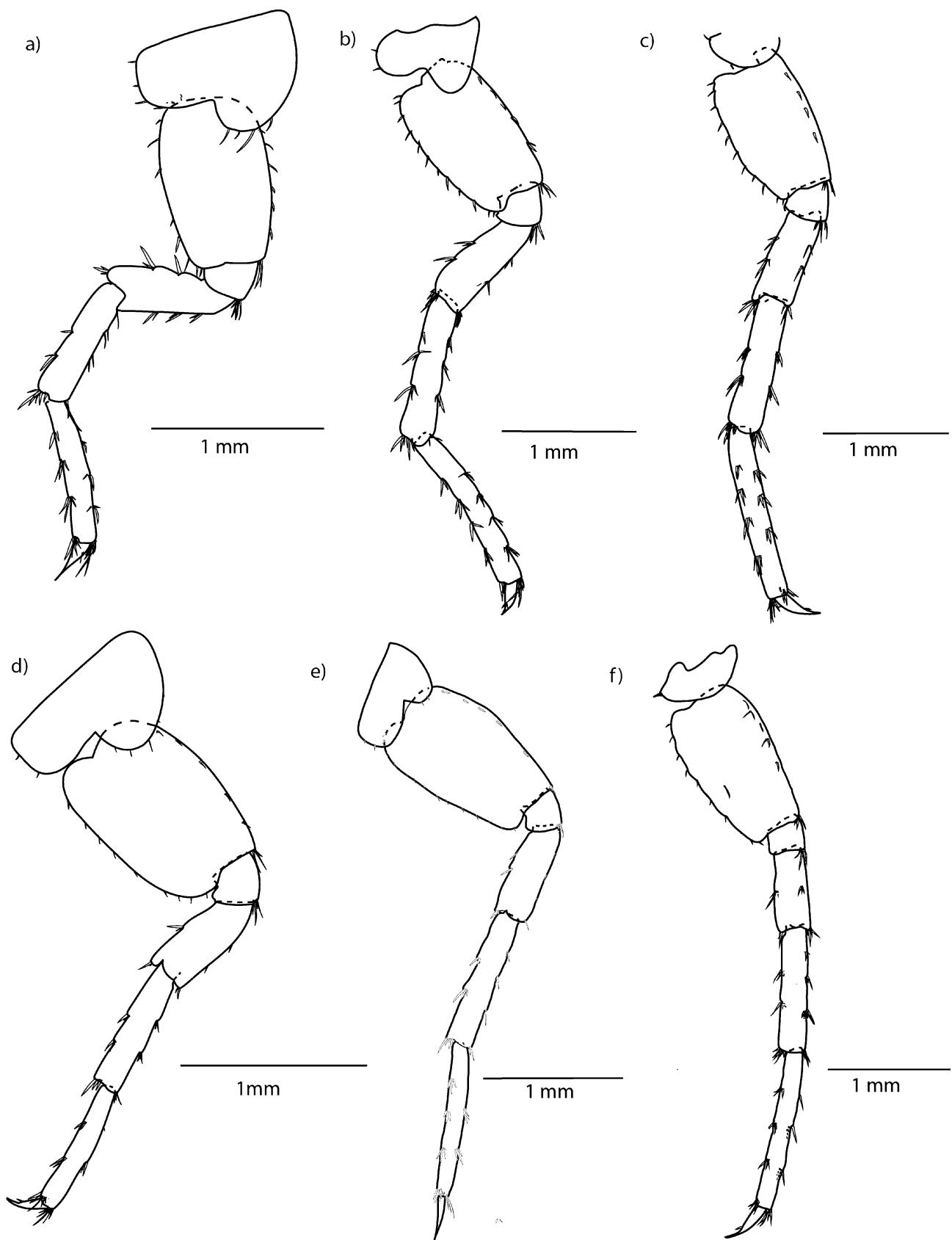


FIGURE 8. Pereopods V, VI and VII of *N. zagorae* sp. n. (a, b, c) and *N. boskovi*c (d, e, f).

