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An Integrated Morphological and Molecular Approach to A New Species Description of Amphipods in the Niphargidae from Two Caves in West of Iran

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Mahmood Mamaghani-Shishvan, Somayeh Esmaeili-Rineh, and Cene Fišer (2017) The cave-dwelling fauna of Zagros region (Northern Iran) has been insufficiently studied. Most of the known cave-specialized species (15 out of 21) belong to the genus *Niphargus* Schiödte, 1849, yet species composition of this genus is not resolved. In this research, we studied *Niphargus* specimens from two recently sampled caves, Shoei and Darvish-Olya Caves, from Kurdistan Province in Iran. The specimens belong to a single, yet undescribed species, which can be diagnosed using mitochondrial (COI) and nuclear sequences (28S rDNA gene sequences) as well as several morphological traits. Based on combined evidence from morphology and molecular characters, we described and named new species, *N. kurdistanensis* sp. nov.. The newly described species is phylogenetically nested within the clade comprising Iranian species. We provide a new and revised identification key for *Niphargus* from the Middle East region.

Key words: 28S rDNA, COI, *Niphargus*, Amphipoda, Morphological characters, Kurdistan, Iran.

BACKGROUND

Caves and other subterranean habitats harbor numerous animals specialized for life in the darkness, so called troglobionts (Culver and Pipan 2009). The species richness of these animals presumably peaks in extensively karstified regions at mid-latitudes, particularly where is high productivity on the surface (Culver et al. 2006; Eme et al. 2015, 2017). This hypothesis, however, was largely shaped using the data from Europe and North America, and its applicability to other continents remains to be evaluated. From geographic point of view northern part of Iran falls into the species-rich mid-latitude belt. This karstic region spreads over 2000 square kilometers, but harbors only few troglobiotic species (Karaman 1998; Hekmatara et al. 2013; Esmaeili-Rineh and Sari 2013). This number might classify as species-rich a single cave but not the entire region (Culver and Sket 2000). The low number of troglobionts most likely suggests that subterranean fauna of Iran received only little attention and is heavily underexplored.

Among aquatic species dominates the amphipod genus *Niphargus*, the largest genus of subterranean amphipods in the world, distributed in Western Palearctic (Väinölä et al. 2008). Until now, there have been 15 species recorded from Iran; all but except one are endemic in the country (Karaman 1998; Hekmatara et al. 2013; Esmaeili-Rineh and Sari 2013; Esmaeili-Rineh et al. 2015a, 2016, 2017a b). The genus classifies among key faunistic elements of European groundwater

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(Zagmajster et al. 2014), and may be a good pointer of regional groundwater diversity in Europe, but possibly also in Iran. However, species inventory of this genus is far from complete: new species are regularly discovered from all parts of the genus range.

Recent cave explorations in northern Iran have revealed new records of this genus; many of these appear to be new species. In this study, we took further steps towards a phylogeny-grounded revision of the genus and towards the global understanding of the cave fauna of Iran. We phylogenetically identified a new species and morphologically described samples from two localities in Iranian Kurdistan, namely Shoei and Darvish-Olya Caves.

MATERIALS AND METHODS

The specimens were collected using a small hand net in stream water from Shoei and Darvish-Olya caves in Kurdistan Province, Iran (Fig. 1). The distance between the caves is about 82 km in straight line. The samples were analyzed molecularly and morphologically as follows.

Morphological details were scrutinized according to the long list of potentially variable morphological traits (Fišer et al. 2009a). Specimens were partly dissected and mounted on slides in a Euparal® medium. Digital photos were taken with an Olympus LABOMED iVu 7000 camera fitted on an LABOMED Lx500 stereomicroscope. Measurements and counts were made using the computer program ProgRes CapturePro 2.7. The specimens used for the present study are deposited at the Zoological Collection, Razi University (ZCRU).

The morphology of herein described species was compared to previously described species. In order to ease the identification of Middle East species, we also updated and revisited the identification key (Esmaeili-Rineh et al. 2015a). The identification key was constructed using DEscription Language for TAxonomy (DELTA; Dalwitz et al. 2003). We incorporated new data into pre-existing database (Esmaeili-Rineh et al. 2015a, 2017b) and automatically generated dichotomous identification key. Whenever possible, we used sexually non-dimorphic characters.

For the molecular analyses, we extracted the total genomic DNA from a part of an animal using Tissue Kits (GenNetBio™) following the manufacturer's instructions (Seoul, South Korea). Mitochondrial COI was amplified using the modified primer pair LCO1490-JJ and HCO2198-JJ (Astrin and Stüben 2008). Amplification and sequencing of the first fragment of 28S ribosomal DNA (rDNA) were performed using the forward primer from Verovnik et al. (2005) and the reverse primer from Zakšek et al. (2007). Polymerase chain reactions (PCRs) in a final volume of 25 μl contained optimized amounts of PCR water, 12.5 μl of Master Mix kit (Sinaclon, Iran), 0.2 μl of each primer (10 μM), and 50-100 ng of genomic DNA template. For COI gene amplification, an initial denaturation step at 94°C for 3 minutes was followed by 36 cycles of 40 seconds at 94°C, 40 seconds at 52.5°C and 2 min at 65°C with a final extension step for 8 minutes at 65°C. Cycling parameters for the 28S rDNA gene were as follows: initial denaturation of 94°C for 7 minutes, 35 subsequent cycles of 94°C for 45 seconds, 55°C for 30 seconds and 72°C for 1 minutes, and a final extension of 72°C for 7 minutes. Purification of PCR products and sequencing were commercially performed by Macrogen Inc. (Korea). Sequencing was performed with both primers mentioned in above.

In order to identify the phylogenetic position of the newly discovered materials, the acquired sequences (GenBank accession numbers are MG008301-MG008303 for 28S gene and MG008304-MG008306 for COI gene) were analyzed within the data set of Esmaeili-Rineh et al. (2015b, 2017a) (see Table S1, all samples included in analyses). All sequences were edited and aligned using ClustalW (Thompson et al. 1994), as implemented in the Bioedit program sequence alignment editor (Hall 1999) using the default settings.

Phylogenetic reconstruction was performed using the Bayesian inferences in Mr Bayes, version 3.1.2 (Ronquist and Huelsenbeck 2003). Bayesian analyses were run for five million generations, with four chains, and trees sampled every 1000 generations, under GTR + G and TrN + I + G models (jModelTest, version 0.1.1, Posada 2008) for 28S and COI genes, respectively. The first 1250 sampled trees were discarded as burn-in, and the subsequent tree likelihoods were checked for convergence in Tracer 1.5.0 (Rambaut and Drummond 2009). A fifty percent majority rule consensus tree was computed using the remaining trees and visualized by FigTree v1.4.0 software. Data on analyzed species are available in the Electronic Supplement of Esmaeili-Rineh et al. (2015b, 2017a b).

