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Phylogeny and biogeography of three new species of *Niphargus* (Crustacea: Amphipoda) from Greece

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ABSTRACT

Three new freshwater Amphipods of the family Niphargidae G. Karaman, 1962 are described from subterranean waters of Greece, *Niphargus aitolosi* sp. nov., *Niphargus karkabounasi* sp. nov. and *Niphargus koukourasi* sp. nov. Phylogenetic analysis based on 28S rDNA and H3 genes suggests that *N. aitolosi* sp. nov. belongs to *N. longicaudatus* species-complex. Species from that complex are distributed along both sides of Adriatic Sea and share roughly similar morphology, including the newly described species from Greece. *Niphargus karkabounasi* sp. nov., the second new species, belongs to a clade distributed broadly across Europe and the Middle East. Many species of this group, including *N. karkabounasi,* are small and likely live in small crevices. The third species, *Niphargus koukourasi* sp.nov. is phylogenetically related and morphologically similar to *N. sanctinaumi* and *N. maximus* from Lake Ohrid on the border of the Former Yugoslav Republic of Macedonia and Albania. In addition, this group of species may be related to species collected from Iran.

1. Introduction

The amphipod genus *Niphargus* Schiöde, 1849 is the largest genus among freshwater Amphipods (Väinöla et al., 2008), distributed in all types of subterranean waters of the Western Palearctic (Fišer et al., 2009a) and one of the key taxa of European groundwater fauna (Zagmajster et al., 2014). The revision of the genus is far from complete and new species are constantly discovered and described (e.g., Karaman et al., 2010; Hekmatara et al., 2013).

Nevertheless, two generalities seem to be arising. Firstly, phylogenetic analyses suggest that morphological differentiation does not follow phylogenetic relationships. Closely related species may be morphologically either very different (Fišer et al., 2008; Trontelj et al., 2012), or morphologically indistinguishable from each other (e.g., Trontelj et al., 2009; Meleg et al., 2013). Molecular analyses revealed also the second important characteristic of *Niphargus*, i.e., that small distributional ranges are rather rule than exception (Trontelj et al., 2009). Their poor dispersal ability is reflected also on a higher taxonomic level, as many clades are related to distinct biogeographic regions (Fišer et al., 2008).

In general a high degree of endemism suggests that efficient registration of extant species requires high sampling effort across the entire genus range. In *Niphargus*, however, this is still far from reality. Many areas within the genus range harbour only few species and it is likely that some of these areas have not been sufficiently explored (Hekmatara et al., 2013). The unexplored areas may contain the missing puzzle pieces needed for reconstruction of species diversity and evolutionary history of the genus. This study is one of the steps needed to complete description of diversity of this important genus.

From a biological point of view, Greece is considered as one of the most biologically diverse countries of Europe (Kryštufek and Reed, 2004; Griffiths, 2006). It is the crossroad between Europe, Mediterranean, Black Sea and the Middle East (Bănărescu, 2004) and demonstrates a complex isolated freshwater system, especially in the west and the south (Economidis, 1995). Despite the numerous phylogeographic studies in the area, many taxa remain unstudied and need revision (Poulakakis et al., 2014). Furthermore, Greek freshwaters exhibit high richness of epigean species and high endemism (Médail and Quézel, 1999; Oikonomou et al., 2014), which reflects its habitat diversity but it could also be the reflection of absence of massive Pleistocene extinctions (Hewitt, 2011). Broad comparative analyses suggest decline of subterranean species richness in southern-most parts of Europe (Zagmajster et al., 2014). Indeed, up to date only seven *Niphargus* species (Karaman, 1934;

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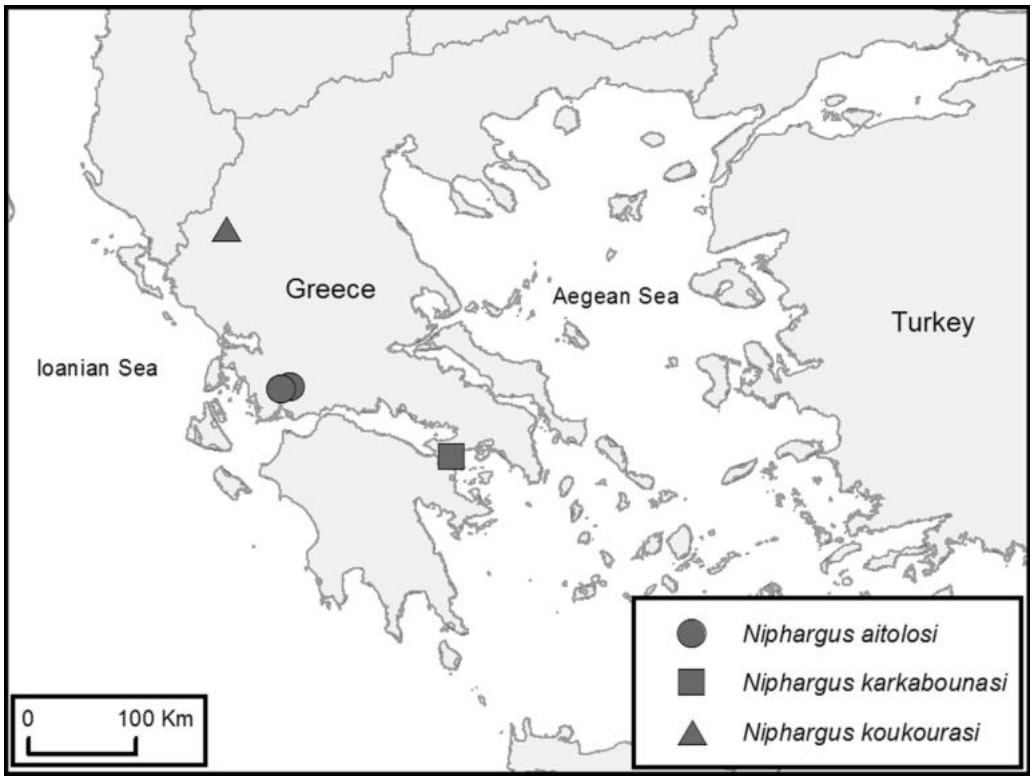

Fig. 1. Distribution map of each of newly described species. Location on the map is representing locality of individual used in phylogenetic analysis.

Karaman, 1950a; Fišer et al., 2006) have been reported from Greece. It seems that the diversity of subterranean fauna is low compared to the rich epigean fauna, implying that this area might be underexplored. In addition, phylogenetic position and origin of these species is largely unknown. Early studies of groundwater fauna based on morphology indicate that Greek and Italian groundwater species may be related to each other (Pesce, 1981, 1985; Fišer et al., 2008) however, relationships towards Middle East fauna have not been established yet.

The present contribution is aiming at describing the three new *Niphargus* species, and presenting their phylogenetic relationship to *Niphargus* species that had been collected in the past decade.

2. Materials and methods

2.1. Samples, DNA extraction, amplification and sequencing

The specimens used for the present study were collected during April and May of 2004 (Fig. 1). Afterwards, the samples were fixed and stored in 96% ethanol until DNA extraction. Genomic DNA was extracted from one pereiopod using the GenElute Mammalian Genomic DNA kit (Sigma). The rest of the animal was kept as a morphological voucher and stored in the Zoological collection (Department of Biology, Biotechnical Faculty, University of Ljubljana).

The first 28S rDNA fragment was amplified using the forward primer from Verovnik et al., 2005 and the reversed primer from Zakšek et al., 2007 and the second part of the 28S rDNA fragment was amplified using primers from Fišer et al., 2013. For sequencing of the second part of the 28S rDNA fragment additional newly designed primer set 5′-TGTCAACAGTGATTGAACATGG-3' and 5′-GCGTTAAGCAGAAAAGAAAACTC-3' was used to get a complete overlap of sequences. The histone H3 gene was amplified using primers H3NF and H3NR from Colgan et al., 2000. PCR was performed under the cycling settings described in Fišer et al., 2013. PCR products were purified using the Exonuclease I and Alkaline Phosphatase (Fermentas, Germany) according to manufacturer's instruction. The fragments were sequenced in both directions using PCR amplification primers by Macrogen Europe (Amsterdam, The Netherlands). Contigs were assembled and edited using Geneious Pro 5.5.6. (Biomatters).

2.2. Morphological analyses

For morphological analyses, specimens were partly dissected in glycerol, and mounted on slides in a glycerol-gelatine medium. Digital photos were taken with an Olympus DP10 camera mounted to an Olympus SZX9 stereomicroscope. Measurements and counts were made using the computer program Olympus DP–soft. Details were examined using a Zeiss microscope with magnifications 100–400×.

Details on measures (landmarks, details on individual characters) have been presented in Fišer et al. (2009b). The following abbreviations are used throughout the text: aI, II–antenna I and II; ul–upper lip; mdR, L–right and left mandible; md-plp – mandibular palp; mxI-II – maxilla I-II; lb – labium; mxpe – maxilliped; gpI, II – gnathopod I and II; pp III–VII – pereopod III–VII; plp – pleopod; ep – epimeral plate; upI–III – uropod I–III, T – telson.All data included in the description, along with photos taken in due process of research, are deposited on the website http://niphargus.info/morpho-database/and anyone using data on *N. aitolosi, N. karkabounasi* and *N. koukourasi* is kindly requested to cite this paper.

2.3. Phylogenetic analyses

To recover phylogenetic relationships of niphargid species from Greece, a dataset of niphargid taxa was compiled, using available

sequences from previous studies (Fišer et al., 2008, 2013; Trontelj et al., 2009, 2012; Altermatt et al., 2014). Based on two previous studies (Fišer et al., 2008; Trontelj et al., 2009) the selection of taxa was made in such a manner that the final dataset includes representatives of all clades of the genus and to keep the number of missing data at minimum levels. The result was a dataset counting one outgroup taxon (*Synurella ambulans*), three *Niphargus* species from Greece and 61 *Niphargus* species collected between Great Britain and Iran. List of taxa and sequences with accession numbers used in the analyses are listed in Table S1.

The obtained 28S rDNA sequences varied considerably in length: about 800 to 930 bp for the first part and 1250 to 1470 bp for the second part of 28 S. To account for the long indels, the sequences were aligned using the E-INS-i option for sequences with multiple conserved domains and long gaps in MAFFT ver. 6 (Katoh and Toh, 2008). The large differences were mainly due to simple sequence repeat insertions in some species, therefore low homology regions with long gaps were removed using Gblocks (Talavera and Castresana, 2007) under the least restrictive settings possible. All histone 3 gene sequences were of equal length (331 bp). All three sequence alignments were concatenated and analyzed as a single dataset in further phylogenetic analysis. Altogether, concatenated alignments of all three gene fragments (2818 nucleotides), was used for Bayesian inference using MrBayes 3.2 (Ronquist and Huelsenbeck, 2003). A general time-reversible model with a proportion of invariant sites and a gamma distribution of rate heterogeneity (GTR + I + Γ) assuming six discrete gamma categories was chosen as the most appropriate model according to AIC and BIC criteria, using ModelGenerator (Keane et al. (2006)). Two parallel searches with four chains each were run for two million generations, sampled every 100th generation. After discarding the first 25% of the sampled trees, final topologies were constructed according to the 50% majority rule.