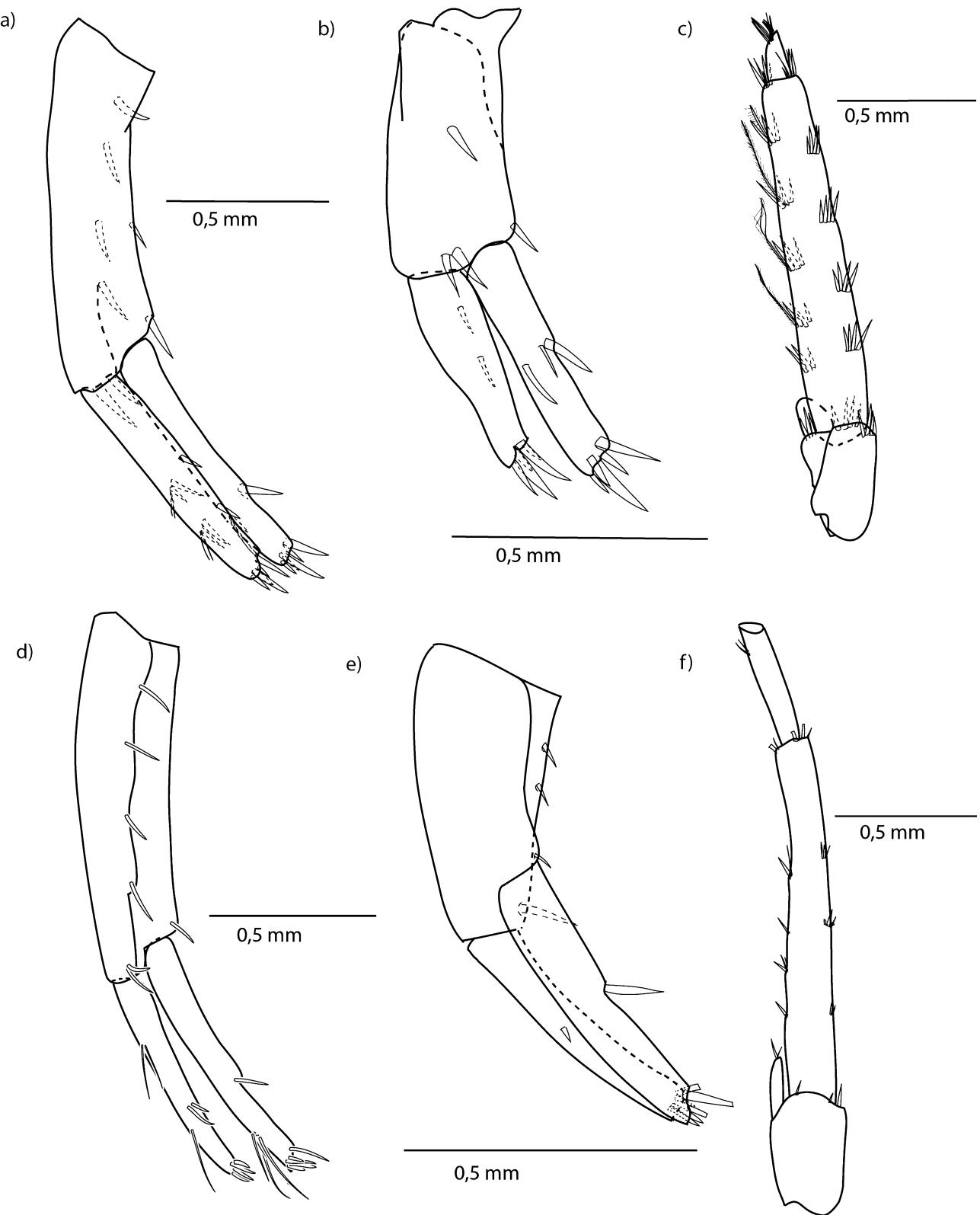


FIGURE 9: Uropods I, II and III of *N. zagorae* sp. n. (a, b, c) and *N. boskovici* (d, e, f). Uropod III of *N. boskovici* is distally damaged; unfortunately it was the only adult male in analysis.