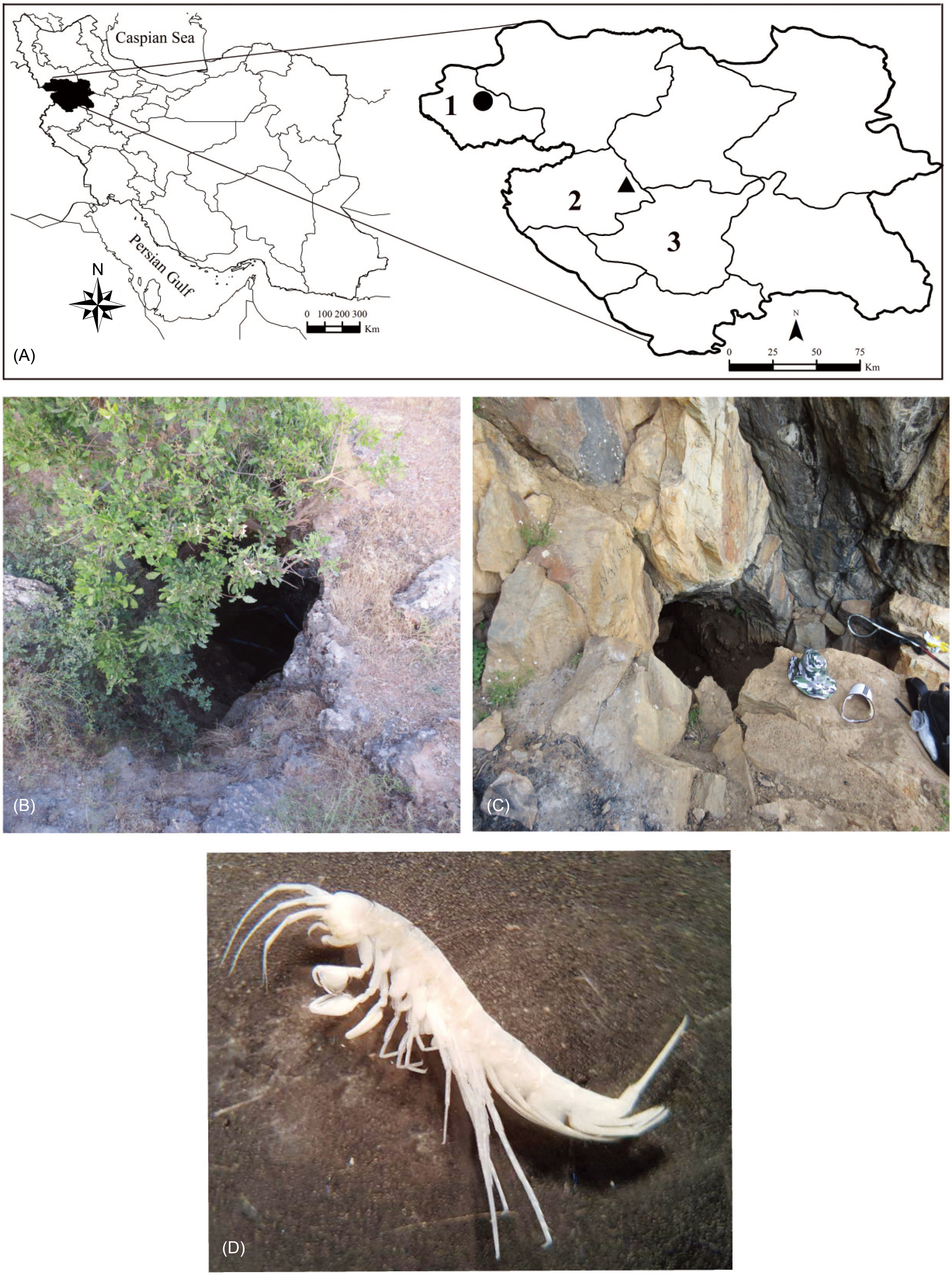

Fig. 1. (A) The sampling localities in Kurdistan Province, Iran (study area). (●), Shoei Cave; (▲), Darvish-Olya Cave; the numbers demarcate the main cities namely: 1, Baneh; 2, Marivan; 3, Sanandaj. (B) Entrance of Shoei cave. (C) Entrance of Darvish-Olya cave. (D) *Niphargus kurdistanensis* sp. nov., holotype, lateral view. Specimen collected from Shoei cave.

To assess divergence from other, already described Iranian species of *Niphargus*, we calculated corrected genetic distances using Kimura two-parameter (K2P) model (Kimura 1980) as implemented in MEGA ver. 5 (Tamura et al. 2011).

Abbreviations used in the figures are as follows: AI - antennae I (antennulae); AII - antenna II (antenna); EP I-III - epimeral plates I to III; GN I-II - gnathopods I-II; H - head; LMND - left mandible; LB- labium (paragnaths); MX I - maxilla I (maxillula); MX II - maxilla II (maxilla); MXP - maxilliped; MNDP -mandibular palp; P III-VII - pereopods III-VII; PGN I-II - propodus of gnathopods I-II; PL I-III - pleopods I-III; RMND - right mandible; T - telson; U I-III -uropods I-III.

RESULTS

SYSTEMATICS

Order Amphipoda Latreille 1816
Suborder Senticaudata Lowry and Myers, 2013
Family Niphargidae Bousfield, 1977
Genus *Niphargus* Schiödte, 1849

***Niphargus kurdistanensis* sp. nov.**
urn:lsid:zoobank.org:pub:C6AAC178-B80A-4DF5-8C86-011BCEB80B5A

Material examined and type locality

Holotype: male specimen (12 mm) from Shoei Cave, Kurdistan Province, Iran. Holotype and three paratypes from the same locality are stored under catalogue number ZCRU Amph.1076 in the Zoological Collection, Razi University, Iran (ZCRU).

Type locality: Shoei Cave, 12 km to Baneh City (near to Iraq border), Kurdistan Province, Iran; (36°0'18"N, 45°53'45"E).

Material examined: One male specimen (holotype) from Shoei Cave, 12 km to Baneh City (near to Iraq border), Kurdistan Province, Iran; (36°0'18"N, 45°53'45"E); collected by V. Akmali in June 2015; catalogue number ZCRU Amph. 1076. Three male specimens were collected from Darvish-Olya Cave, 75 km to Marivan City, Kurdistan Province, Iran; (35°37'14"N, 46°37'59"E) by M. Mamaghani in November 2016; male specimen (7 mm) record with catalogue number ZCRU Amph.1077 in the Zoological Collection, Razi University, Iran.

Diagnosis: Telson is deeply cleft; each lobe bears four apical robust setae. The palpus of maxilla I is short and not reaching the tip of the outer lobe. The outer plate of maxilla I bears seven robust setae with none or 1 lateral projection. Posterior margins of epimeral plates II-III armed with robust spiniform setae. Laterally, the urosomites I to III bear two, two and one robust setae, respectively. The propodus of gnathopods I to II have one and two robust setae with lateral projections on outer surface in palmar corner, respectively.

Description of holotype: Total length of specimen 12 mm. Body strong and stout. Head length 8% of body length (Fig. 2). Antennae I (Fig. 2A) 0.5 of body length. Peduncular articles 1-3 progressively shorter; peduncular articles 2:3 in ratio 1.38:1; main flagellum with more than 12 articles; accessory flagellum biarticulated, reaching 1/3 of article 4 of main flagellum, with three and two simple setae, respectively (Fig. 2A). Peduncular article 4 of antennae II slightly longer than article 5, with eight and four groups of simple setae, respectively; flagellum with nine articles. Length of flagellum : length of peduncle articles 4 + 5 as 0.5:1 (Fig. 2B).

Labium (Fig. 3D) with inner lobes and setae on the tip of the lobes. Inner plate of maxilla I with two long simple setae; outer plate with seven robust setae with 0-0-1-1-0-1-0 lateral projection; palp biarticulated, shorter than outer lobe, with three long distal simple setae (Figs. 2D-E). Both plates of maxilla II with numerous distal simple setae and two lateral simple setae (Fig. 3E).

Left mandible having pars incisiva with five teeth, lacinia mobilis with four teeth and six setae with lateral projections between lacinia and triturative molar (Fig. 2F). Right mandible with pars incisiva having four teeth, lacinia mobilis pluritooth and six setae with lateral projections between lacinia and triturative molar (Fig. 2G). Mandibular palp articles 1:2:3 ratios as 1:1.8:1.8. The proximal article with no setae, the second article with 13 setae along inner margin and the third article with one group of three A-setae, four groups of B-setae, no C-setae, 25 D-setae and five E-setae (Fig. 2H).

Maxilliped with short inner plate bearing four distal robust setae intermixed with three distal simple setae; outer plate exceeding half of the palp article 2, with 12 robust setae along inner margin and eight simple setae distally. Maxilliped palp article 3 with one proximal, inner and outer group of long simple setae at outer margin; palp terminal article with one simple seta at outer margin and two setae at the base of nail, nail shorter than