3. Results

3.1. Species descriptions

3.1.1. Niphargus aitolosi sp.nov.

3.1.1.1. Material examined. Lake Lysimachia, Padanassa, Agrinio, Hellas; 24.4.2004; leg. C. Fišer & R. Verovnik (six males, one female, type series; vouchers NB994-NC000). Sample was collected in the soil between the roots of the fallen tree at the coast of the lake; Lake Trichonida, Padanassa, Agrinio, Hellas; 24.4.2004; leg. C. Fišer & R. Verovnik (one male and one female; vouchers NC001-NC002). Sample was collected in a tiny spring near the coast, specimens were found in decaying leaves.

3.1.1.2. Type locality. Lake Lysimachia, Padanassa, Agrinio, Hellas.

3.1.1.3. Type series. Holotype female 9.8 mm (no 2; voucher NB995). Paratypes include one male of length 10.6 mm, all collected on the type locality.

3.1.1.4. Etymology. The species (pronounced as etolósi) is named in honour of the ancient King Aiotlos, of the region of Aitolia.

3.1.1.5. Diagnosis. Mid-sized *Niphargus* of slender appearance and acute to subrounded epimeral plates. Pleon segments with up to 4 setae along postero-dorsal margin and single dorso-lateral seta on urosomite I. Telson with apical, lateral, mesial and dorsal spines, telson lobes are narrowing only slightly. Outer lobes of maxilla I with seven spines denticulated along inner side. Inner lobes of maxiliped have more than three spines. Appendages short, dactyli of pereopods III–VII with only one spiniform seta at the base of the nail. Gnathopods small and quadrate, with several setae along outer margin of dactylus. Endopodite of uropods I and exopodite of uropods III sexually dimorphic and remarkably elongated in males.

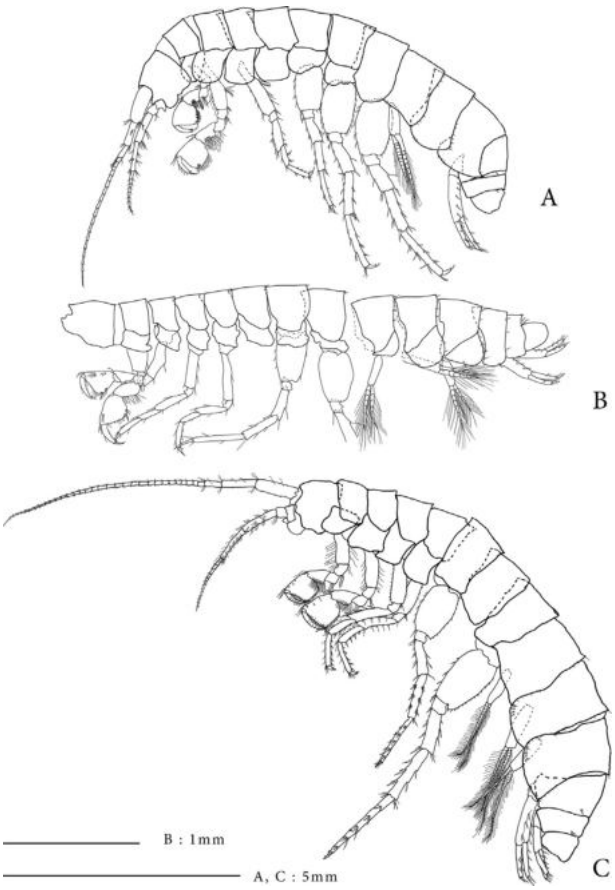

Fig. 2. *A. Niphargus aitolosi* sp. nov. (holotype: female, 9.6 mm; type locality: 38.5611°N, 21.3694°E, Lake Lysimachia, Padanassa, Agrinio, Hellas); B. *Niphargus karkabounasi* sp. nov. (holotype: male, 3.5 mm; type locallity: 37.5000°N, 23.5000°E, Agioi Theodoroi, Korinthos, Peloponnese, Hellas); C. *Niphargus koukourasi* sp. nov (holotype: male, 14.5 mm; type locallity: 39.2555° N, 20.5028° E, Springs of Louros River, Vouliasta, Ioannina, Hellas).

3.1.2. Description of type series

Head and trunk (Fig. 2A): Body length up to 13.1 mm. Head length up to 10% of body length; rostrum absent. Pereonites I–VI without setae, pereonite VII postero ventrally with up to 5 setae.

Pleonites I–III with up to 4 setae along the entire dorso-posterior margin. Epimeral plate I (Fig. 3A) slightly inclined; along posterior margin 5 spiniform setae. Epimeral plate II (Fig. 3B) slightly inclined, posterior and ventral margins sinusoid and convex, respectively; ventro-postero-distal corner slightly pointed; along ventral margin 2 spiniform setae; along posterior margin 5–7 thin setae. Epimeral plate III (Fig. 3C) inclined, posterior and ventral margin concave and slightly convex, respectively; ventro-postero-distal corner pointed; along ventral margin 3 spiniform setae; along posterior margin 5–8 thin setae.

Urosomite I postero-dorso-laterally with 1 weak setae; urosomite II postero-dorso-laterally with 2 setae; urosomite III without setae. At the base of uropod I single strong spinifrom seta.

Telson (Fig. 3H) *length*: width ratio is 1: 0.90–0.95; cleft is 0.60–0.70 of length; telson margins straight and narrow apically. Telson spiniform setae (per lobe): 3 apical spiniform setae of up to 0.35 telson length; 0–2 mesial setae; lateral margins up to 2 setae; dorsal surface with one group up to 2 spiniform setae.

Antennae (Fig. 4; A,B): Antenna I 0.30–0.45 of body length. Flagellum with up to 22 articles; each article with 1 aesthetasc.

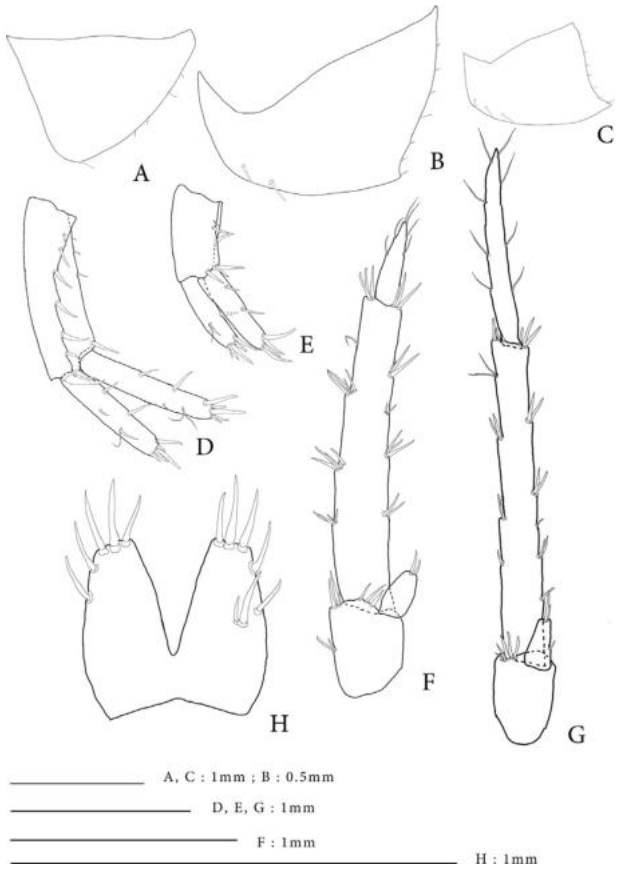

Fig. 3. *Niphargus aitolosi* A. epimeral plate I; B. epimeral plate II; C. epimeral plate III; D. uropod I; E. uropod II; F. uropod III male; G. uropod III female; H. telson (drawings A–E, G, H: female holotype; drawing F: male paratype).

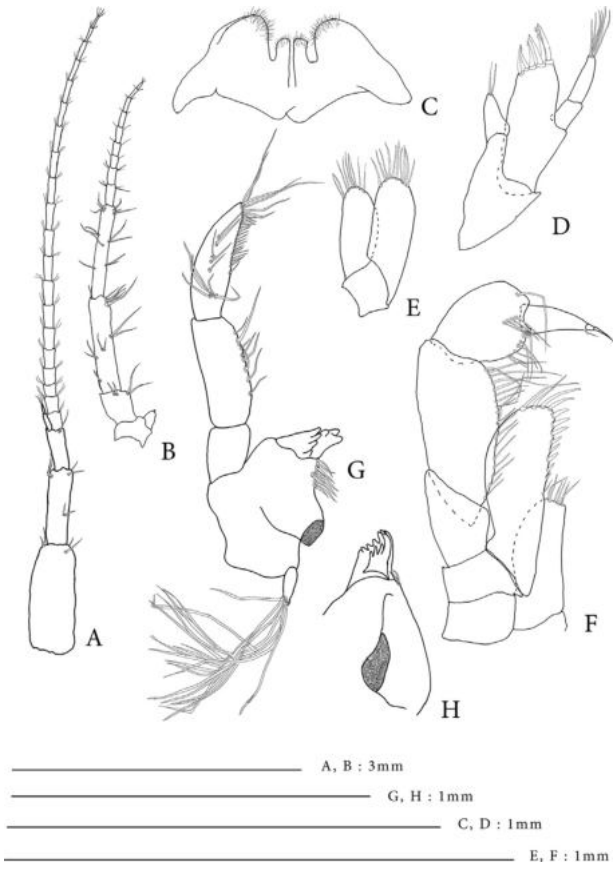

Fig. 4. *Niphargus aitolosi* A. antenna I; B. antenna II; C. labrum; D. maxilla I; E. maxilla II; F. maxilliped; G. right mandible; H. left mandible (drawings A–H: female holotype).

Peduncle articles in ratio 1: (0.70–0.85): (0.35–0.45). Proximal article of peduncle dorso-distally slightly produced. Accessory flagellum biarticulated; distal article equal to one half of proximal article length.

Length ratio antenna I: antenna II as 1: (0.50–0.60). Flagellum of antenna II with 7–9 articles; each article with several setae, many also with a single elongate sensilla of unknown function. Peduncle articles lengths 4: 5 is 1: (0.9–0.95); flagellum 0.70–0.75 of summed length of peduncle articles 4 and 5.

Mouthparts (Fig. 4; C–H): Labrum typical; inner lobes of labium longer than half of the outer lobes.

Left mandible: Incisor with 5 teeth, lacinia mobilis with 4 teeth; between lacinia and molar a row of thick serrated setae, long seta at the base of molar. Right mandible: incisor processus with 4 teeth, lacinia mobilis with several small denticles, between lacinia and molar a row of thick serrated setae. Ratio of mandibular palp article 3: article 2 (distal) is 1: (1.05–1.20). Proximal palp article without setae; the second article with up to 8 setae; distal article with one group of 3–5 A setae; 3–5 groups of B setae; 15–27 D setae and 3–4 E setae.