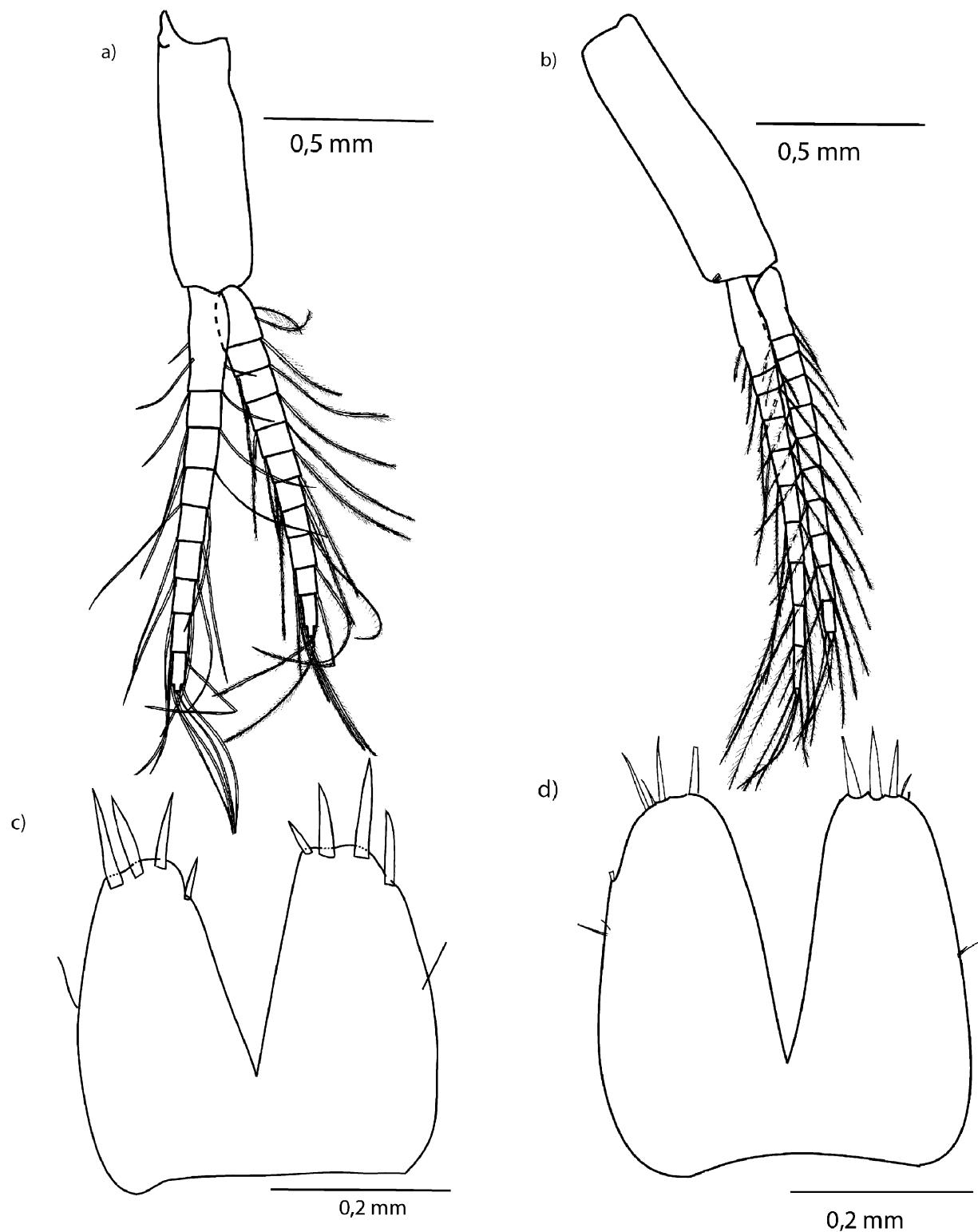


FIGURE 10. Pleopods and telson of *N. zagorae* sp. n. (a, c) and *N. boskoviци* (b, d).

Gnathopod II (Fig. 6d) coxa with 7 [6–8] setae distoventrally, width: length ratio as 1:0.87 [1:(0.87–0.97)]. Article 2 length: width as 1:34 [1:(0.30–0.35)]. Article 3 with one row of 4 [2–4] posteroventral setae. Length of article 5 is 0.86 [0.86–0.91] of article 6 length. Article 5 with proximal bulb; 1 group of setae disto-anteriorly; setae also on bulb and along posteromesial margin. Article 6 of gnathopod II larger than article 6 of gnathopod I (circumference of gnathopods I:II as 0.78:1 [0.78–0.85:1]); rectangular in shape, anterodistal angle strongly

inclined and palm longer as length of article. Anterior margin with 2 [2–3] groups of setae (in total 5 [4–6] setae) and anterodistal group of 8 [6–8] setae. Posterior margin with 9 [7–9] rows of setae. Palmar corner with long palmar spine, small smooth inner spine and 2 [1–2] outer denticulated spines. Outer surface proximal to palmar spine with 2 long setae; inner surface with several groups of small setae. Dactylus with 6 [4–6] single setae along outer margin, inner margin with small setae; nail 0.28 [0.28–0.32] of dactylus length.

Pereopods III–IV (Fig. 7a, c). Pereopods III:IV as 1:0.98 [1:(0.94–0.98)]; coxal plates III–IV width:length as 1:0.89 [1:(0.82–0.89)] and 1:1.10 [1:(0.97–1.10)], respectively; ventral margins with 6–9 [4–9] setae. Each dactylus with 2 tiny setae at the base of nail and 1 plumose seta dorsally; nail IV 0.50 [0.45–0.52] of dactylus length, dactylus IV 0.48 [0.42–0.48] of propodus length.

Pereopods V–VII (Fig. 8a–c). Coxae V–VI with anterior lobe, posterior margin with 2 and 3 [1–3] setae, distal seta may be spine-like. Coxa VII semicircular, 1 seta posteriorly. Lengths of pereopods V:VI:VII as 1:1.25:1.38 [1:(1.25–1.30):(1.33–1.38)]; pereopod VII 0.49 [0.42–0.49] of body length. Articles 2 ovoid, with small disto-posterior lobe, respective widths:lengths as 1:0.68 [1:(0.69–0.76)], 1:0.60 [1:(0.60–0.73)], 1:0.63 [1:(0.63–0.71)]. Bases (articles 2) of pereopods V–VII with 9 [9–10], 12 [9–12], 12 [9–12] posterior setae and 6 [5–6], 5 [5–6], 6 [5–6] anterior groups of setae and spines, respectively. Each dactylus with 2 tiny setae at the base of nail, or with 1 tiny seta and 1 tiny spine; 1 plumose seta dorsally; nail VII 0.33 [0.33–0.38] of dactylus length, dactylus VII 0.28 [0.28–0.31] of propodus length.

Gills oval shaped.

Pleopods (Fig. 10a) inner ramus longer than outer, each ramus of 10 and 11 [9–11] articles, 2 retinaculae on each pleopod.

Uropods I–III. Uropod I (Fig. 9a) basipodite with 6 lateral and 2 [2–3] mesial spines. Endopodite with 2 groups of totally 7 [6 or 7] spines and setae and 5 apical spines, exopodite with 4 [2–4] groups of totally 11 [2–11] spines and 5 apical spines. Endopodite:exopodite lengths as 1:1.08 [1:(1.03–1.09)].

Uropod II (Fig. 9b) endopodite:exopodite lengths as 1:1.14 [1:(1.08–1.17)].