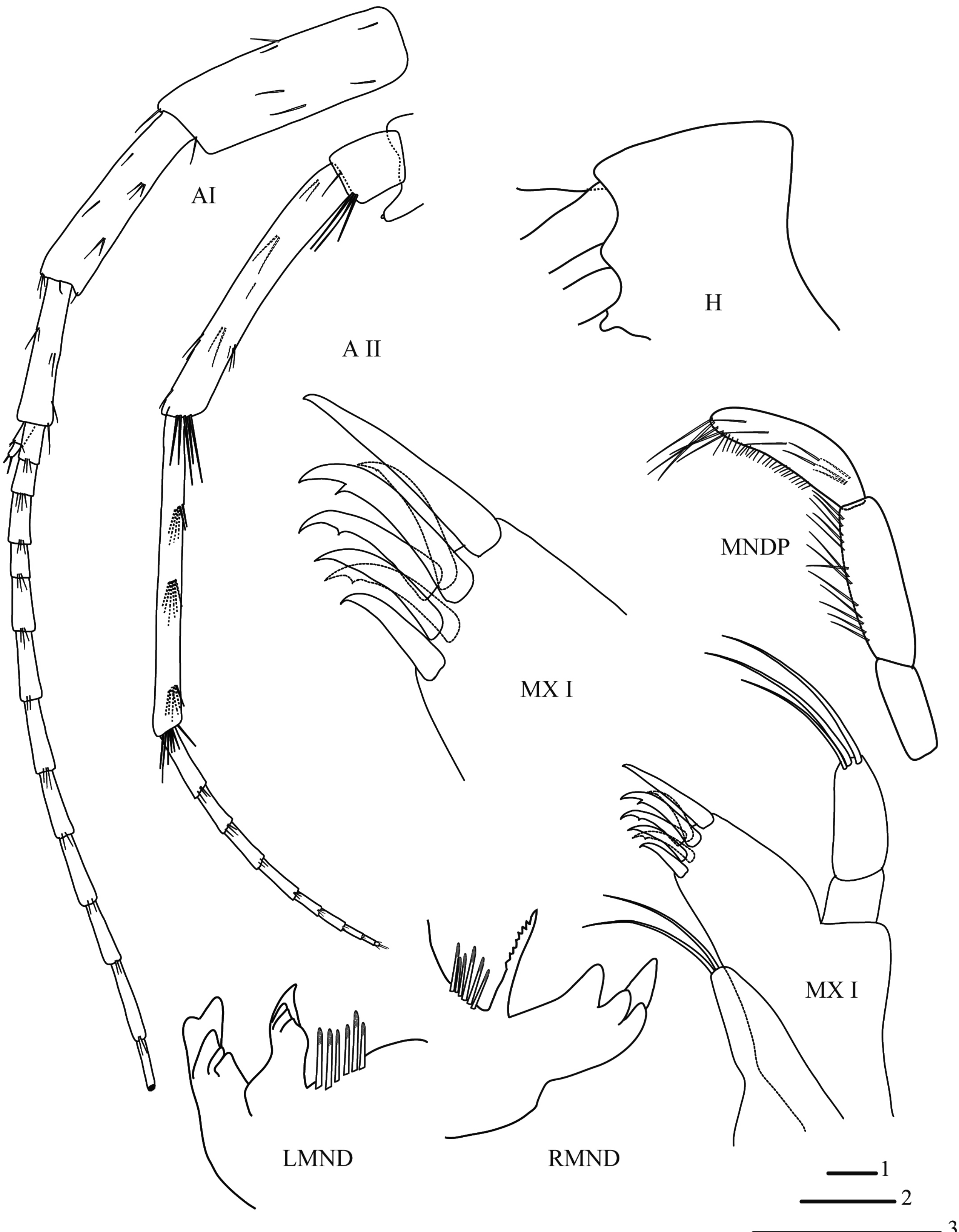

Fig. 2. *N. kurdistanensis* sp. nov., male 12 mm (holotype, ZCRU Amph.1076). (A) AI; (B) AII; (C) H; (D-E) MX I; (F) LMND; (G) RMND; (H) MNDP. Scale bars: 1 = 0.25 mm (LMND, RMND); 2 = 0.5 mm (H, MX I, MNDP); 3 = 1 mm (AI-AII).

pedestal (Fig. 3C).

Coxa of gnathopod I almost quadratic slightly rounded, with eight setae along antero-ventral margins. Basis with setae in groups and single setae along anterior and posterior margins; posterior margins of ischium and merus with one posterior group of setae each. Carpus 0.5 of basis length and 0.63 propodus length. Carpus with one group of seven setae antero-distally, and rows of setae on the posterior bulk. Propodus of gnathopod I, trapezoid shape and broader than long; anterior margin with 18 setae in four groups in addition to antero-distal group of five simple setae. Palm convex, with one strong palmar robust seta, one supporting robust seta without lateral projections on inner surface, and two robust setae with lateral projections on outer surface; two setae under supporting robust setae in palmar corner. Dactylus reaching posterior margin of propodus, outer and inner margins of dactylus with one and three simple setae, respectively. Nail length 0.27 of total dactylus length (Fig. 3A).

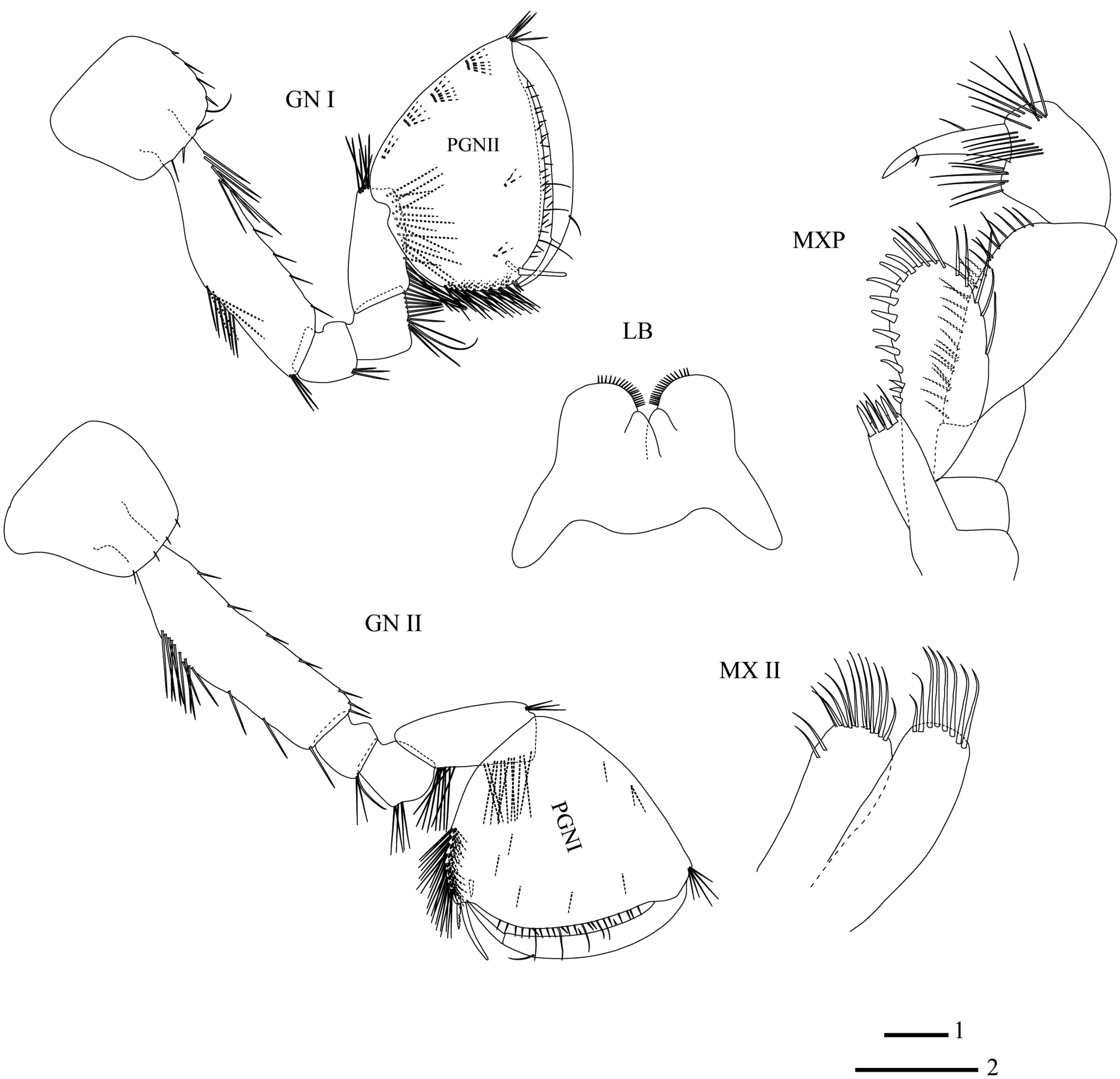

Fig. 3. *N. kurdistanensis* sp. nov., male 12 mm (holotype, ZCRU Amph. 1076). (A) GN I; (B) GN II; (C) MXP; (D) LB; (E) MX II. Scale bars: 1 = 0.5 mm (LB, MXP, MX II); 2 = 1 mm (GN I-II).

Coxa of gnathopod II trapezoid, broader than long, ventral margin with four simple setae. Basis with setae on anterior and posterior margins; ischium and merus with posterior group of setae. Carpus with one group of three setae antero-distally, a bulge with long simple setae; carpus 0.62 of basis length and 0.72 of propodus length. Propodus broader than long; anterior margin with three setae in two groups in addition to antero-distal group of five simple setae. Palm slightly convex, with one strong long palmar robust seta, one short supporting robust seta on inner surface and one robust seta with lateral projections on outer surface. Dactylus not reaching posterior margin of propodus, outer and inner dactylar margins with one and five simple setae, respectively; nail short, 0.25 of total dactylus length (Fig. 3B).