Maxilla I distal palp article with 4–6 apical setae. Outer lobe of maxilla I with 7 uni-toothed stout setae apically; inner lobe with 2 setae.

Maxilla II inner lobe slightly smaller than outer lobe with ratio 0.85/1; both of them setose apically and subapically.

Maxilliped palp article 2 with 12 rows of setae along inner margin; distal article with a dorsal seta and a group of small setae at the base of the nail. Maxilliped outer lobe with 7–10 flattened thick setae mesially and 5–7 serrated setae apically and subapically; inner lobe with 4–5 flattened thick setae apically and 7–8 serrated setae.

Coxal plates, gills and oostegites (Figs. 5 and 6)*:* Coxal plate I of parallelogram shape, antero-ventral corner subrounded; anterior and ventral margin of coxa I with 5–10 setae. Coxal plate II depth: width is 1: 0.95–1.20; anterior and ventral margin with 8 setae. Coxal plate III depth: width is 1: 0.95–1.17; anterior and ventral margin with 5–10 setae. Coxal plate IV depth: width is 1: 1.05–1.25; posteriorly slightly concave (approx 0.08 of coxa width); along antero-ventral margin 6–10 setae. Coxal plates V–VI: anteriorly developed lobe; posterior margin with 2–4 setae each. Coxal plate VII half-egg shaped with 1 posterior seta. Gills II–VI ovoid and reach up to the mid of basis, oostegites large ovoid, with long setae.

Gnathopod I (Fig. 5A): Ischium with 1 submarginal seta and 3–5 postero-distal setae. Carpus 0.50–0.60 of basis length and 0.75–0.90 of propodus length. Anterior margin of carpus with the distal group of setae; carpus posteriorly with transverse rows of setae proximally, a row of lateral setae; postero-proximal bulge well developed (approximately almost 1/4 of carpus length), positioned proximally. Propodus trapezoid, palm convex and slightly inclined. Along posterior margin 4–5 rows of denticulated setae. Anterior margin with 7–13 setae in 2–3 groups in addition to antero-distal group counting 7–13 setae. Proximally to palmar spine is a group of 2–4 facial setae; several groups of short facial setae on the inner surface present. Palmar corner rounded and with strong palmar spiniform seta, single supporting spiniform seta on inner surface and 3–4 single thick spiniform setae on outer side. Nail length 0.30–0.35 of total dactylus length; along anterior margin 4–6 seta; along inner margin a row of short setae.

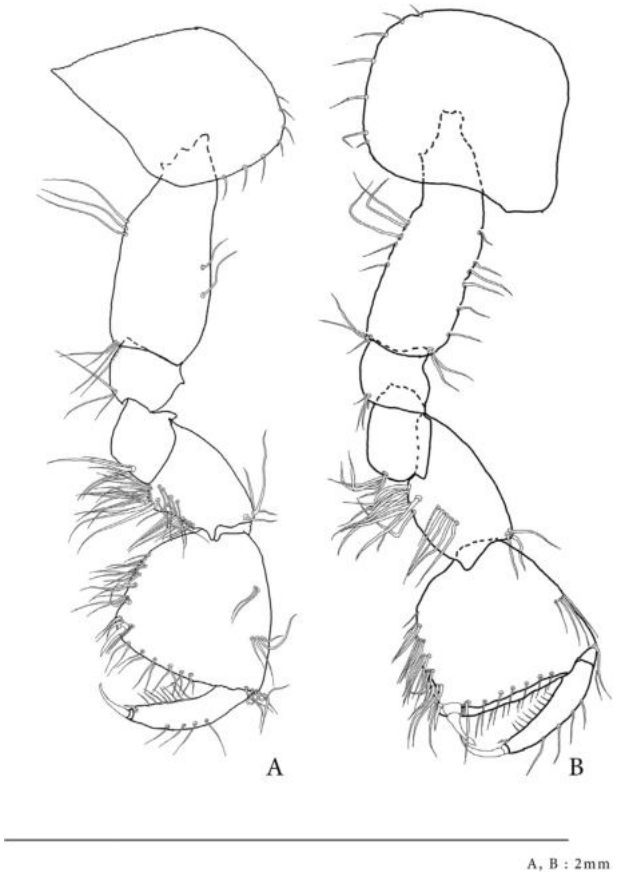

Fig. 5. *Niphargus aitolosi*: A. gnathopod I; B. gnathopod II (drawings A, B: female holotype).

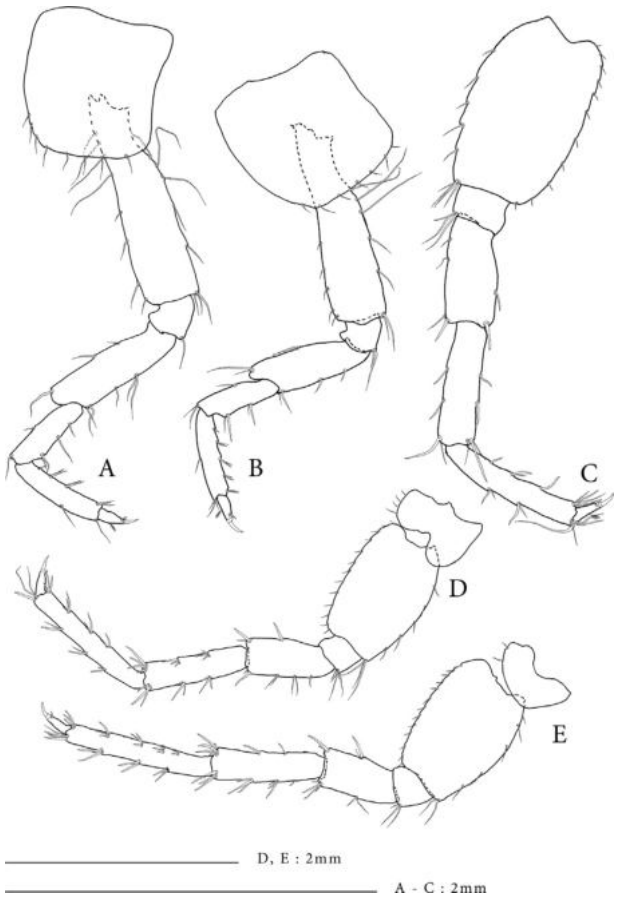

Fig. 6. *Niphargus aitolosi* A. pereopod III; B. pereopod IV; C. pereopod V; D. pereopod VI; E. pereopod VII (drawings A–E: female holotype).

Gnathopod II (Fig. 5B): Basis width: length is 1: 0.35–040. Ischium with 3–5 postero-distal setae in a single row. Carpus 0.55–0.65 of basis length and 0.90–1.00 of propodus length. Anterior margin of carpus with distal row of setae; carpus posteriorly with transverse rows of setae proximally and a row of lateral setae; postero-proximal bulge large, about 0.25 of carpus length, positioned proximally. Propodus small-sized (circumference measures 0.13–0.18 of body length) and slightly larger than propodus of gnathopod I [lenght ration is 1: (1.05–1.15)]. Propodus rectangular, palm convex and slightly inclined. Posterior margin with 6–7 rows of setae. Anterior margin with 4–8 setae in 2–4 groups in addition to 5–10 antero-distal setae. Proximal to palmar spine with a group of 2–4 facial setae and several individual setae along inner surface. Palmar corner with strong palmar spiniform seta, single supporting spiniform seta on inner surface and 3–4 single thick-spiniform setae on outer side. Nail length 0.25–0.35 of total dactylus length. Along anterior margin 4–5 seta; along inner margin few short setae.

Pereopods III-IV (Fig. 6; A,B): Lengths of pereopods III: IV equal to ratio 1: (0.9–0.95). Dactylus IV 0.45–0.55 of propodus IV; nail length 0.45–0.55 of total dactylus length. Dactyli III-IV with one dorsal plumose seta; at the base of nail one spiniform setae.

Pereopods V–VII (Fig. 6; C–E): Lengths of pereopods V: VI: VII is 1: (1.30–1.35): (1.30–1.45). Pereopod VII length 0.35–0.45 of body length.

Bases V–VII length: width is 1. (0.65–0.75), (0.65–0.70) and (0.65–0.75), respectively; posterior margins straight to convex, with small distal lobes; posteriorly 9–16, 9–14 and 10–15 thin flexible setae respectively; anteriorly 5–7, 5–7 and 4–6 groups of slender spiniform setae respectively. Dactylus VII length 0.30–0.35 of propodus VII length; nail length 0.30–0.40 of total dactylus length. Dactyli V–VII with 1 dorsal plumose seta and 1 spinform seta at the base of nail.

Pleopods: Pleopods I–III with 2 hooked retinacles. Bases of pleopods II-III with distinct simple flexible setae.Pleopod II rami of 9–14 articles each.

Uropods (Fig. 3; D–G): Uropod I protopodite with 5–9 dorsolateral spiniform setae and 2–5 dorso-medial spiniform setae. Exopodite: endopodite lengths is 1: (1–1.15–1.25 females) or 1: (1.90–2.05 in males); rami straight. Endopodite with 3–9 setal groups with 4–16 stout and flexible sete; apically 3–6 stout spiniform setae.Exopodite with 3–4 setal groups with 4–8 stout and flexible sete; apically with 5 stout spiniform setae.

Uropod II exopodite: endopodite lengths is 1: 1.15–1.35.

Uropod III up to 0.20 (females) or 0.33 (males) of body length.Protopodite with 2–7 spiniform lateral seta and 6–10 apical spiniform setae. Endopodite 0.45–0.80 of protopodite length, apically with 2–4 spiniform and flexible setae; laterally with 1–5 setae. Exopodite of uropod III rod-shaped, distal article 0.30 (female) or 0.80 (in males) of the proximal article length. Proximal article with 4–7 groups of spiniform setae along inner margin and 4–7 groups of spiniform setae along outer margin. Distal article with up to 6 setae groups along both margins; apically with 2–4 setae.

3.1.2.1. Variability. Smaller individuals are less spiny and in body proportions more similar to females. Males differ from females in body size (males being larger) and proportion of the uropods I and III. Inner ramus of uropod I is substantionally elongated in males, almost as twice as long as in females. Similarly, uropod III is longer, with subtstantionally elongated articles of expopodite. Notable is also the difference between two populations we analyzed. All spec-

imens from Lake Trichonida have subrounded epimeral plate III, whereas specimens from Lake Lisimachia have distinctly pointed ventro-posterior corner.