Uropod III (Fig. 9c) basipodite with no [0–1] lateral spines and 7 [7–10] apical spines. Exopodite proximal article with 6 [5–6] and 5 [4–6] groups of spines, setae and plumose setae along inner and outer margin respectively. Apical article of exopodite with no [0–1] setae laterally and 8 [5–8] setae apically. Endopodite with 0–1 lateral spines and 3 or 4 apical spines and setae. Distal article of exopodite as 0.14 [0.13–0.20] (females) of proximal article, total uropod III as 0.19 [0.17–0.21] of body length (females).

Remarks. *Niphargus zagorae* sp. n. is similar to *N. boskovicci*, but differs from the latter: i) by the presence of spine on urosomite III; ii) slightly larger and more inclined propods of gnathopods II; iii) at bases of nails of dactyls of pereopods III–IV only setae are present; and iv) pereopod V is proportionally longer when compared to pereopods VI and VII.

The material we studied was limited, and it is not clear whether males are sexually differing from the females.

Niphargus boskovicci S. Karaman, 1952

(Figs 1, 2, 3c–d, 4c–d, 5 b, d, f, h–j, 6b, c, 7c–d, 8d–f, 9d–f, 10b, d)

Niphargus (*Orniphargus*) *boskovicci* S. Karaman, 1952: 45, figs 1–10.

Niphargus boskovicci *boskovicci* S. Karaman, 1960: 81.—G. Karaman & Ruffo 1986: 523.

Niphargus boskovicci S. Karaman, 1953: 145 (9), figs 5–11.—G. Karaman 2014b: 211.

Material examined. Bjelušica near Zavala, Bosnia and Hercegovina, 2 males and 2 females, date of collection 1975.

Type locality. Vjetrenica Cave, Bosnia and Hercegovina.

Distribution. Bjelušica, Zavala, Popovo polje; Duboki do, Njeguši, Kotor; Jama kod Gornje Bijenje, Gornja Bijenja, Nevesinje; Krušnica spring, Gornji Vakuf, Rama; Poganjača, Grebci, Trebinje; Reznica, Grebci, Trebinje; Vjetrenica, Zavala, Popovo polje (Fig. 1).

Diagnosis. Small species with slender body, posterior margin of pleon segments with 5 or more setae, epimeral plates angulate; telson narrow, only with apical and lateral spines. Propodi of gnathopods I–II rectangular and mid-sized, dactyli of gnathopods with several single setae along outer margin. Pereopods V–VII progressively longer. Dactyls of pereopods III–VII with a tiny seta and tiny spine at the base of nail. Pleopods with 2 retinaculae each.

Uropod I sexually non-dimorphic (both rami subequal), uropod III sexually dimorphic, with distal article of exopodite elongated in males.

Description. *Body* (Fig. 3b, 9d). Body elongated, 7.1–10.9 mm long. Head 0.09–0.11 of body length, without rostrum. Pereonite VII with 1 or 2 posteroventral setae, pleon segments I–III with 3–7 posterodorsal setae. Epimeral plates rectangular, with posterior and distal margins convex. Epimeral plates II–III with 4–7 setae posteriorly; subventrally 1 and 2 spines, respectively. Urosomites I–III with 1, 1, 0 dorsolateral spines, respectively, on each side of body. Uropod I with strong seta at base. Telson (Fig. 10d) length:width ratio as 1:0.9–1.05, telson cleft 0.70–0.75 of telson length. Telson with 3–5 apical spines (per lobe), 0–2 lateral spines (per lobe), neither mesial nor dorsal spines observed. Laterally 2 plumose setae on each lobe. Longest apical spines as long as 0.20–0.33 of telson length.

Antennae I. Antenna I (Fig. 4c) 0.34–0.40 of the body length. Peduncle segments in ratio 1:(0.65–0.85):(0.35–0.40), flagellum of 17–20 articles, each bearing one seta and 1 aesthetasc. Accessory flagellum biarticulated, distal article about 1/3–1/2 length of basal article.

Antenna II (Fig. 4d) 0.48–0.57 of antenna I length. Lengths of peduncle articles 4:5 as 1:(0.77–0.93). Flagellum II with 10–13 articles, each bearing short seta and another chemosensory seta.

Mouthparts (Fig. 5b, d, i, h). Left mandibular pars incisiva with 5 teeth and lacinia mobilis with 4 teeth, pars molaris triturative. Right mandible (fig. 5b) with 4 dentate incisor blades, multidenticulated lacinia mobilis and 6 or 7 plumose setae between lacinia and triturative molar.

Mandibular palp tri-articulate, basal article without setae, middle article with 5–7 long setae along inner margin, distal article with 1 group of 3–5 A setae, 3–4 groups of B setae, 15–19 D setae and 3–5 E setae. Palp articles 2 and 3 in ratio 1:1.20–1.28.

Maxilla I (Fig. 5d) with 2 or 3 setae on inner lobe, outer lobe with 7 spines; inner most spine pluridentated, other spines with up to 2 denticles. Palp biarticulated, with 5–7 distal and subdistal seta. Maxilla II (Fig. 5f) with subequal lobes, each with a group of long apical and subapical seta. Labium (Fig. 5i) with inner lobes.