Coxa III rectangular, length to width ratio as 1.16:1; antero-ventral margin with eight simple setae. Coxa IV quadrate, antero-ventral margin with seven simple setae, posterior concavity shallow and approximately 0.1 of coxa width (Figs. 4A-B). Coxa V with anterior lobe, with four and one simple setae on anterior and posterior lobes, respectively. Coxa VI with anterior lobe, with one simple seta on posterior lobe. Coxa VII half-ovoid, with one simple posterior seta (Figs. 4C-E).

Pereopod III:IV lengths in ratio as 1.18:1 (Figs. 4A-B). Dactylus IV short, length of dactylus 0.33 of propodus, nail shorter than pedestal (Fig. 4B). Pereopods V:VI:VII length ratios as 1:1.08:1.16. Pereopod VII 0.5 of body length. Pereopod bases V-VII each with eight groups of robust setae along anterior margins and 10-10-9 simple setae along posterior margins, respectively (Figs. 4C-E). Postero-ventral lobe of ischium in pereopods V-VII weakly developed. Ischium, merus and carpus in pereopods V-VII with several groups of robust and simple setae along anterior and posterior margins; propodus of pereopod VII longer than these in V-VI, dactyli of pereopods V-VII with one robust and one short simple seta at the base of nail on inner margin, nail length of pereopod VII 0.29 of total dactylus length (Figs. 4C-E).

Pereonites I-VII without setae. Pleonites I-III each with numerus simple setae along dorsal surface. Epimeral plates I-III (Fig. 5G) with angular postero-ventral corners, anterior and ventral margins convex; postero-ventral margins of plates I-III with three, five and six robust setae posteriorly, respectively. Epimeral plates II-III with two and three robust setae along of ventral margins, respectively. Peduncle of pleopods I-III with two-hooked retinacles. Peduncle of pleopod III with two simple setae along of inner margin (Figs. 5A-C); rami of pleopods I-III each with 10 to 16 articles (Figs. 5A-C).

Urosomites I-III with two, two and one robust setae dorso-laterally, respectively. Urosomite I with one robust seta at the base of uropod I. Peduncle of uropod I with seven and four large robust setae along dorsolateral and dorsomedial margins, respectively. Inner ramus of uropod I slightly longer than outer ramus (ratio 1.07:1); inner ramus with five groups of robust setae laterally and five robust setae distally; outer ramus with six groups of six robust and simple setae laterally and five robust setae distally (Fig. 5D). Inner ramus in uropod II longer than outer, both rami with lateral and distal long robust setae (Fig. 5E). Uropod III long, almost 0.45 of body length. Peduncle of uropod III with two robust setae. Outer ramus biarticulated, distal article measures 0.15 of the proximal article. The proximal article of outer ramus bearing seven and six groups of robust setae along inner and outer margins, respectively (Fig. 5F); distal article with simple setae laterally and four simple setae distally. Inner ramus short, with three robust distal setae and one simple lateral seta. Telson two times longer than broad, lobes slightly narrowing; each lobe with four robust setae distally, with one long robust and two plumose setae laterally, with one robust seta mesially (Fig. 5H).

Description of specimen from Darvish-Olya cave: Total length of specimen 7 mm. Body strong and stout. Head length 14% of body length (Fig. 6). Antennae I (Fig. 6A) 0.6 of body length. Peduncular articles 1-3 progressively shorter; peduncular articles 2:3 in ratio 1.47:1; main flagellum with 15 articles; accessory flagellum biarticulated and reaching 2/3 of article 4 of main flagellum, with one and two simple setae, respectively (Fig. 6A). Length ratio antenna I:II as 1:0.68. Peduncular article 4 slightly longer than article 5, with three and five groups of simple setae, respectively; flagellum with six articles. Length of flagellum : length of peduncle articles 4 + 5 as 0.61:1 (Fig. 6B).

Labium (Fig. 7D) with inner lobes and setae on the tip of the lobes. Inner plate of maxilla I with two long simple setae; outer plate with seven robust setae with 0-0-1-1-1-0-1 lateral projection; palp biarticulated, shorter than outer lobe, with two long distal simple setae (Figs. 6D-E). Both plates of maxilla II with numerous distal simple setae (Fig. 7E).

Left mandible having pars incisiva with five

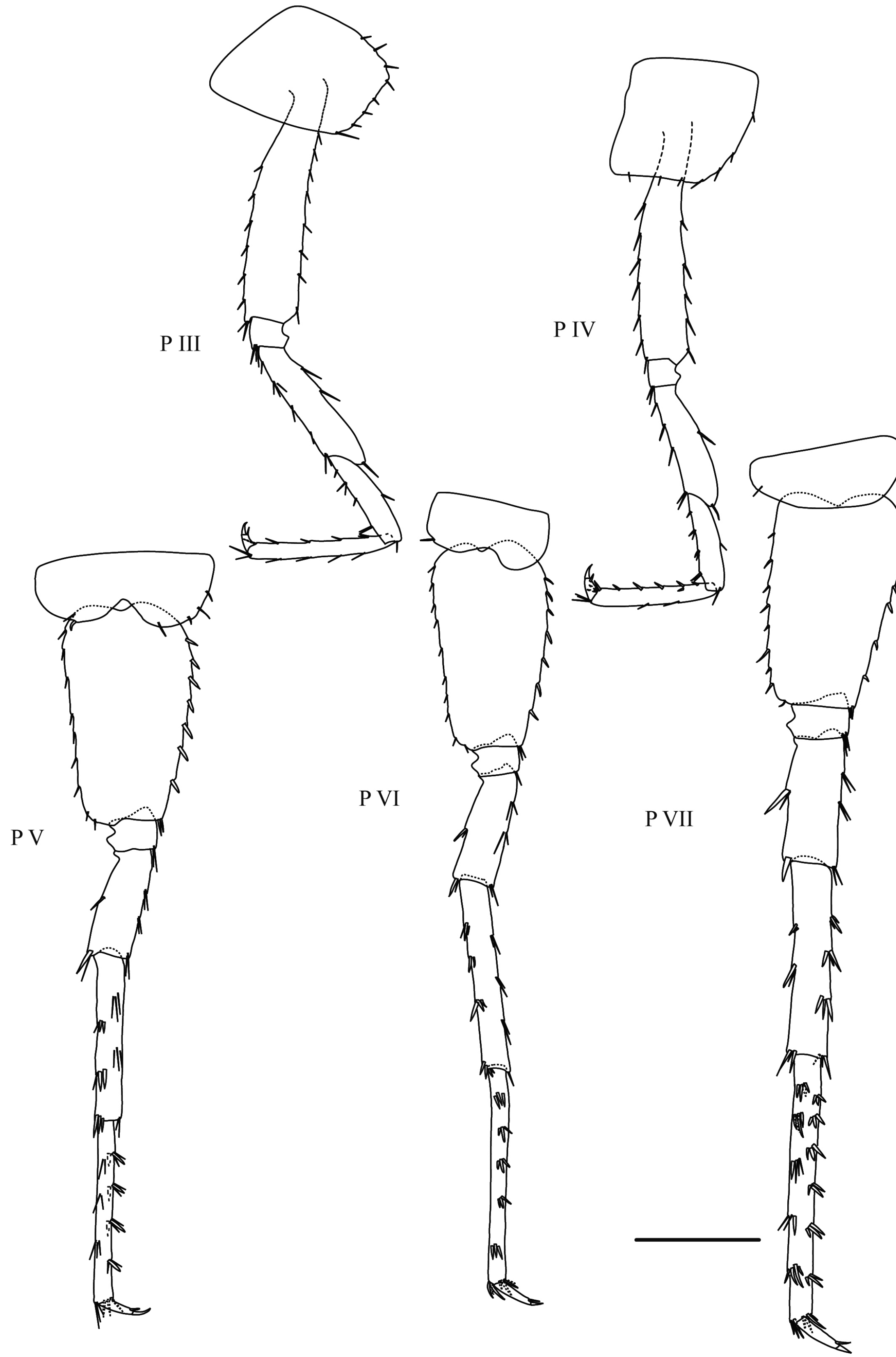

Fig. 4. *N. kurdistanensis* sp. nov., male 12 mm (holotype, ZCRU Amph. 1076). (A) P III; (B) P IV; (C) P V; (D) P VI; (E) P VII. Scale bars: 1 mm (P III- P VII).