3.1.2.2. Remarks and affinities. Aside from morphology, species status of this species is supported with monophyly on 28S gene marker. As this marker is rather conservative, it is likely that populations of *N. aitolosi* have been evolving independently for a long time. Mid-body size, small gnathopods, relatively short pereopods, sexually dimorphic uropods I and III and the shape of epimeral plate II suggests this species resembles species from *N. longicaudatus* Costa, 1851 species-complex. Present phylogenetic analysis suggests that this species is nested within the complex of morphologically cryptic or 'semi-cryptic' species, which contains species from both sides of the Adriatic Sea (Table 1). On the other hand, the group requires a thorough revision (Ruffo and Stoch, 2006). A number of species within the complex may be expected (Stoch, pers. comm.); however, in this study the comparisons between described species and populations are limited. Newly described species differ from *N. longicaudatus* (Italy, vicinity of Napoli, type population, Karaman, 1986) and *N. versluysi* (Greece; Karaman, 1950a) by spinal pattern on dactyli of pereopods VII; the latter species have in these articles more than one spine. In addition, population in the vicinity of Napoli (Italy) have consistently more spines on epimeral plate II and no lateral spines on telson (unpublished data). Populations from the Gargano region (Fig. 15, Italy, own observations) and the Island of Cres (Fig. 15, Croatia, own observations) have fewer flattened spines on inner lobes of maxiliped (mostly three). Another species form the complex described from Bosnia and Herzegovina, *Niphargus cvijici* S. Karaman, 1950 differs from herein described species in shape of telson; in *N. cvijici* it is broader with lobes more narrowing apically (Karaman, 1950b). Recently described *N. frasassianus* Karaman et al., 2010 (Frasassi Cave System, Italy) differs from Greek species in spines on outer lobe of maxilla I. The Italian species has spines with denticles along both sides of spines, whereas herein described species has tiny denticles only along inner side of tooth (Karaman et al., 2010). *Niphargus pasquinii* Vigna- Taglianti, 1966 (Fig. 15) differs from the described species by larger number of pectinate spines on outer lobe of maxilla I (Vigna- Taglianti, 1966).

Although we could check the material on our own, these observations deserve a critical remark that the number of collected and observed individuals per population was low (7–10) and that the variation of all above listed populations may be sampled incompletely. The entire monophylum requires extensive revision. The reported observations suggest that several species may not be easily distinguished using morphological traits alone and that future identification of species within the complex may largely depend on a well-established barcoding system.

3.1.3. Niphargus karkabounasi sp. nov.

3.1.3.1. Material examined. Agioi Theodoroi, Korinthos, Peloponnese, Hellas; 21.4.2004; leg. C. Fišer & R. Verovnik. Sample was collected in approximately 20 m deep well.

3.1.3.2. Type locality. Agioi Theodoroi, Korinthos, Peloponnese, Hellas.

3.1.3.3. Type series. Holotype is 3.5 mm long male (no 1; voucher NC003). Paratypes include males of lengths 3.0 (no 2; voucher NC004), 3.2 (no 4; voucher NC006), and 3.4 mm (no 5; voucher NC007), respectively, and a female of length 4 mm (no 3; voucher NC005), all collected on type locality.

3.1.3.4. Etymology. The species is named in honour of Assistant Professor Spyridon Karkabounas for his contribution to the anticancer research. (Laboratory of Physiology, Division of Clinical and Basic Functional Sciences Department of Medicine, Faculty of Health Sciences, University of Ioannina, University Campus, 45110, Ioannina, Hellas).

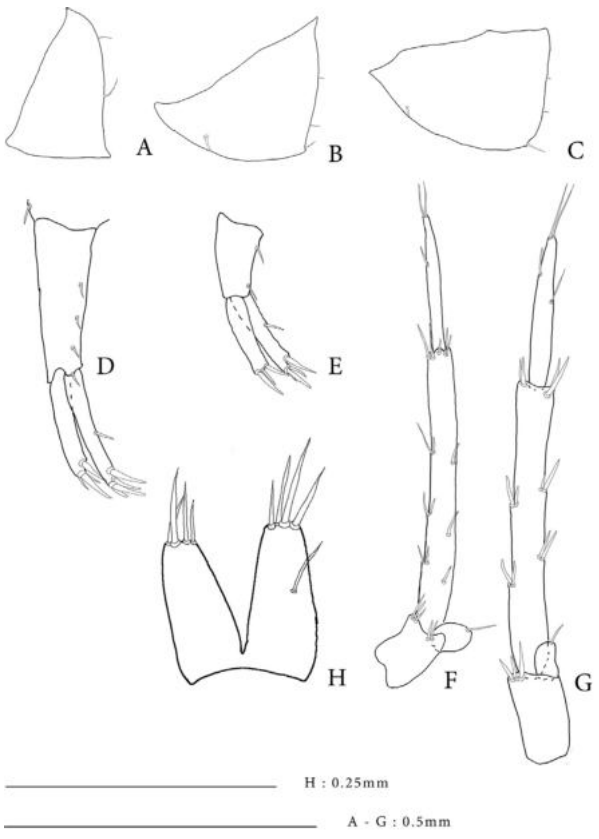

Fig. 7. *Niphargus karkabounasi* A. epimeral plate I; B. epimeral plate II; C. epimeral plate III; D. uropod I; E. uropod II; F. uropod III male; G. uropod III female; H. telson (drawings A–F, H: male holotype; drawing G: female paratype).

3.1.3.5. Diagnosis. Small and slender *Niphargus*, with subrounded epimeral plates. Appendages short, dactyli with spine at the base of nail. Pleopods with two retinacles. Uropod I sexually non-dimorphic and uropod III sexually dimorphic; males have elongated expopodite articles. Propodi of gnathopods small and triangular, the first and the second pair of subequal size**,** gnathopod dactylus with a single seta along outer margin.

3.1.4. Description of type series.

Head and trunk (Fig. 2B): Body length up to 4 mm. Head length up to 11% of body length; rostrum absent. Pereonites I–IV without setae. Pereonites V–VII with one simple seta on ventro-posterior corner.

Pleonites I–III with up to 3 setae along the entire dorso-posterior margin. Epimeral plate I (Fig. 7A) slightly inclined; posterior and ventral margins concave and slightly sinusoid; ventro-postero-distal corner distinct and slightly produced; along posterior margin 2 thin setae. Epimeral plate II (Fig. 7B) slightly inclined, posterior and ventral margins concave and slightly sinusoid, respectively; ventro-postero-distal corner distinct but not produced; along ventral margin 1 spiniform setae; along posterior margin 3 thin setae. Epimeral plate III (Fig. 7C) inclined, posterior and ventral margins straight and slightly convex, respectively; ventro-postero-distal corner distinct but not produced; along ventral margin 1 spiniform seta; along posterior margin 3 thin setae.

Urosomite I postero-dorso-laterally with 2 weak setae; urosomite II postero-dorso-laterally with 1 thin seta and 1 spiniform

Table 1
Comparison of *N. aitolosi* with morphologically similar species.

Species	Epimeral plate II (number of ventral spines)	Telson (lateral spines)	Telson (shape of lobes apically)	Maxilliped (flattened spines on inner lobe)	Maxilla I (number of pectinate spines on outer lobe)	Maxilla I (type of denticulation on outer lobe)	Pereopod VII (number of spines on dactylus)	Source of information
N. aitolosi sp.n.	2	2	Broad	4–5	7	Few denticles along inner margin	1	Present study
N. frasassianus Karaman, Borowsky, Dattagupta (2010)	2 –3	0	Broad	3	7	Few denticles along both sides	1	Karaman et al. (2010)
N. pasquinii Vigna-Taglianti (1966)	2	1 –2	Broad	3–4	9 –13	Densely pectinate	2 –3	Personal observation[a]
N. cvijici Karaman S. 1950	2	0	Narrow	2–3	7	Few denticles along inner margin	1	Karaman (1950b)
N. versluysi Karaman S. 1950	2	0	Broad	?	7	Few denticles along inner margin	1 –2	Karaman (1950a)
N. longicaudatus (Neaples) A. Costa 1851	2 –3	0	Broad	3–5	7	Few denticles along inner margin	2 –3	Personal observation[b], Karaman (1986)
N. longicaudatus (Cres)[e] A. Costa 1851	2	1 –2	Broad	2	7	Few denticles along inner margin	1	Personal observation[c]
N. longicaudatus (Gargano)[e] A. Costa 1851	1 –2	1 –2	Broad	3	7	Few denticles along inner margin	1	Personal observation[d]

[a] Sorgenti di S. Vittorino, Rieti, Lazio, ITA; 20. 3. 2004; leg. C. Fišer, 7 individuals.
[b] Monte Faito-Vico Equense, Neaples, ITA, 26.3.2004; leg. C. Fišer, 10 individuals.
[c] Spring Retec, Lubenice, Cres, CRO, 30.4. 2004; leg. B. Sket; 9 individuals.
[d] Sorgente di Laura, Gargano, ITA, 31. 3. 2004; leg. C. Fišer; 7 individuals.
[e] *N. longicaudatus* is a complex of several species that needs to be revised. Populations in the table likely deserve species status, see text.

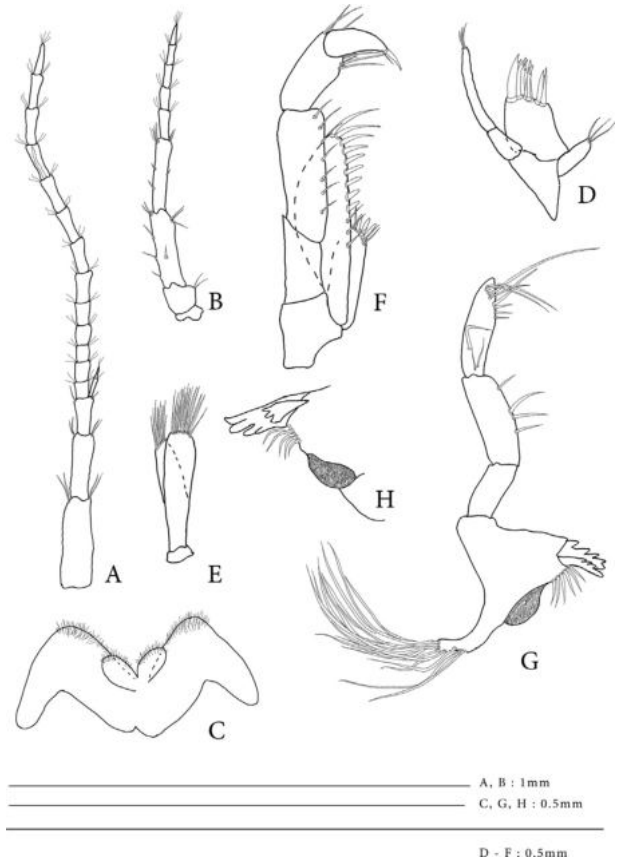

Fig. 8. *Niphargus karkabounasi* A. antenna I; B. antenna II; C. labrum; D. maxilla I; E. maxilla II; F. maxilliped; G. left mandible; H. right mandible (drawings A–F: male holotype; drawings G, H: female paratype).