Maxilliped (Fig. 5h) inner lobe with 3 or 4 strong flattened spines and 6–8 strong plumose setae apically and subapically. Outer lobe with 8–10 strong flattened mesial spines and 4 or 5 plumose apical setae. Dactylus with setae at base of nail.

Gnathopods. Gnathopod I (Fig. 6b) coxa of rhomboid shape with 6–11 setae distoventrally. Article 2 length: width as 1:0.44–0.48. Article 3 with 1 row of 5–9 posteroventral setae. Length of article 5 0.76–0.81 of article 6 length. Article 5 with proximal bulb; 1 group of setae distoanteriorly; setae also on bulb and along posteromesial margin. Article 6 rectangular in shape, anterodistal corner slightly inclined. Anterior margin with 2 or 3 groups of setae (in total 10–12 setae) and anterodistal group of 9–12 setae. Posterior margin with 5 or 6 rows of setae. Palmar corner with long palmar spine, small smooth inner spine and 2 or 3 outer denticulated spines. Outer surface proximal to palmar spine with 2 long setae; inner surface with several groups of small setae. Dactylus with 5 or 6 single setae along outer margin, inner margin with small setae; nail 0.33–0.35 of the dactylus length.

Gnathopod II (Fig. 6c) coxa with 6–9 setae ventrodistally, width: length ratio as 1:0.97–1.05. Article 2 length: width as 1:0.31–0.36. Article 3 with one row of 4 or 5 posteroventral setae. Length of article 5 is 0.90–0.98 of article 6 length. Article 5 with proximal bulb; 1 group of setae anterodistally; setae also on bulb and along posteromesial margin. Article 6, larger than article 6 of gnathopod I (circumference of gnathopods I:II as 0.85–0.93:1); rectangular in shape, anterodistal angle inclined and palm longer as length of article. Anterior margin with 2 or 3 groups of setae (in total 7–9 setae) and anterodistal group of 7–12 setae. Posterior margin with 5–8 rows of setae. Palmar corner with long palmar spine, small smooth inner spine and 2 outer denticulated spines. Outer surface proximal to palmar spine with 1 or 2 long setae; inner surface with several groups of small setae. Dactylus with 3–7 setae along outer margin; inner margin with small setae; nail 0.30–0.34 dactylus length.

Pereopods III–IV (Fig. 7b, d). Pereopods III:IV as 1:0.95–0.98; coxal plates III–IV width:length as 1:0.92–0.96 [females], 1.05–1.07 [males]) and 1:0.92–1.11 [females], 1.15–1.16 [males], respectively; ventral margins with 5–9 setae. Dactyli with tiny spine and tiny seta at base of nail, 1 plumose seta dorsally; nail IV 0.43–0.51 of dactylus length, dactylus IV 0.36–0.40 of propodus length.

Pereopods V–VII (Fig. 8d–f). Coxae V–VI with anterior lobe, posterior margin with 1–3 setae, distal seta may be spine-like. Coxa VII semicircular, 1 seta posteriorly. Lengths of pereopods V:VI:VII as 1:(1.32–1.40):(1.39–1.5); pereopod VII 0.39–0.44 of body length. Article 2 ovoid, with small posterodistal lobe, respective widths:lengths as 1:(0.71–0.79), 1:(0.63–0.73), 1:(0.58–0.71). Bases (articles 2) of pereopods V–VII with 8–15, 9–

11, 9–12 posterior setae and 4–5, 5–6, 5 anterior groups of setae and spines, respectively. Each dactylus with tiny seta and tiny spine; 1 plumose seta dorsally; nail VII 0.30–0.37 of dactylus length, dactylus VII 0.30–0.31 of propodus length.

Gills oval shaped.

Pleopods (Fig. 10a) inner ramus longer than outer, each ramus of 8–12 articles, 2 retinaculae on each pleopod.

Uropods I–III. Uropod I (Fig. 9d) basipodite with 6 or 7 lateral and 0–2 mesial spines. Endopodite with 2 groups of 6 or 7 spines and setae, and 5 apical spines. Exopodite with 2 or 3 groups of 7 spines and 5 apical spines. Endopodite:exopodite ratio as 1:1.04–1.11.

Uropod II (Fig. 9e) endopodite:exopodite ratio as 1:1.04–1.30.

Uropod III (Fig. 9f) basipodite with 1–3 lateral spines and 2–6 apical spines. Exopodite proximal article with 4–6 and 4 groups of spines, setae and plumose setae along inner and outer margin respectively. Apical article of exopodite with 4 setae laterally and 4–6 setae apically. Endopodite with 0 or 1 lateral spines and 3 or 4 spines and setae apically. Distal article of exopodite as 0.45–0.52 (males) of proximal article. Uropod III length 0.23–0.25 of body length (males).

Remarks. The species is sexually dimorphic, males having an elongated distal article of uropod III. It seems, however, that males are rare. Most of samples in our hand contained only females and it seems that females also dominated samples of S. Karaman (1952, 1953). While this article was processed, type series was re-described; the two descriptions are similar (G. S. Karaman 2014c).