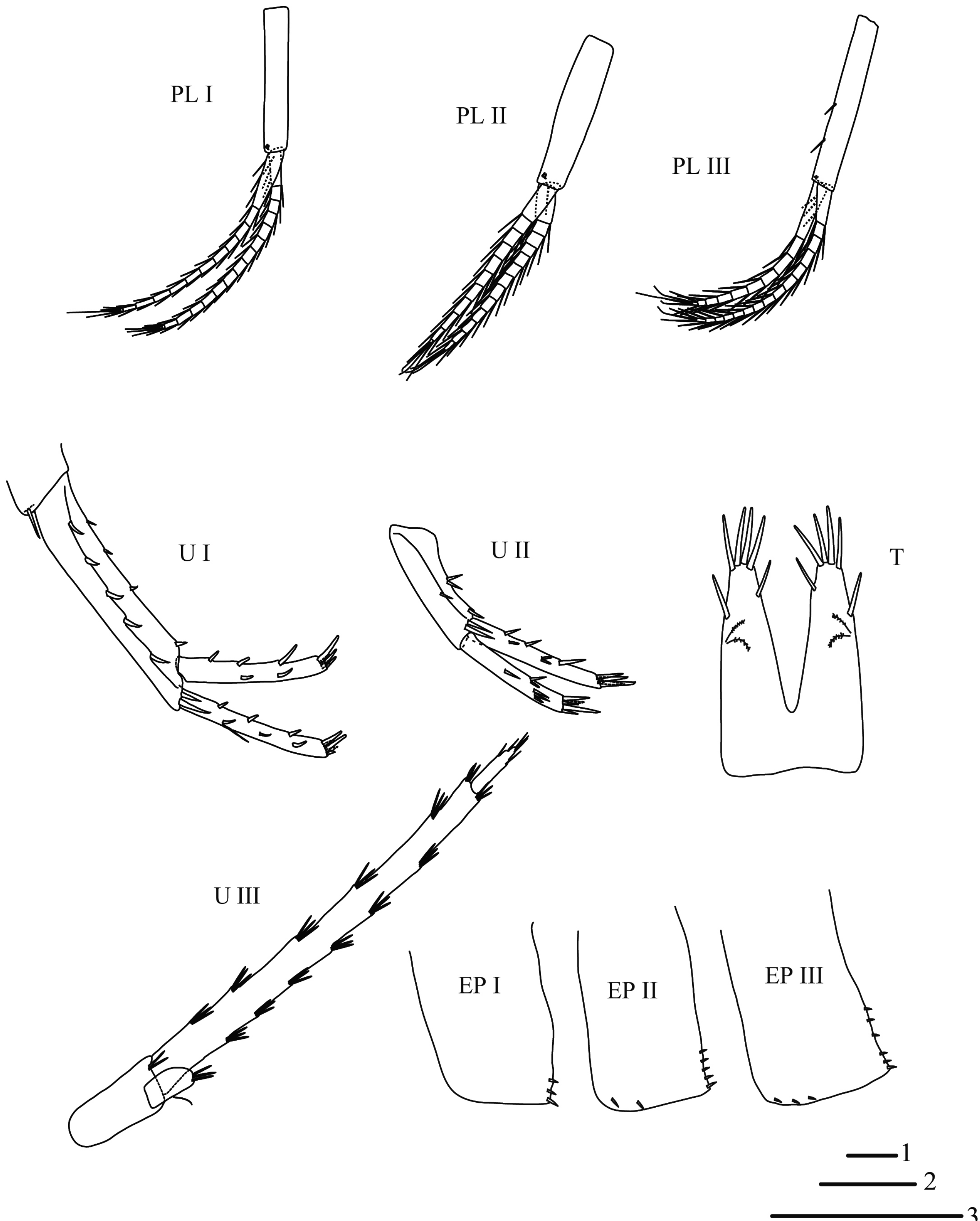

Fig. 5. *N. kurdistanensis* sp. nov., male 12 mm (holotype, ZCRU Amph. 1076). (A) PL I; (B) PL II; (C) PL III; (D) U I; (E) U II; (F) U III; (G) EP I-III; (H) T. Scale bars: 1 = 0.5 mm (EP I-III, T); 2 = 1 mm (PL I-III, U I-II); 3 = 2 mm (U III).

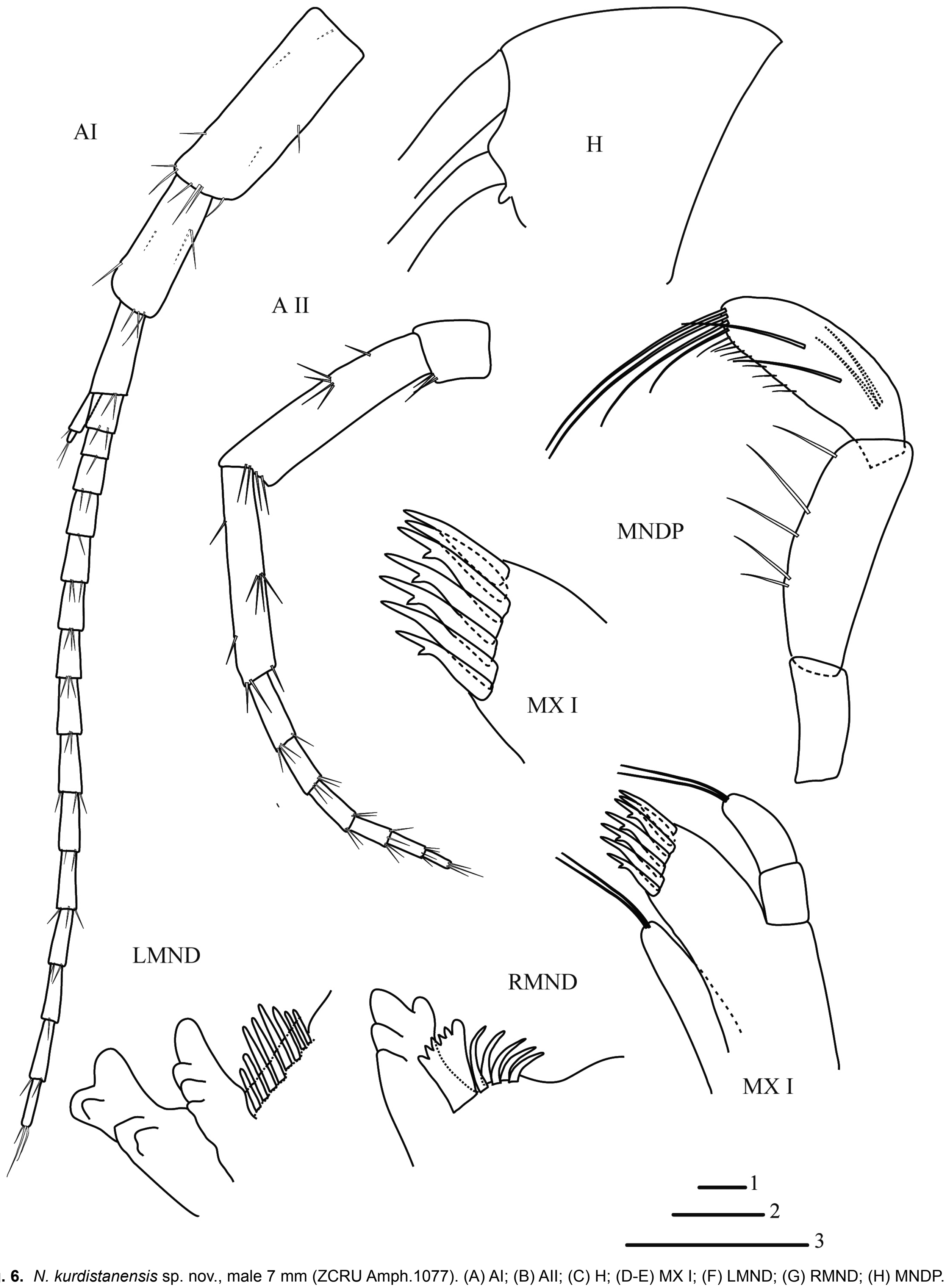

Fig. 6. *N. kurdistanensis* sp. nov., male 7 mm (ZCRU Amph.1077). (A) AI; (B) AII; (C) H; (D-E) MX I; (F) LMND; (G) RMND; (H) MNDP. Scale bars: 1 = 0.25 mm (LMND, RMND); 2 = 0.5 mm (H, MX I, MNDP); 3 = 1 mm (AI-AII).