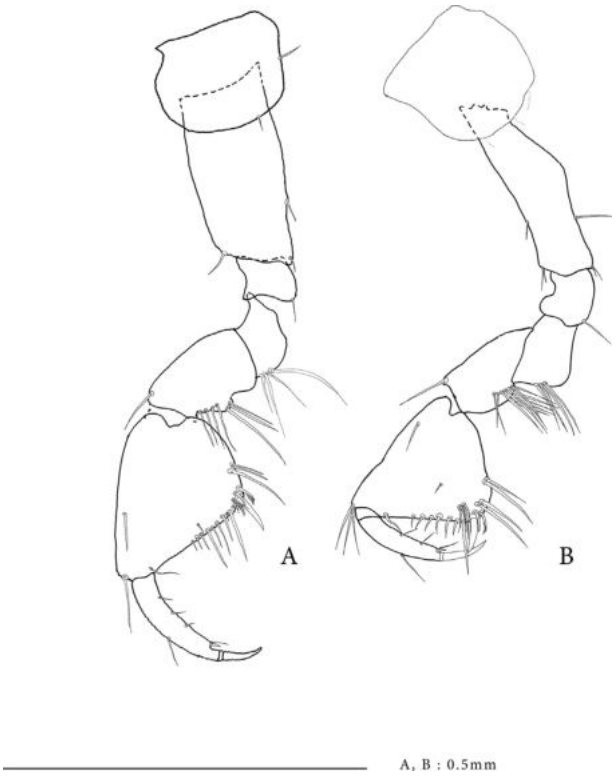

Fig. 9. *Niphargus karkabounasi* A. gnathopod I; B. gnathopod II (drawings A, B: male holotype).

seta; urosomite III without setae. At the base of uropod I single strong spinifrom seta.

Telson (Fig. 7H) length: width ratio is 1: (0.8–0.95); cleft is (0.70–0.80) of length; telson margins straight and narrow apically. Telson spiniform setae (per lobe): 3–4 apical spiniform setae of between 0.30–0.40 telson length; lateral and mesial margins without setae; dorsal surface with 1 simple, flexible seta.

Antennae (Fig. 8; A,B): Antenna I 0.40–0.65 of body length. Flagellum with up 12–14 articles; 1 aesthetasc. Peduncle articles in ratio 1: (0.65–0.75): (0.40–0.50). Proximal article of peduncle dorsodistally slightly produced. Accessory flagellum biarticulated; distal article shorter than one half of proximal article length.

Length ratio antenna II: antenna I ranging from 0.55 to 0.71. Flagellum of antenna II with 5 articles; each article with setae and elongate sensilla of unknown function. Peduncle articles lengths 5:4 ranging from 0.85 to 0.95; articles 4 + 5 length: flagellum length ranging from 1.64 to 1.77.

Mouthparts (Fig. 8; C–H): Labrum typical; inner lobes of labium slightly longer, than half of the outer lobes.

Left mandible: Incisor with 5 teeth, lacinia mobilis with 4 teeth; between lacinia and molar a row of thick serrated setae, long seta at the base of molar. Right mandible: incisor processus with 4 teeth, lacinia mobilis with several small denticles, between lacinia and molar a row of thick serrated setae. Ratio of mandibular palp article 2: article 3 (distal) equal to 1: (1.00–1.10). Proximal palp article without setae; second article with 2–4 setae; distal article with 1–2 A setae; 0 of B setae; 4–5 D setae and 4 E setae.

Maxilla I distal palp article with 3 apical setae. Outer lobe of maxilla I with 7 uni-toothed stout setae apically; inner lobe with 2–3 setae.

Maxilla II inner lobe smaller than outer lobe with ratio 1: 0.80; both of them setose apically and subapically.

Maxilliped palp article 2 with 6–7 rows of setae along inner margin; distal article with a group of small setae at the base of nail. Maxilliped outer lobe with 6–7 flattened thick setae mesially and 3–5 serrated setae apically and subapically; inner lobe with 2–3 flattened thick setae apically and 3 serrated setae.

Coxal plates, gills and oostegites (Figs. 9 and10): Coxal plate I of parallelogram shape, antero-ventral corner subrounded; anterior and ventral margin with 1–2 setae. Coxal plates II and III width: depth 1: 0.85–1.00; ventral margin with 1–4 setae. Coxal plate IV width: depth ratio 1: (0.70–0.85); posteriorly slightly concave (<0.10 of coxa width); along antero-ventral margin 1–3 setae. Coxal plates V and VI: anteriorly developed lobe; posterior margin with 1–2 seta. Coxal plate VII half-egg shaped with 1 posterior seta. Gills II–VI ovoid and reach up to the mid of basis, oostegites large ovoid, with long setae.

Gnathopod I (Fig. 9A): Ischium with 1–2 submarginal seta. Carpus length is 0.60–0.70 of basis length and 0.60–0.80 of propodus length. Anterior margin of carpus with the distal seta; carpus posteriorly with transverse rows of setae proximally, a row of lateral setae; postero-proximal bulge large (1/3 of carpus length), positioned proximally. Propodus almost triangular, palm convex and inclined. Along posterior margin 1–2 rows of simple setae. Anterior margin with 1 seta in addition to antero-distal group with 1 seta group of 2 facial setae proximal to palmar spiniform seta; several groups of short setae on the inner surface present. Palmar corner rounded and with strong palmar spiniform seta, single supporting spiniform seta on inner surface and 1 denticulated thick spiniform seta on outer side. Nail length 0.20–030 of total dactylus length; along anterior margin 1 seta; along inner margin a row of short setae.

Gnathopod II (Fig. 9B): Basis width: length equal to (0.30–0.35): 1. Ischium with 1–2 submarginal setae. Carpus length (0.55–0.60) of

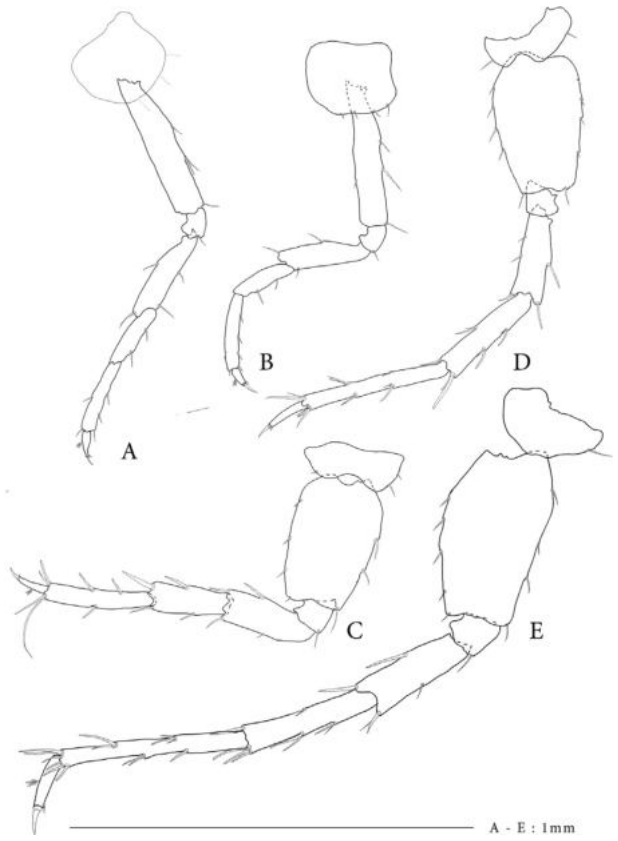

Fig. 10. *Niphargus karkabounasi* A. pereopod III; B. pereopod IV; C. pereopod V; D. pereopod VI; E. pereopod VII (drawings A–E: male holotype).

basis length and (0.75–0.80) of propodus length. Anterior margin of carpus with distal seta; carpus posteriorly with transverse rows of setae proximally and a row of lateral setae; postero-proximal bulge large (1/3 of carpus length), positioned proximally. Propodus small (circumference measures 0.15–0.20 of body length) and approximately equal in size to propodus of gnathopod I [length ratio 1: (0.95–1.05)]. Propodus distinctly triangular, palm convex and distinctly inclined.Posterior margin with 2 rows of simple setae. Anterior margin with 1 single seta in addition to 3 antero-distal setae. Group of 1–4 facial setae proximal to palmar spiniform seta; individual surface setae present. Palmar corner with strong palmar spiniform seta, single supporting spiniform seta on inner surface and 1 denticulated thick-spiniform setae on outer side. Nail length 020–0.30 of total dactylus length. Along anterior margin 1 seta; along inner margin 2–3 short setae.

Pereopods III and IV (Fig. 10; A,B): Lengths of pereopods III: IV equal to ratio 1: (0.95–0.97). Dactylus IV 0.30–0.40 of propodus IV; nail length 0.20–0.30 of total dactylus length. Dactyli III and IV with 1 dorsal plumose seta; at the base of nail with tiny seta and 1 spiniform setae.

Pereopods V–VII (Fig. 10; C–E): Lengths of pereopods V: VI: VII is 1: 1.00: (1.50–1.60). Pereopod VII length 0.40–0.50 of body length.

Bases V–VII length: width equal to 1: (0.6–0.68), (0.63–0.67) and (0.55–0.65), respectively; posterior margins straight to convex, with small distal lobes; posteriorly (4–6), (4–6) and (5–6) thin flexible setae respectively; anteriorly (3–5), (4–5) and (3–5) groups of slender spiniform setae respectively. Dactylus VII length 0.35–40 of propodus VII length; nail length 0.20 of total dactylus length. Dactyli V–VII: at the base of nail 1 spinform setae.

Pleopods: Pleopods I–III with 2 hooked retinacles. Pleopod II rami of 4–6 articles each.

Uropods (Fig. 7; D–G): Uropod I protopodite with 3–4 dorsolateral spiniform setae. Endopodite: Exopodite: 1.13–1.28; rami straight. Endopodite with 1 individual stout setae and 4 apical spiniform setae. Exopodite with 4 apical spiniform setae.

Uropod II exopodite: endopodite lengths equal to 1: 1.00–1.30.

Uropod III up to 0.30 of body length.Protopodite with 2–5 apical spiniform setae. Endopodite 0.55 of protopodite length, with 1 apical seta.Exopodite of uropod III rod-shaped, distal article to the proximal article length ranging from 0.36 to 0.50. Proximal article with 2–3 groups of short, spiniform setae along inner margin and 2–3 groups of short, spiniform setae along outer margin. Distal article with 1–3 setae groups along inner margin; apically with 2–4 setae.

3.1.4.1. Variability. Males differ from females in having elongated distal article of uropod III.

3.1.4.2. Remarks and affinities. Monophyly on 28S gene fragment supports species hypothesis of *Niphargus karkabounasi*. The species belongs to a clade that is broadly distributed and includes species from all Balkan Peninsula (Table 2). Most of the species from the clade are small, and may include species specialized for life in interstitial waters (inc. members of so called *jovanovici*, *kochianus* and *transitivus* groups; see e.g., Fišer et al., 2008 for discussion and brief morphological characterization of these polyphyletic species-groups). Sexually dimorphic uropod III, sexually nondimorphic uropod I, subrounded epimeral plates and small gnathopods make this species superficially similar to species broadly known under the name *N. aquilex* agg. Schioedte 1855; most stabile difference between these species and *N. karkabounasi* seems to be the number of hooks in retinacles, herein described species having only two in contrast to other species from this complex. In addition, the triangular shape and semi-equal size of propodi of gnathopods seems to be unique among *Niphargus* species. To our knowledge, equally sized propods of gnathopods have small slender species living in interstitial water. These species, however, have remarkably larger gnathopods with propodus of almost almond shape. Herein described species has roughly triangular propods of gnathopods, similarly to *N. lattingere* G. Karaman, 1983 and *N. minor* Sket, 1956. In the latter, however, margins of propodi of gnathopods are more convex. In addition, both species clearly differ by angular epimeral plates with straight margins (Karaman, 1983a,b).