Conclusions

We described a new species of *Niphargus*, redescribed its closest relative and revised the distribution of two morphologically similar and phylogenetically closely related species. Morphological differences between the two species are small and comprise the presence of a seta on urosomite III, setal ornamentation of pereopods III–IV, proportions in gnathopods II and proportions between peropods V–VII. The differences in morphology are small and would not have been recognized without the help of molecular data. The support for species hypotheses is based mainly on molecular markers and seems to be strong enough to treat the two as separate species. The study contributes to a broader knowledge of overlooked species living in epikarstic systems and supports the general progress towards integrative taxonomy (Padial *et al.* 2010; Schlick Steiner *et al.* 2010)

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APPENDIX 1. List of species used in phylogenetic analysis. Voucher number refers to collection of Department of Biology, BF, UL. The label 'GB' means publicly available sequences from GenBank.

Species	Voucher ID	Locality name	Genbank accession number		
			28S	H3	COI
<i>Carinurella paradoxa</i>	NA738	Torre, Ruda, Monfalcone, ITA	NA	KR905901	KR905829
<i>Niphargobates orophobata</i>	NA546	Planinska jama, Kačja vas, Planina, SLO	KR905879	KR905900	NA
<i>Niphargus aberrans</i>	NA025	Planinska jama, Kačja vas, Planina, SLO	EF617260	NA	NA
<i>Niphargus aitolosi</i>	NA081	Lisimachia, Klisorevmata, Agrinio, GRE	EU693310	KP133142	NA
<i>Niphargus aquilex</i>	NA029	Marden, Marden, Wes Sussex, GB	EF617264	KP300936	NA
<i>Niphargus bajuvaricus</i>	NA024	Well A96, Lobau, Wien, AUT	EF617259	JQ815476	NA
<i>Niphargus balcanicus</i>	NA070, NB422	Vjetrenica, Zavala, Popovo polje, BIH	EF617280	JQ815507	KR905796
<i>Niphargus bilecanus</i>	NA182	Ljelješnica, Poraslica, Dabarsko polje, BIH	JQ815550	NA	KR905826
<i>Niphargus boskovic</i>	NA036	Bjelušica, Zavala, Popovo polje, BIH	EF617271	JQ815502	KR905781
<i>Niphargus brachytelson</i>	NA071	Lukova jama pri Zdihovem, Suhor, Kočevje, SLO	EU693293	JQ815489	KR905797
<i>Niphargus carniolicus</i>	NA017	Jama pod gradom Luknja, Prečna, Novo mesto, SLO	EF617252	JQ815501	KR905776
<i>Niphargus caspary</i>	NA073	Tuebingen, Tuebingen, Tuebingen: GER	EU693291	KJ566712	NA
<i>Niphargus cf. arbiter</i>	NA050	Spring in port Vrbnik, Vrbnik, Krk, CRO	EF617286	KR905885	KR905786
<i>Niphargus cf. stygius</i>	NA152	Valeni (wells), Ploiesti, Prahova, ROM	KJ566693	KJ566720	KR905821
<i>Niphargus croaticus</i>	NA076	Suvaja, Lušci Palanka, Sanski most, BIH	EU693297	JQ815490	KT007331
<i>Niphargus cvijici</i>	NA147	Popovo polje, Ravno, Ravno, BIH	JQ815554	JQ815516	NA
<i>Niphargus dalmatinus</i>	NA060	Biba spring, Vrana, Pakoštane, CRO	EF617296	JQ815484	KR905790
<i>Niphargus decui</i>	NA154	Limanu springs, Mangalia, Dobrogea, ROM	KF719272	KJ566720	KR905821
<i>Niphargus dimorphopus</i>	NA125	Gulpen, Gulpen, Limburg, NED	EU693296	EU693253	NA
<i>Niphargus dobati</i>	NA013	Rakov Škocjan, Zelše, Cerknica, SLO	EF617247	JQ815499	KR905774
<i>Niphargus dobrogicus</i>	NA140	Well N to Limanu, Mangalia, Dobrogea, ROM	KR905871	KR905891	KR905816
<i>Niphargus elegans</i>	NA061	San Pancrazio, San Pancrazio, Verona, ITA	EF617297	JQ815485	KR905791
<i>Niphargus factor</i>	NA078	Vjetrenica, Zavala, Popovo polje, BIH	EU693298	JQ815508	KR905798
<i>Niphargus fongi</i>	NA018	Dolga jama pri Koblarjih, Koblarji, Kočevje, SLO	EF617253	JQ815472	NA
<i>Niphargus frasassianus</i>	na	Grotte di Frasaasi, Fabriano, Perugia, ITA	GU973411	NA	GU973034
<i>Niphargus gallicus</i>	na	str. Bisericii 39, Dulceşti, Mangalia, ROM	KF290033	NA	KF290225
<i>Niphargus glenniei</i>	na	Plympton farm catchpit, Plymouth, Devon, GB	KC315617	NA	KC315644
<i>Niphargus grandii</i>	NA080	Torre, Ruda, Monfalcone, ITA	EU693300	KJ566715	KR905799

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APPENDIX 1. (Continued)