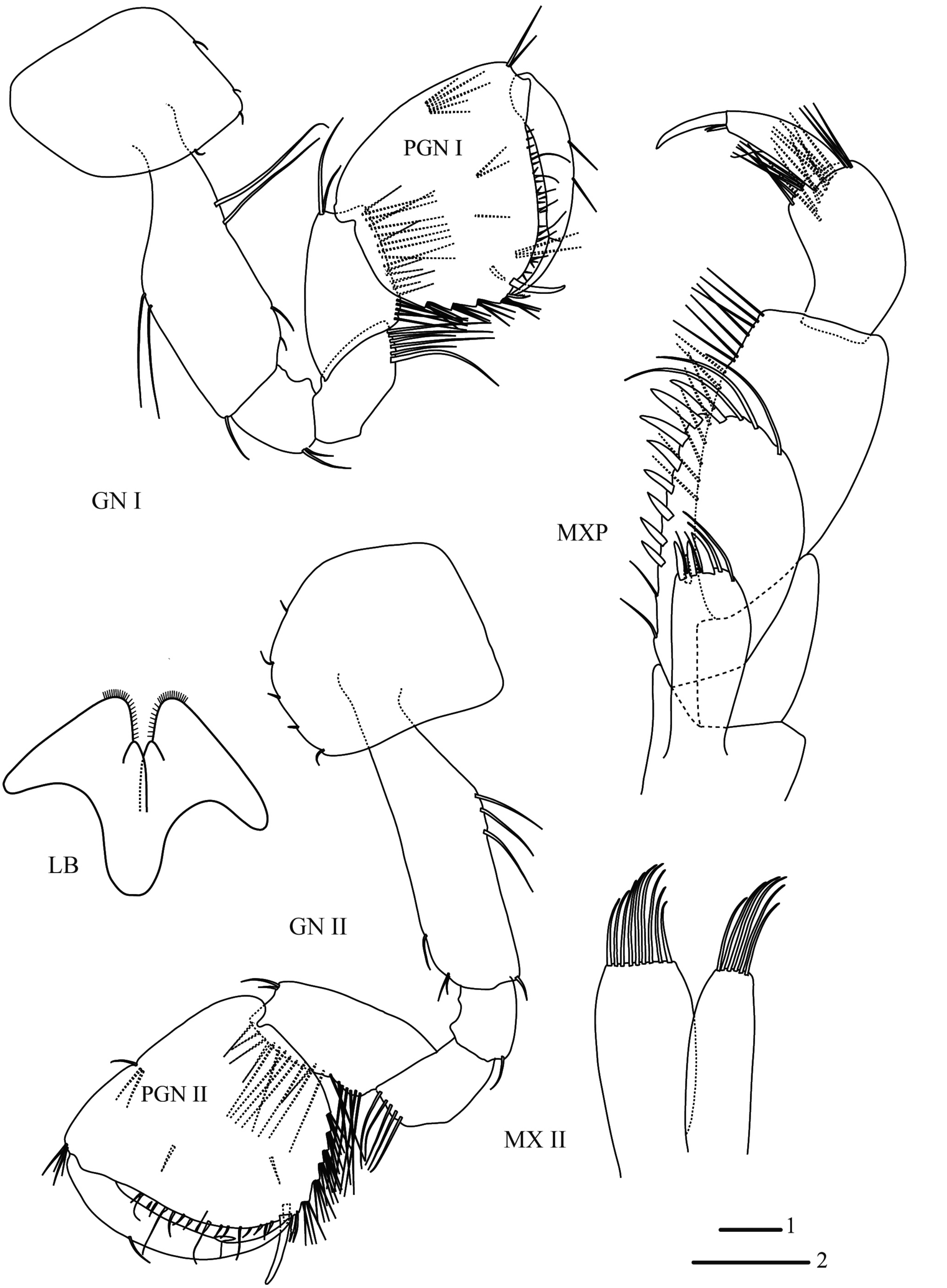

Fig. 7. *N. kurdistanensis* sp. nov., male 7 mm (ZCRU Amph. 1077). (A) GN I; (B) GN II; (C) MXP; (D) LB; (E) MX II. Scale bars: 1 = 0.5 mm (LB, MXP, MX II); 2 = 1 mm (GN I-II).

teeth, lacinia mobilis with four teeth and eight setae with lateral projections between lacinia and triturative molar (Fig. 6F). Right mandible with pars incisive having four teeth, lacinia mobilis pluritooth and five setae with lateral projections between lacinia and triturative molar (Fig. 6G). Mandibular palp articles 1:2:3 ratios as 1:1.87:1.85. The proximal article has no setae, the second article with five setae along inner margin and the third article with one group of two A-setae, two groups of B-setae, no C-setae, nine D-setae and four E-setae (Fig. 6H).

Maxilliped with short inner plate bearing three distal robust setae intermixed with four distal simple setae; outer plate exceeding half of the palp article 2, with eight robust setae along inner margin and three simple setae distally. Palp article 3 of maxilliped with one proximal, inner and outer group of long simple setae at outer margin; palp terminal article with one simple seta at the base of nail, nail shorter than pedestal (Fig. 7C).

Coxa of gnathopod I trapezoid, broader than long, antero-ventral margins with four simple setae. Basis with setae on anterior and posterior margins; ischium and merus with posterior group of setae. Carpus with one group of two setae antero-distally, a bulge with long simple setae; carpus 0.71 of basis length and 0.8 of propodus length. Propodus broader than long; anterior margin with five setae in one group in addition to antero-distal group of two simple setae. Palm slightly convex, with one strong long palmar robust seta, one short supporting robust seta on inner surface and one robust seta with lateral projections on outer surface. Dactylus reaching posterior margin of propodus, outer and inner margins with a row of two and two simple setae, respectively; nail short, 0.3 of total dactylus length (Fig. 7A).

Coxa of gnathopod II subrounded-quadratic, with five simple setae along antero-ventral margins. Basis with setae in groups and single seta along anterior and posterior margins; posterior margins of ischium and merus with one posterior group of setae each. Carpus 0.69 of basis length and 0.79 propodus length. Carpus with one group of two setae antero-distally, and rows of setae on the posterior bulk. Propodus of gnathopod II larger than propodus of gnathopod I, trapezoid shape and broader than long; anterior margin with three setae in one group in addition to antero-distal group of four simple setae. Palm convex, with one strong palmar robust seta, one supporting robust seta without lateral projections on inner surface, and two robust setae with lateral projections on outer surface. Dactylus reaching posterior margin of propodus, outer and inner margins of dactylus with one and two simple setae, respectively. Nail length 0.32 of total dactylus length (Fig. 7B).

Coxa III rectangular, length to width ratio as 1.06:1; ventral margin with four simple setae. Coxa IV rectangular, antero-ventral margin with five simple setae, posterior concavity shallow and approximately 0.1 of coxa width (Figs. 8A-B). Coxa V with anterior lobe, with one simple seta on anterior lobe. Coxa VI with anterior lobe, with one simple seta on anterior lobe. Coxa VII with one simple seta (Figs. 8C-E).

Pereopod III:IV lengths ratio as 1.06:1 (Figs. 8A-B). Dactylus IV short, length of dactylus 0.4 of propodus, nail shorter than pedestal (Fig. 8B). Pereopods V:VI:VII length ratios as 1:1.07:1.2. Pereopod VII 0.42 of body length. Pereopod bases V-VII with five, six and five groups of robust setae along anterior margins, respectively and with seven, seven and six simple setae along posterior margins, respectively (Figs. 8C-E). Postero-ventral lobe of ischium in pereopods V-VII weakly developed. Ischium, merus and carpus in pereopods V-VII with several groups of robust and simple setae along anterior and posterior margins; propodus of pereopod VII longer than these in V-VI, dactyli of pereopods V-VII with one robust and one short simple seta at the base of nail on inner margin, dactylus of pereopod VI with one simple seta on outer margin, nail length of pereopod VII 0.2 of total dactylus length (Figs. 8C-E).

Pereonites I-VII without setae. Pleonites I-III each with 3-4 simple setae along dorsal surface. Epimeral plates I-III (Fig. 9G) with angular postero-ventral corners, anterior and ventral margins convex; postero-ventral margins of plates I-III with two, three and four robust setae posteriorly, respectively. Epimeral plates II-III with two robust setae along of ventral margins each. Peduncle of pleopods I-III with two-hooked retinacles. Peduncle of pleopod I with one simple seta at distal part of outer margin. Peduncle of pleopod III with one simple seta along of inner margin (Figs. 9A-C); rami of pleopods I-III each with seven to nine articles (Figs. 9A-C).