3.1.5. Niphargus koukourasi sp.nov.

3.1.5.1. Material examined. Springs of Louros River, Vouliasta, Ioannina, Hellas; 25.4. 2004; leg.C. Fišer & R. Verovnik; type series, 4 individuals. In sampled excavations along the coast of the spring lake.

3.1.5.2. Type locality. Springs of Louros River, Vouliasta, Ioannina, Hellas.

3.1.5.3. Type series. Holotype is 14.5 mm long (no 1; voucher NC008). Paratypes include one male 14.5 mm (no 3; voucher NC009) and two females 14.3 (no 2; voucher NC010) and 13.6 mm (no 4; voucher NC011), respectively.

3.1.5.4. Etymology. The species is named in honour of the zoologist, retired Professor of Zoology Athanasios Koukouras (School of Biology, Faculty of Sciences, Aristotle University of Thessaloniki, Hellas).

3.1.5.5. Diagnosis. Niphargus species that in general appearance most closely resembles *N. sanctinaumi*, from which it differs by smaller body size, but higher number of articles in flagellum of antenna I, much fewer number of dorsal spines on telson and more

Table 2
Comparison of *N. karkabounasi* with morphologically similar species.

Species	Epimeral plates II-III	Telson-lateral spines	Gnathopods–propodi	Number of hooks in retinacles	Source
N. karkabounasi sp.n.	Subrounded	Absent	Triangular, straight margins; propodus I > propodus II	2	Present study
N. aquilex (GB) Schioedte 1855	Subrounded	Absent	More trapezioid; propodus I < propodus II	3–4	Karaman (1980)
N. aquilex (ITA) Schioedte 1855	Subrounded	Absent	More trapezioid; propodus I < propodus II	3–4	Karaman (1982)[a]
N. carniolicus Sket 1960	Subrounded	1–2	More trapezioid; propodus I < propodus II	2–3	Karaman (1989)
N. dobati Sket 1999	Subrounded	Absent	More trapezioid	3–4	Sket (1999)
N. fongi Fišer and Zagmajster 2009	Subrounded	1–2	More trapezioid; propodus I < propodus II	4–7	Fišer and Zagmajster (2009)
N. tauri Schellenberg 1933	Subrounded	2–3	More trapezioid; propodus I < propodus II	5–6	Karaman (1973)
N. minor Sket 1956	Angular	1–2	Triangular, strongly convex margins; propodus I < propodus II	3	Karaman (1983a)
N. lattingere Karaman G. 1983	Angular	Absent	Triangular, strongly convex margins; propodus I < propodus II	2	Karaman (1983b)[a]

[a] All material cited in the references above has been revised personally. Exceptions are *N. aquilex*[2], Karaman (1982) and *N. lattingere*, Karaman (1983).

densely setose posterior margins of pereopods V–VII. Uropods I sexually not dimorphic, exopodite of uropod III elongated only in males.

3.1.6. Description of type series

Head and trunk (Fig. 2C): Body length up to 14.54 mm. Head length up to 12% of body length; rostrum absent. Pereonites I–VII without setae.

Pleonites I-III with up to 5 setae along the entire dorso-posterior margin. Epimeral plate I (Fig. 11A) slightly inclined; ventro-postero-distal corner distinct but not produced; along posterior margin 6 spiniform setae. Epimeral plate II (Fig. 11B) slightly inclined, posterior and ventral margins concave and convex, respectively; ventro-postero-distal corner distinct but not produced; along ventral margin 3–4 spiniform setae; along posterior margin up to 5–8 thin setae. Epimeral plate III (Fig.11C) inclined, posterior and ventral margin straight and slightly convex, respectively; ventro-postero-distal corner distinct but not produced; along ventral margin with 4–5 spiniform setae; along posterior margin 6–7 thin setae.

Urosomite I postero-laterally with 1 strong seta; urosomite II postero-laterally with 1–2 simple setae; urosomite III without setae. At the base of uropod I single strong spinifrom seta.

Telson (Fig. 11H) length: width ratio equal to 1: (0.85–0.95); cleft is 0.70–0.75 of length; telson margins straight and narrow apically. Telson spiniform setae (per lobe): 4–5 apical spiniform setae of up to 0.50–0.58 telson length; mesial and lateral margins with 1–2 spiniform setae; dorsal surface with 2–3 spiniform setae in 1–2 groups. Pairs of simple setae inserted mid-laterally.

Antennae (Fig. 12A,B): Antenna I 0.55–0.60 of body length. Flagellum with 31–33 articles; up to 3 aesthetascs on flagellar articles. Peduncle articles in ratio 1: (0.85–0.90): (0.40–0.50). Proximal article of peduncle dorso-distally slightly produced. Accessory flagellum biarticulated; distal article shorter than one half of proximal article length.

Length ratio antenna I: antenna II equal to 1: (0.40–0.45). Flagellum of antenna II with 13–14 articles; each article with setae and elongate sensilla of unknown function. Lengths of peduncle articles 4: 5 ratio 1: (0.90–0.95); flagellum 0.70–0.85 of joint length of peduncle articles 4 + 5.

Mouthparts (Fig. 12 C–H): Labrum typical; inner lobes of labium longer than half of the outer lobes.

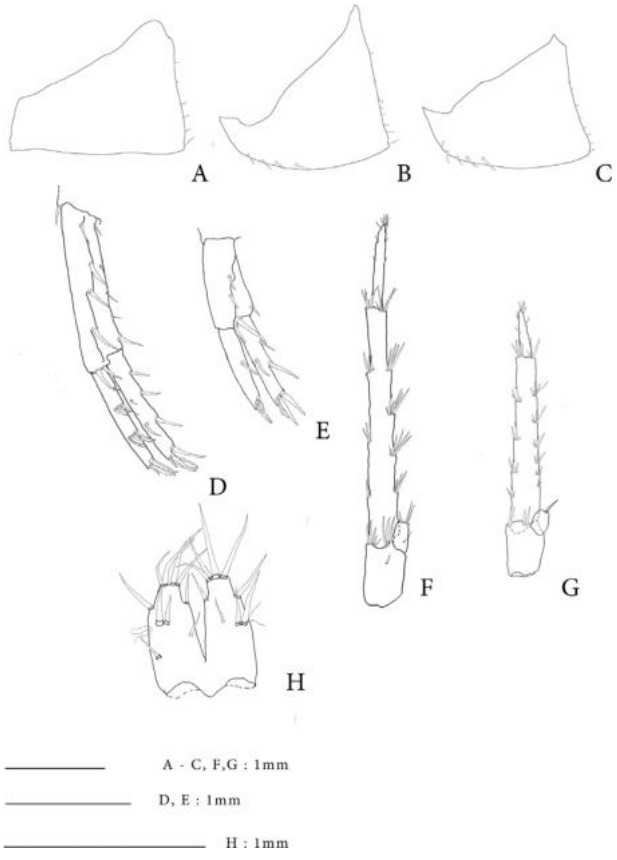

Fig. 11. *Niphargus koukourasi* A. epimeral plate I; B. epimeral plate II; C. epimeral plate III; D. uropod I; E. uropod II; F. uropod III male; G. uropod III female; H. telson (drawings A–D, F: male holotype; drawing E: male paratype no 3; drawing G: female paratype no 4).

Left mandible's incisor with 5 teeth, lacinia mobilis with 4 teeth; between lacinia and molar a row of thick serrated setae, long seta at the base of molar. Right mandible: incisor processus with 4 teeth, between lacinia and molar a row of thick serrated setae. Ratio of mandibular palp article 2: article 3 (distal) equal to 1: (1.05–1.25). Proximal palp article without setae; the second article with 15–18

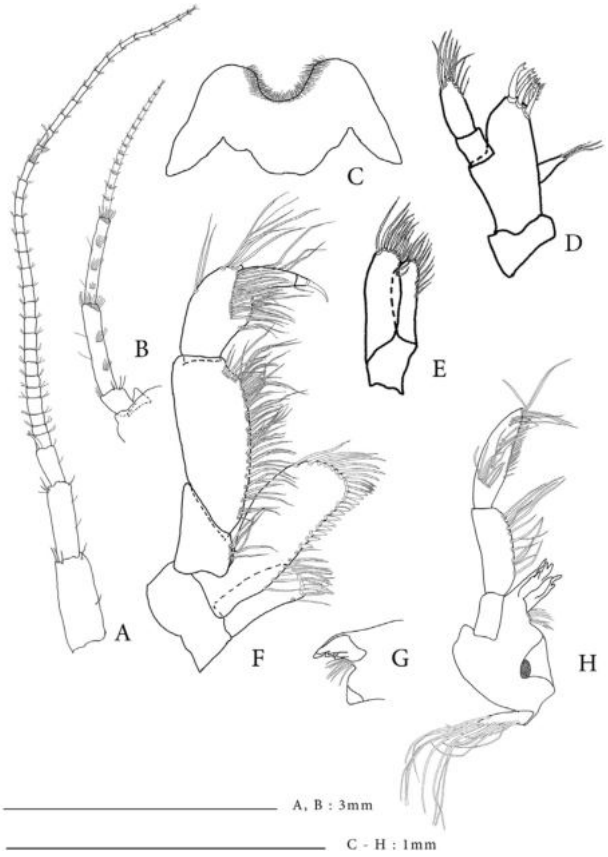

Fig. 12. *Niphargus koukourasi* A. antenna I; B. antenna II; C. labrum; D. maxilla I; E. maxilla II; F. maxilliped; G. left mandible; H. right mandible (drawings B, D–H: male holotype; drawing A: male paratype no 3; drawing C: female paratype no 4).