Species	Voucher ID	Locality name	Genbank accession number		
			28S	H3	COI
<i>Niphargus hadzii</i>	NA082	Izvir pod orehom, Verd, Vrhnik, SLO	EU693301	KR905887	KR905800
<i>Niphargus hercegovinensis</i>	NA151	Žira, Turkovići, Ravno, BIH	JQ815549	JQ815517	KR905820
<i>Niphargus hrabei</i>	NA083	Stream near the road W from Lupoglav, Lupoglav, Zagreb, CRO	EU693302	KJ566716	KR905801
<i>Niphargus hvarensis</i>	NA038	Trsteno, Dubrovnik, Dubrovnik, CRO	EF617273	JQ815479	KR905782
<i>Niphargus ictus</i>	na	Grotte di Frasaasi, Fabriano, Perugia, ITA	GU973415	NA	GU973012
<i>Niphargus illidžensis</i>	NA084	Vrelo Bosne, Iliđa, Sarajevo, BIH	EU693304	JQ815491	KR905802
<i>Niphargus irlandicus</i>	na	Carrigacrump Quarry, Coyne, Cork, GB	KC315618	NA	KC315647
<i>Niphargus karamani</i>	NA085	Fram 119 (well), Fram, Maribor, SLO	EU693305	KR905888	KR905803
<i>Niphargus karkabounasi</i>	NA217	Agioi Theodoroi, Korinthos, Peloponnese, GRE	KR905877	KR905898	NA
<i>Niphargus kenki</i>	NA086	Spring near to Sodna vas 25, Sodna vas, Podčetrtek, SLO	KR905869	NA	KR905804
<i>Niphargus kochianus</i>	NA090	Saint Albans, Hertfordshire, Hertfordshire, GB	EU693308	JQ815492	NA
<i>Niphargus kolombatovici</i>	NA964, NA963	Žira, Turkovići, Ravno, BIH	JQ815553	JQ815522	KT007386
<i>Niphargus krameri</i>	NA040	Fojba, Šestani, Pazin, CRO	EF617275	JQ815503	NA
<i>Niphargus kusceri</i>	NB422	Njegoševa pećina, Njeguši, Kotor, MNE	JQ815443	KR905929	KR905767
<i>Niphargus labacensis</i>	NA022	Tomačevo (interstitial waters), Ljubljana, Ljubljana, SLO	EF617257	JQ815474	KR905777
<i>Niphargus lessiniensis</i>	NA064	Grotta del Aqua, Ponte de Veja, Monte Lessini, ITA	EF617300	JQ815488	NA
<i>Niphargus liburnicus</i>	NB018	Grotta Andrea, Iamiano, Doberdob, ITA	KT007478	NA	KT007418
<i>Niphargus likanus</i>	NA523	Jama v kamnolomu, Vinica, Črnomelj, SLO	JQ815441	JQ815498	KR905828
<i>Niphargus longicaudatus</i>	NA006	Retec (source), Lubenice, Island of Cres, CRO	EF617240 EF617240	KJ566705	KR905772
<i>Niphargus longicaudatus</i>	NA007	Stream near the road Monte Faito-Vico Equense, Casola, Napoli, ITA	EF617241	JQ815469	NA
<i>Niphargus longidactylus</i>	NA021	Sneberje (Sava freatic waters), Ljubljana, Ljubljana, SLO	EF617256	JQ815473	NA
<i>Niphargus longiflagellum</i>	NA093	Podpeška jama, Videm, Grosuplje, SLO	EU693311	JQ815520	KR905805
<i>Niphargus lourensis</i>	NA094	Louros spring, Vouliasta, Ioannina, GRE	EU693312	NA	NA
<i>Niphargus lunaris</i>	NA095	Bubanj vrelo, Dolac Donji, Trilj, CRO	EU693313	KR905889	KR905807
<i>Niphargus miljeticus</i>	NA500	Vodice, Babino polje, island of Mljet, CRO	KR905878	KR905899	NA
<i>Niphargus montanarius</i>	na	Grotte di Frasaasi, Fabriano, Perugia, ITA	GU973419	NA	GU973003

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APPENDIX 1. (Continued)