Urosomites I-III with two, two and one dorso-lateral robust setae, respectively. Urosomite I with one robust seta at the base of uropod I. Peduncle of uropod I with six and three large robust setae along dorsolateral and dorsomedial margins, respectively. Inner ramus of uropod I slightly longer than outer ramus (ratio 1.05:1); inner ramus with two groups of robust setae laterally and five robust setae distally; outer ramus with two groups of robust setae laterally and five robust setae distally (Fig. 9D). Inner ramus in uropod II longer than outer, both rami with lateral and distal long robust setae (Fig. 9E). Uropod III long, almost 0.45 of body length. Peduncle of uropod III with five robust setae. Outer ramus biarticulated, distal article measures 0.22 of the proximal article. Proximal article of outer ramus bearing five groups of robust setae along each inner and outer margins (Fig. 9F); distal article with simple setae laterally and four simple setae distally. Inner ramus short, with three robust distal setae. Telson slightly longer than broad, lobes slightly narrowing; each lobe with four robust setae distally, with two plumose setae laterally (Fig. 9H).

Etymology

The name "kurdistanensis" refers to Kurdistan Province (Iran), where the species was found.

Phylogenetic position of the new species and its genetic distinctness

We sequenced and analyzed DNA from three individuals, two from Shoei cave and one from Darvish-Olya cave. All three specimens showed unique haplotypes for both 513 base pairs long fragment of COI gene and for 810 base pairs long fragment of 28S ribosomal DNA. Phylogenetic analyses using 57 cogeners consistently placed a new species into a clade comprised of exclusively Middle East species. Its accurate position

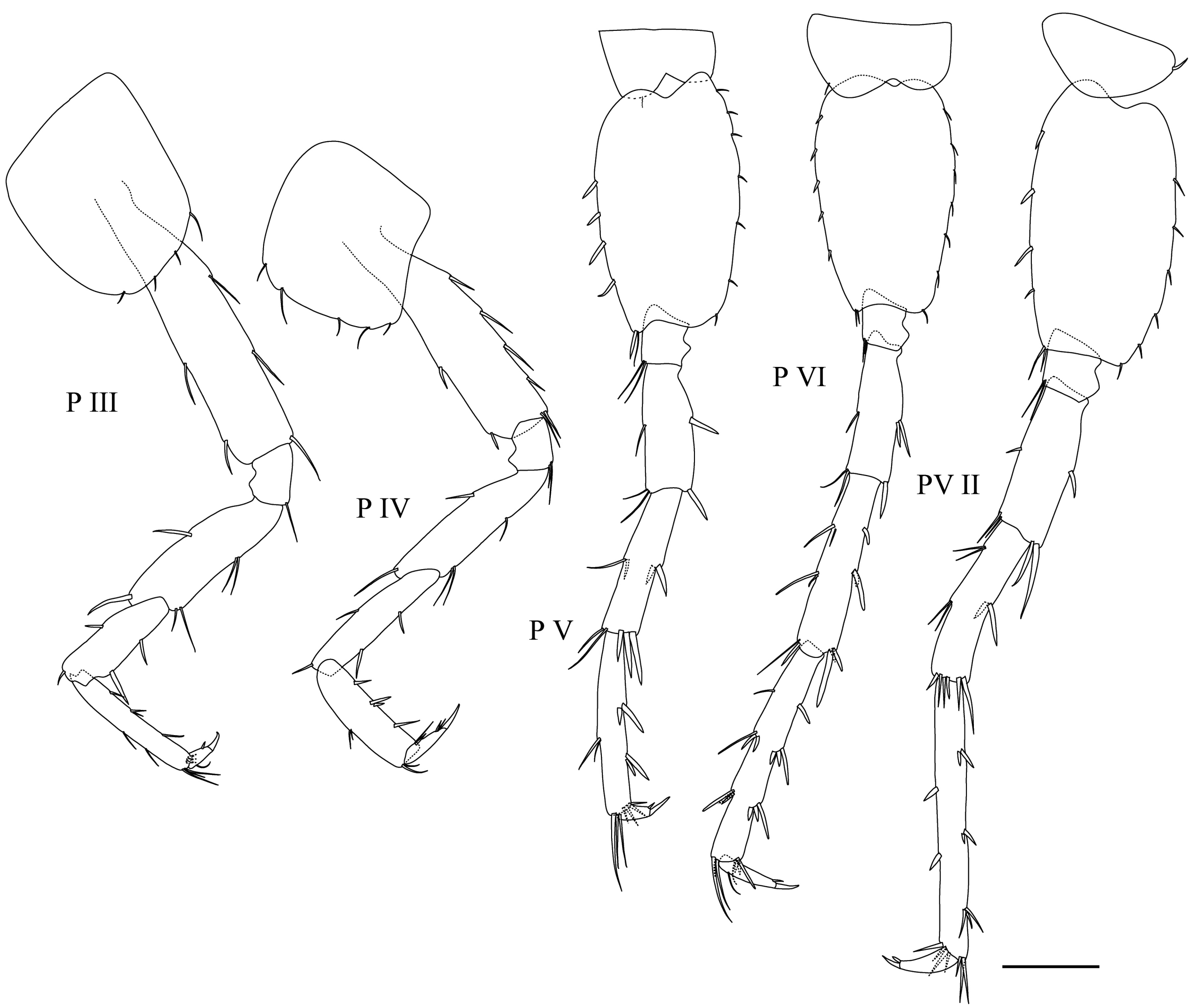

Fig. 8. *N. kurdistanensis* sp. nov., male 7 mm (ZCRU Amph. 1077). (A) P III; (B) P IV; (C) P V; (D) P VI; (E) P VII. Scale bars: 1 mm (P III- P VII).

within the clade however remains unknown. The phylogenetic relationships within the clade remained mainly unresolved in a phylogenetic analysis based on 28S fragment. The phylogenetic hypothesis based on COI gene suggests a sister relationship with unknown species from Lebanon, however, the support for this node is weak and should not be treated as reliable hypothesis (Figs. 10-11).

The new species is clearly distinct from all other Iranian species. The Pairwise Kimura two parameter genetic distances between *N. kurdistanensis* sp.nov. and all other species varied between 0.3-9.7% and 9.8-19.9 for 28S