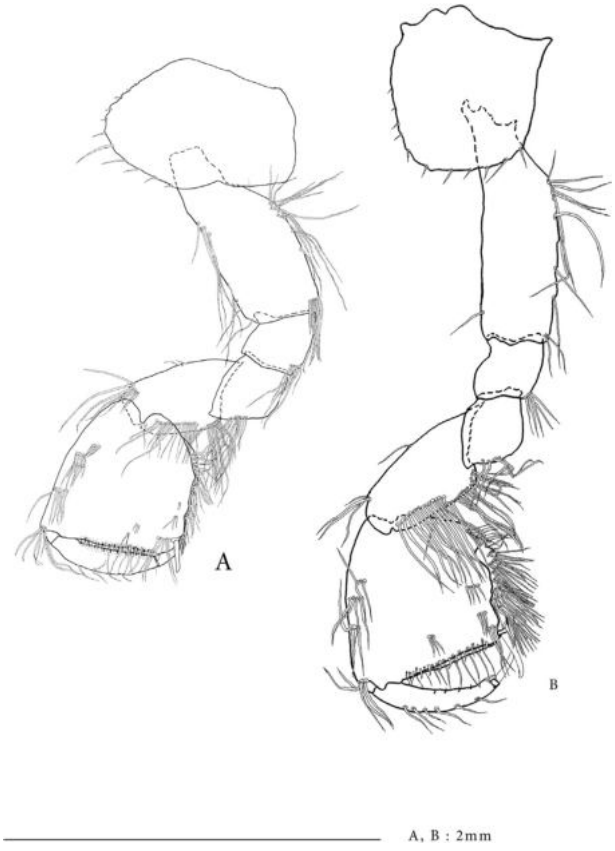

Fig. 13. *Niphargus koukourasi* A. gnathopod I; B. gnathopod II (drawing A: male holotype; drawing B: male paratype no 3).

setae; distal article with one group of 6–8 A setae; 4–5 groups of B setae; 25–31 D setae and 7–10 E setae.

Maxilla I distal palp article with 8–10 apical setae. Outer lobe of maxilla I with 1 pluri- and 6 uni-toothed stout setae apically; inner lobe with 3 apical and subapical setae.

Maxilla II inner lobe 0.75 of outer lobe's length; both of them setose apically and subapically.

Maxilliped palp article 2 with 5–6 rows of setae along inner margin; distal article with a dorsal seta and a group of small setae at the base of the nail. Maxilliped outer lobe with 12–13 flattened thick setae mesially and 7 serrated setae apically and subapically; inner lobe with 4–5 flattened thick setae apically and 9 serrated setae.

Coxal plates, gills and oostegites (Figs. 13 and 14): Coxal plate I deep and of parallelogram shape, antero-ventral corner subrounded; anterior and ventral margin of coxa I with 11 setae. Coxal plate II width: depth equal to 1: (1.00–1.20); anterior and ventral margin with 9–11 setae. Coxal plate III width: depth equal to 1: (1.00–1.20); anterior and ventral margin with 10–15 setae. Coxal plate IV width: depth ratio equal to 1: 1.00–1.15; posteriorly slightly concave (0.05–0.09 of coxa width); along antero-ventral margin 9–15 setae. Coxal plates V and VI: anteriorly developed lobe; posterior margin with 2–3 setae. Coxal plate VII half-egg shaped with 1 posterior seta. Gills II–VI ovoid and reach up to mid of basis, oostegites large ovoid, with long setae.

Gnathopod I (Fig. 13A): Ischium with 10–12 postero-distal setae. Carpus 0.55–0.70 of basis length and 0.80–0.90 of propodus length. Anterior margin of carpus with distal group of setae and additional group of setae; carpus posteriorly with transverse rows of setae proximally, a row of lateral setae; postero-proximal bulge large (1/3 of carpus length), positioned proximally. Propodus trapezoid, palm

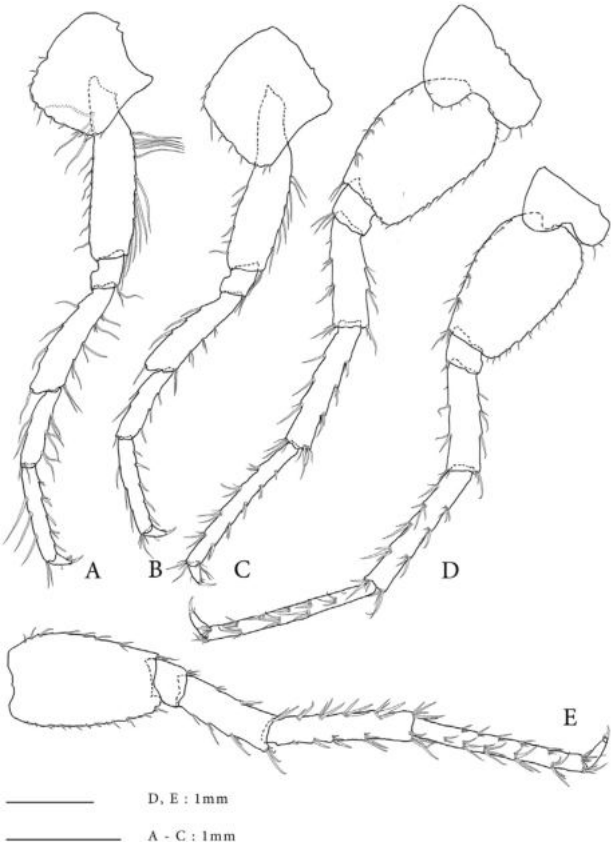

Fig. 14. *Niphargus koukourasi* A. pereopod III; B. pereopod IV; C. pereopod V; D. pereopod VI; E. pereopod VII (drawings A–E: male holotype).

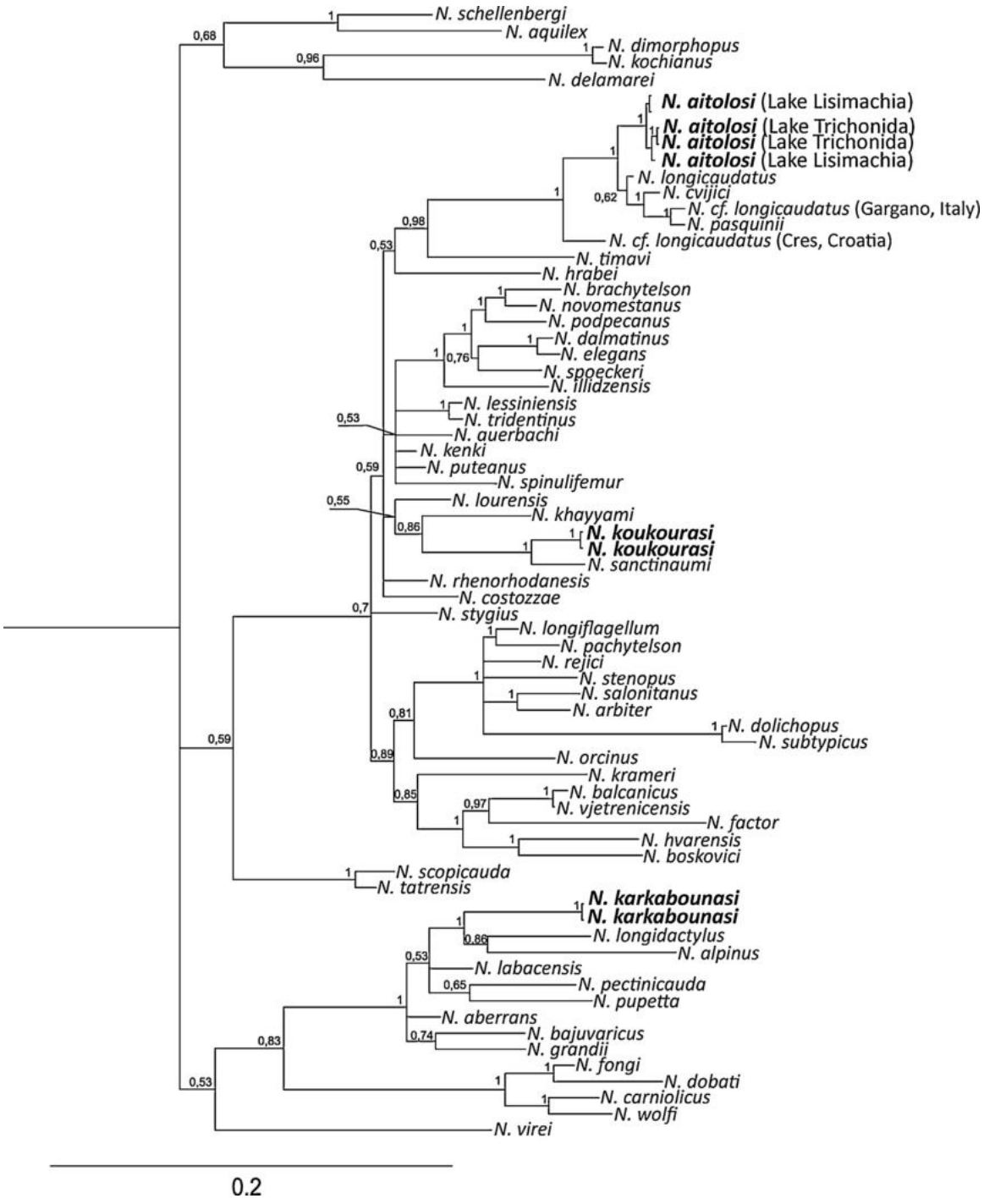

Fig. 15. Bayesian tree of 64 Niphargus species, based on two fragments of 28S gene and histone 3 gene. Numbers on branches indicate posterior probabilities. Outgroup (Synurella ambulans) is not presented.

convex and slightly inclined. Along posterior margin 7–9 rows of denticulated setae. Anterior margin with 18–24 setae in 3–4 groups in addition to antero-distal group with 13–14 setae. Group of 4–6 facial setae proximally to palmar spiniform seta; several groups of short setae present, on the inner surface. Palmar corner slightly produced and with strong palmar spiniform seta, 3 single supporting spiniform setae on inner surface and 3 denticulated thick spiniform setae on outer side. Nail length 0.22–0.30 of total dactylus length; along anterior margin 5–7 setae; seta presented as single or in pairs; along inner margin a row of short setae.