Species	Voucher ID	Locality name	Genbank accession number		
			28S	H3	COI
<i>Niphargus multipennatus</i>	NA169	Tomačovo (interstitial waters), Ljubljana, Ljubljana, SLO	KJ566700	KJ566721	KR905825
<i>Niphargus novomestanus</i>	NA096	Tominčev studenec, Žužemberk, Žužemberk, SLO	EU693314	JQ815509	KR858496
<i>Niphargus orcinus</i>	NA099	Križna jama, Bločice, Lož, SLO	EU693315	JQ815510	KR905808
<i>Niphargus pachytelson</i>	NA100	Podpeška jama, Videm, Grosuplje, SLO	EU693316	JQ815511	KR905809
<i>Niphargus pasquinii</i>	NA010	Sorgenti San Vittorino, San Vittorino, Castel Sant'Angelo, ITA	EF617244	JQ815471	KR905773
<i>Niphargus pectinicauda</i>	NA023	Tomačovo (interstitial waters), Ljubljana, Ljubljana, SLO	EF617258	JQ815475	KR905778
<i>Niphargus podgoricensis</i>	NA166	Spring at Dobro polje, Dobro polje, Podgorica, MNE	KR905875	KR905896	KR905824
<i>Niphargus podpecanus</i>	NA101	Podpeška jama, Videm, Grosuplje, SLO	EU693317	JQ815512	NA
<i>Niphargus polymorphus</i>	NA047	Obodska pećina, Rijeka Crnojevića, Cetinje, MNE	EF617282	JQ815505	KR905784
<i>Niphargus pupetta</i>	NA102	Tomačovo (interstitial waters), Ljubljana, Ljubljana, SLO	EU693318	KJ566717	NA
<i>Niphargus puteanus</i>	NA066	Gasthof Zur Walba, Pentling, Pentling, GER	EF617302	KJ566709	KR905795
<i>Niphargus rejici</i>	NA048	Podpeško jezero, Jezero, Ig, SLO	EF617283	JQ815481	KR905785
<i>Niphargus rhenorodanensis</i>	NA104	Grotte Cormoran, Torcieu, Lyon, FRA	EU693319	KJ566719	KR905811
<i>Niphargus salonitanus</i>	NA053	Gospa od Stomorije spring, Kaštel Stari, Split, CRO	EF617289	JQ815483	KR905788
<i>Niphargus sanctinaumi</i>	NA105	Sveti Naum spring, Sv. Naum, Ohrid, MAC	EU693320	KP133144	KR905812
<i>Niphargus schellenbergi</i>	NA032	Well near road 800 m SE from Heyd, Heyd, Durbuy, BEL	EF617267	JQ815478	KR905780
<i>Niphargus scopicauda</i>	NA026	Huda luknja pri Gornjem Doliču, Završe, Slovenj Gradec, SLO	EF617261	JQ815477	KR905779
<i>Niphargus slovenicus</i>	NA106	Stražišče, Kranj, Kranj, SLO	EU693322	JQ815493	KR905813
<i>Niphargus sphagnicolus</i>	NA035	Mostec, Rožnik, Ljubljana, SLO	EF617270	NA	KR858495
<i>Niphargus spinulifemur</i>	NA107	Stream NE to Hrastovlje, Hrastovlje, Koper, SLO	EU693323	JQ815494	KR858500
<i>Niphargus speeckeri</i>	NA108	Pivka jama, Veliki otok, Postojna, SLO	EU693324	JQ815513	KR905814
<i>Niphargus stenopus</i>	NA049	Jama pod gradom Luknja, Prečna, Novo mesto, SLO	EF617284	JQ815506	NA
<i>Niphargus steueri</i>	NA950, NB050	Jama pod Krogom, Sočerga, Koper, SLO	KT007494	JQ815523	KT007358
<i>Niphargus stygius</i>	NA110	Jelenska jama, Borovnica, Vrhnik, SLO	KR905870	KR905890	KR905815
<i>Niphargus subtypicus</i>	NA112	Jama pod gradom Luknja, Prečna, Novo mesto, SLO	EU693326	JQ815514	KT007433
<i>Niphargus tatrensis</i>	NA028	Lodowe zrodlo (Icy spring), POL	EF617263	NA	NA
<i>Niphargus timavi</i>	NA114	Labodnica, Trebiciano, Trieste, ITA	EU693327	JQ815495	KR858497

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Species	Voucher ID	Locality name	Genbank accession number		
			28S	H3	COI
<i>Niphargus tridentinus</i>	NA063	Grotta Bus Pursi, Lumezzane, Brescia, ITA	EF617299	JQ815487	KR905793
<i>Niphargus trullipes</i>	NA046	Vjetrenica, Zavala, Popovo polje, BIH	EF617281	JQ815504	KR905783
<i>Niphargus vadimi</i>	NA144	Skelska peščera, Rodnikovo, Krym, UKR	KR905872	KR905892	KR905817
<i>Niphargus vinodolensis</i>	NA062	Ceovići, Bačići, Novi Vinodolski, CRO	EF617298	JQ815486	KR905792
<i>Niphargus virei</i>	NA003	Dorpstraat 7 (well), Reijmerstok, Limburg, NED	EF617237	JQ815467	KR905771
<i>Niphargus vjetrenicensis</i>	NA116	Vjetrenica, Zavala, Popovo polje, BIH	EU693329	JQ815521	KR858499
<i>Niphargus wolfi</i>	NA015	Križna jama, Bločice, Lož, SLO	EF617250	JQ815500	KR905775
<i>Niphargus zagorae</i>	NA212	Kevina jama, Radošić, Split, CRO	KR827044	NA	KR827046
<i>Niphargus zagorae</i>	NA213	Golubinka pod Barišinovcem, Črvrljevo, Šibenik, CRO	KR905876	NA	KR905827
<i>Niphargus zagorae</i>	NB609	Tomina jama, Ivkovići, Trogir, CRO	KR827043	KR905897	KR827045
<i>Niphargus zagrebensis</i>	NA059	Gadina, Loka, Črnomelj, SLO	EF617295	KR905886	KR905789
<i>Nipharus fontanus</i>	na	Little stour river, Littlebourn, Littlebourn / Amersham, Buckinghamshire, BG	EF617304	NA	KC315635
<i>Pontoniphargus racovitzai</i>	na	Pestera de la Movile, Mangalia, Dobrogea, ROM	KF290023	NA	NA
<i>Synurella ambulans</i>	NA002	forest ditch near Dept. Of. Biology, Ljubljana, Ljubljana, SLO	EF617236	NA	KR905770