Gnathopod II (Fig. 13B): Basis width: length equal to 1: (0.30–0.35). Ischium with 6–8 postero-distal setae in one row. Carpus 0.55–0.65 of basis length and 0.90–0.95 of propodus length. Anterior margin of carpus with distal row of setae and one additional group of setae; carpus posteriorly with transverse rows of setae proximally and a row of lateral setae; postero-proximal bulge large (1/3 of carpus length), positioned proximally. Propodus small-sized (circumference measures 0.15–0.17 of body length) and larger than propodus of gnathopod I (1: (0.85–0.88)). Propodus rectangular, palm convex and slightly inclined. Posterior margin with 10–12 rows of denticulated setae. Anterior margin with 11–13 setae in 3

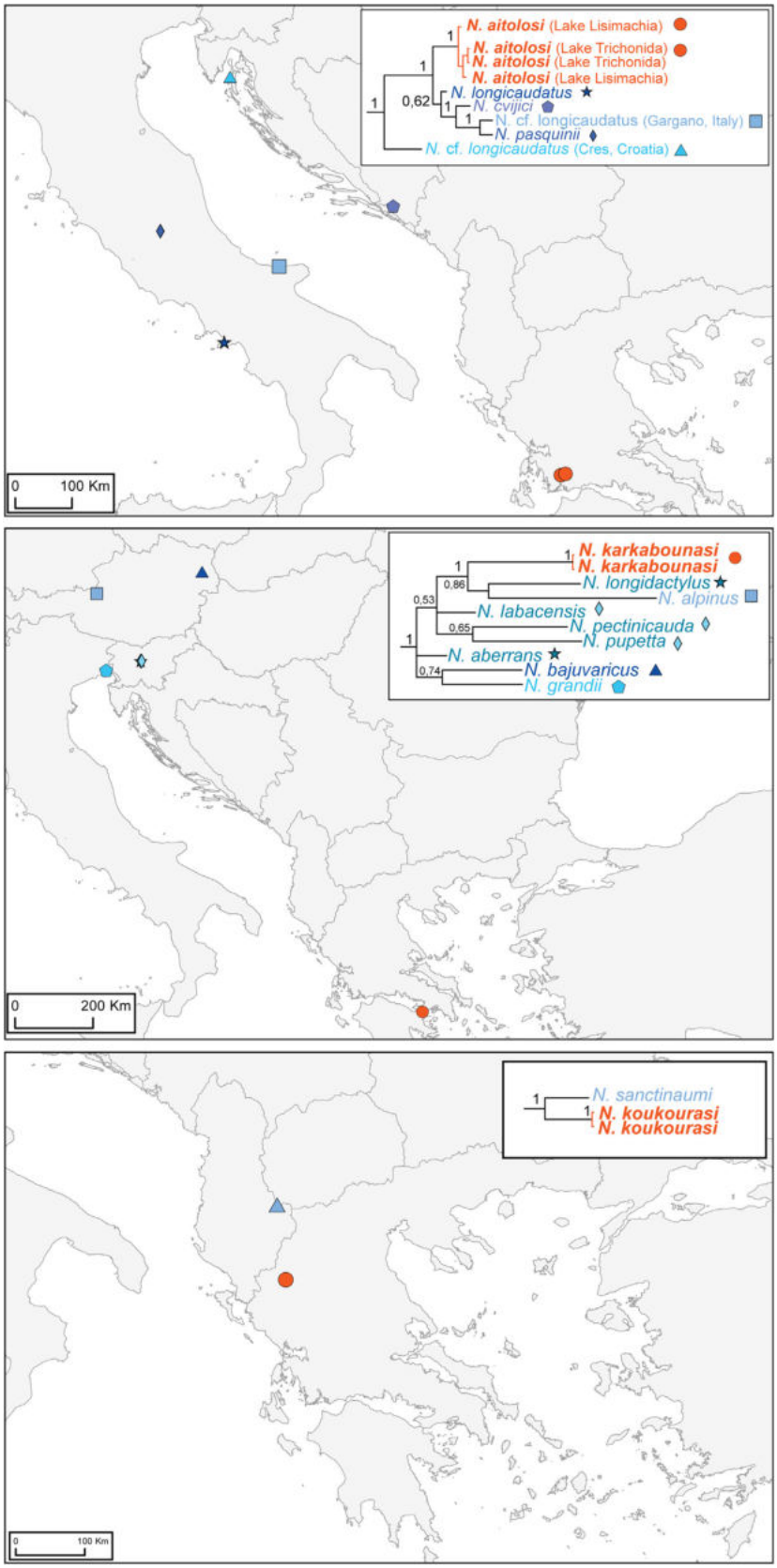

Fig. 16. Biogeographical map- tree distribution among the three new species and their relatives.

Table 3
Comparison of *N. koukourasi* with morphologically similar species.

Species	Telson, number of dorsal spines per lobe	Antenna II–number of flagellar articles	Pereopod VII basis–number of posterior setae	Uropod I (males only)	Source
N. koukourasi sp.n.	<4	13–14	15–22 (13–14 mm)[a]	Both rami subequal	Present study
N. maximus Karaman S. 1929	4–5	12	15 (Body length 19 mm)[a]	Inner ramus as twice as long as outer	Karaman 1943
N. sanctinaumi Karaman S. 1943	10–14	10–11	13–15 (Body length 15–22 mm)[a]	Both rami subequal	Personal observation[b]

[b] Spring, Sv. Naum, Ohrid, MAK; 30. 4. 2004; leg. C. Fišer &R. Verovnik; 3 individuals.

groups in addition to 12 antero-distal setae. Group of 4–6 facial setae proximally of palmar spiniform seta; individual surface setae present. Palmar corner with strong palmar spiniform seta, 1 single supporting spiniform seta on inner surface and 2–3 denticulated thick-spiniform setae on outer side. Nail length 0.20–0.25 of total dactylus length. Along anterior margin 5–7 setae, presented as single or in pairs; along inner margin few short setae.

Pereopods III-IV (Fig. 14 A, B): Lengths of pereopods III: IV equal to ratio 1: (0.94–0.97). Dactylus IV 0.3 of propodus IV; nail length 0.46 of total dactylus length. Dactyli III-IV with no dorsal plumose seta; at the base of nail 1 tiny seta and 1 spiniform seta.

Pereopods V-VII (Fig. 14 C–E): Lengths of pereopods V: VI: VII equal to 1: 1.34: 1.34. Pereopod VII length 0.45–0.50 of body length.

Bases V-VII length: width equal to 1: (0.70–77), (0.64–0.67) and (0.63–0.68), respectively; posterior margins straight to convex, with small distal lobes; posteriorly 16–19, 15–22 and 10–17 thin flexible setae respectively; anteriorly 6–8, 11–12 and 7–9 groups of slender spiniform setae respectively. Dactylus VII length 0.20–0.27 of propodus VII length; nail length 0.25–0.33 of total dactylus length. Dactyli V-VI dorsal plumose seta; at the base of nail 1 tiny and 1 spinform setae; dactylus VII with dorsal plumose seta; at the base of nail 1 spinform seta.

Pleopods: Pleopods I–III with 2 hooked retinacles. Bases of pleopods I and III without setae, pleopod II has seta near retincles. Pleopod II rami of 11–15 articles each.

Uropods (Fig. 11 D–G): Uropod I protopodite with 4–6 dorso-lateral spiniform setae and 3–4 dorso-medial spiniform setae. Exopodite: endopodite lengths equal to 1: (1.05–1.10); rami straight, no differences between the sexes. Endopodite with 4–5 individual spiniform setae and 5 apical spiniform setae. Exopodite with 5–6 spiniform setae in 2–4 groups; apically with 5–6 spiniform setae.

Uropod II exopodite: endopodite lengths equal to 1: 1.05–1.15.

Uropod III 0.20–0.27 of body length. Protopodite with 1–2 lateral seta and 6–8 apical spiniform setae. Endopodite 0.45–0.55 of protopodite length, apically with 3 spiniform setae; laterally with 1 setae. Exopodite of uropod III rod-shaped, distal article 0.35–0.47 (males) or 0.25–0.30 (females) of the proximal article length. Proximal article with 4–6 groups of spiniform setae along inner margin and 3–4 groups of spiniform setae along outer margin. Distal article with 5–6 setae groups along inner margin; apically 3 setae.

3.1.6.1. Variability. Males have slightly more elongated distal article of exopodite of uropod III than females. Other substantional variation was not observed.

3.1.6.2. Remaks and affinities. As in previous species, monophyly on 28 S gene marker supports long-term independent evolution of *Niphargus koukourasi* and additionally justifies its species status. This species is phylogenetically and morphologically related to *N. sanctinaumi* S. Karaman, 1943; a species living in the vicinity of Lake Ohrid in the Former Yugoslav Republic of Macedonia (Table 3

[a] Body size is for an orientation; in general the number of spines increases in larger specimens; herein described species seems to be the most setose one.

). Three *N. sanctinaumi* of lengths 14.7, 16.7 and 22.3 mm has been reviewed. A comparison of samples indicates that both species differ in the number of flagellar articles of antenna II (10–11, *N. s* and 13–14 in *N. k.*), number of dorsal spines on telson (10–14 *N. s.*, <3 *N. k.*) and density of setae on pereopods bases (e.g,. pereopod VI basis has 13–15 and 15–22 posterior setae in *N. s.* and *N. k.* respectively). These traits positively correlate to the body size, however, the species from Lake Ohrid shows less articles and setae although it is larger than species from Greece. In the vicinity of Lake Ohrid lives also the complex *N. maximus* S. Karaman, 1929, which is easily distinguishable from *N. koukourasi* by sexually dimorphic uropod I; in *N. maximus* inner ramus may be as twice as long as outer ramus (Karaman, 1943).

3.1.7. Phylogenetic analyses

Our analyses suggests that Greek *Niphargus* fauna is phylogenetically highly diverse, comprising two broadly distributed clades and one endemic clade with members distributed in southern parts of Balkan Peninsula. *Niphargus* species from Greece are not phylogenetically closely related (Fig. 15). The first species, *N. aitolosi* (see below), belongs to *N. longicaudatus* complex (Fig. 16). This complex is distributed across the Apennine Peninsula and along the Eastern coasts of the Adriatic Sea including islands and mainland. Species within the complex are morphologically similar (see below). It is likely, that the species complex spread across the Balkan and Apennine Peninsulas during the Messinian Salinity crisis (6–5.5 Mio), when the Adriatic Sea and large parts of the Mediterranean Sea dried up (Krijgsman et al., 1999). The second species, *N. karkabounasi*, belongs to a clade the members of which are distributed between Western Europe and Eastern Europe (Fišer et al., 2008; Meleg et al., 2013) as well as in the Crimean Peninsula and in Northern Iran (Fig. 16). Many species from this clade are small and many of them seem to have adapted to live in tiny voids, either in interstitial waters or cracked rocks (Meleg et al., 2013).

The third species, *N. koukourasi*, is closely related to the species found at the shores of Ohrid Lake on the border between Albania and Former Yugoslav Republic of Macedonia (Fig. 16). The phylogenetic position of this clade to the rest of *Niphargus* species is unclear; a weak support (Fig. 15) implies affiliation to *N. khayyami* described from Iran and to the fourth Greek species *N. lourensis* collected in the same area. This relationship is poorly supported by our phylogenetic analysis (posterior probabilities lower than 0.9). Nevertheless, both species are much stouter in general appearance (Fišer et al., 2006; Fišer et al., 2006).

4. Conclusions and perspectives

The present study increased the total number of *Niphargus* species in Greece to ten. Nevertheless, this study should be considered as the first step towards a systematic research of *Niphargus* fauna in Greece. While preparing the manuscript, we were informed of a few new records of *Niphargus* in Greece (F. Stoch, Ş. Sarbu and J.-F. Flot, pers. comm. 2014) indicating that in the near future new species descriptions from that region can be expected, but also that systematic fieldwork is still needed.

Preliminary data suggest high phylogenetic diversity, as all newly discovered species belong to different clades. Evidently,

Greek fauna is related to *Niphargus* taxa in other parts of Europe not only, in Italy. From the biogeographic point of view, Greece in Miocene had been a natural connection between the Middle East (Turkey, Syria, Lebanon, and Iraq) and Europe. Species collected at the eastern most border of the genus range (Iran) form a clade that is unclearly related to European species and sampling in the Eastern Mediterranean may be critically important for recovery of the entire *Niphargus* phylogeny.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jcz.2015.02.002.

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