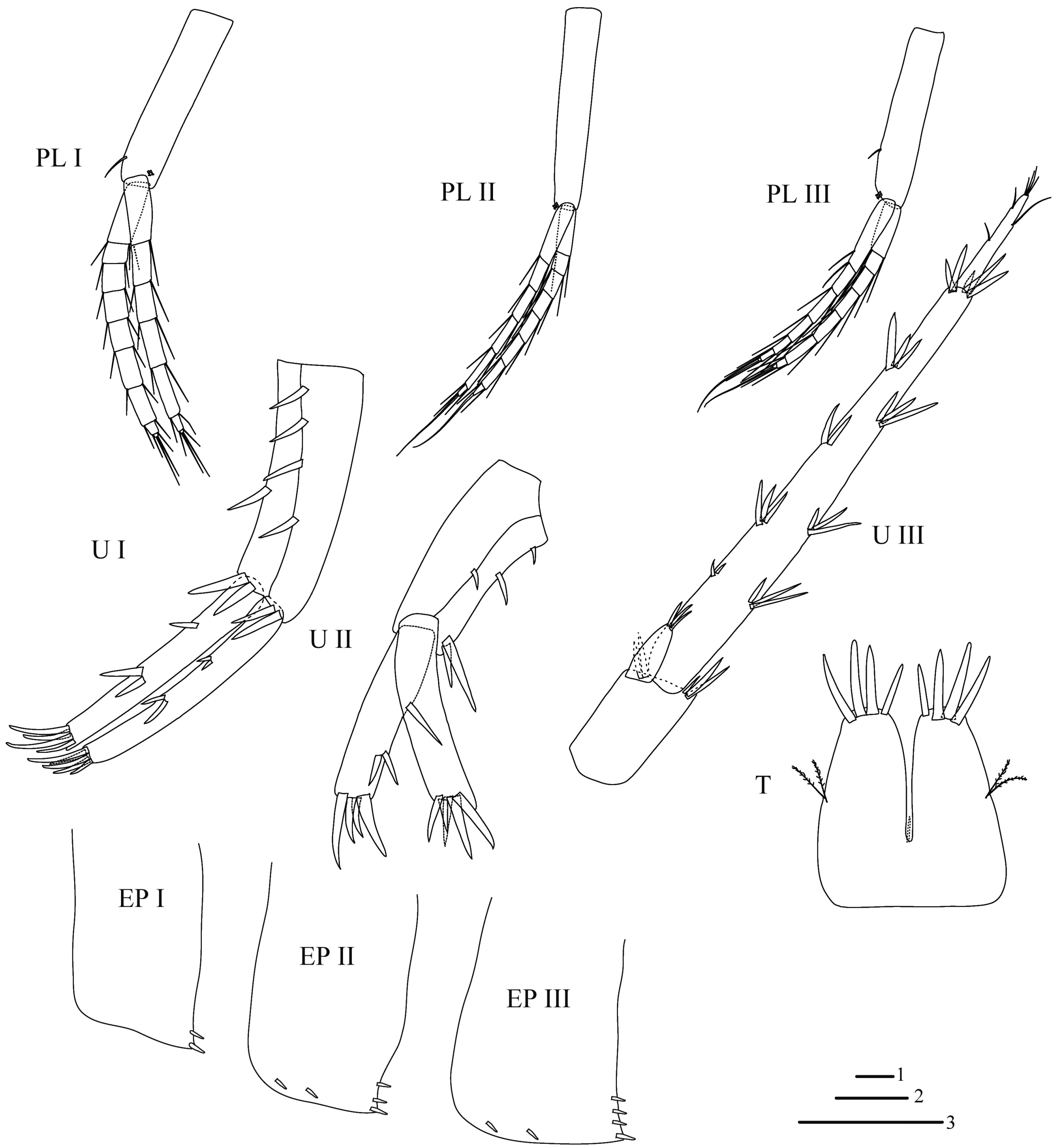

Fig. 9. *N. kurdistanensis* sp. nov., male 7 mm (ZCRU Amph. 1077). (A) PL I; (B) PL II; (C) PL III; (D) U I; (E) U II; (F) U III; (G) EP I-III; (H) T. Scale bars: 1 = 0.5 mm (EP I-III, T); 2 = 1 mm (PL I-III, U I-II); 3 = 2 mm (U III).

and COI, respectively (Table 1). These differences are in a range when amphipod species become reproductively isolated (Lagrue et al. 2014). In addition to morphological distinctness, molecular divergence provides an additional support for the hypothesis that *N. kurdistanensis* sp. nov. deserves an independent species status.

DISCUSSION

Interpopulational variation

The two individuals we described and illustrated differ in some traits that may be taxonomically important. In particular remarkable are differences in the shape of propodus of

Fig. 10. Bayesian consensus tree of 53 *Niphargus* species (52 taxa from Esmaeili-Rineh et al. 2015b, 2017a), based on the 28S ribosomal DNA sequences. Species are identified and named according to available taxonomic descriptions. Posterior probabilities are indicated on main branches.

gnathopod II and in the shape of telson, with specimen from Shoei Cave (holotype) having much larger and hoof-shaped propods, and much narrower telson. The number of specimens at hand do not allow assessment whether these differences are due geographic isolation and reflect some local adaptation (*e.g.* Delić et al. 2016), or due to ontogenetic differences (Fišer et al. 2008). Many *Niphargus* species grow allometrically, and may change body proportions during their lifespan (Fišer et al. 2008). So far, allomotric growth was shown for head length, propods of gnathopods, and telson width-length ratio. It is therefore likely that specimens from Darvish-Olya Cave and Shoei Cave cover variation of juveniles and adults, respectively.

These differences in spite, many taxonomic traits seem to be stable. This particularly refers to the nearly smooth spiniform setae on outer lobe of maxilla I, spiniform setae along posterior margins

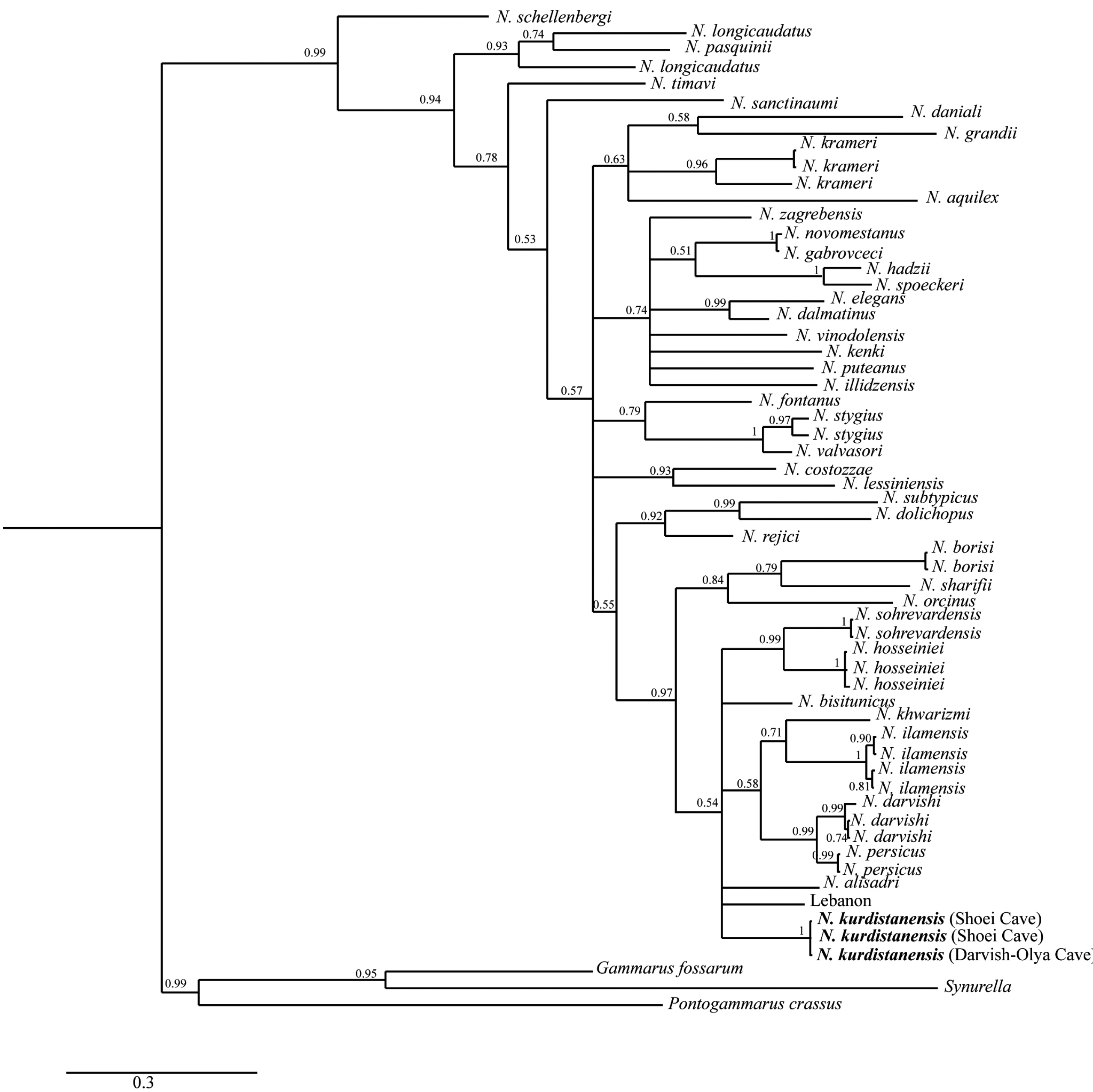

Fig. 11. Bayesian consensus tree of 41 *Niphargus* species (40 taxa from Esmaeili-Rineh et al. 2015b), based on the COI sequences. Species are identified and named according to available taxonomic descriptions. Posterior probabilities are indicated on main branches.

of epimeral plates, dorso-lateral spiniform setae on urosomite III, short maxillar palpus, and four apical spiniform telson robust setae. Except from the latter two, all these characteristics are rare among *Niphargus* species, and their unique combination can be considered as reliable diagnosis.

Comparison with other species from Middle East

The shortest diagnosis of the species comprises four characters, which we discuss in relation to other species from Middle East. A very easily visible character is the elevated number of apical robust setae per telson lobe. There are many species in Europe in which the number of apical robust setae exceeds four per telson lobe. By contrast, an overview of 29 species from the Middle East unveils there are only five species where the number of apical robust setae exceeds the number three: *N. daniali* (Esmaeili-Rineh and Sari 2013) and *N. kurdistanensis* sp. nov. (both from Iran), as well as *N. imitator* (Karaman 2012a), *N. kirgizi* (Fišer et al. 2009b), and some populations of *N. tauri* (Karaman 2012b) (all from Turkey). Most of these species are known by only few representatives and within species variation of this character is difficult to evaluate. In European species, this character often varies within species (*e.g.*, Fišer et al. 2010), and we suggest that this trait, albeit easily visible, needs to be used with care in identification procedure.

The second easily visible character is dorso-lateral robust setae on urosomite III. This trait is rather uncommon in European specimens (see *e.g.* for discussion Petković et al. 2015), but relatively frequent in Middle East. Similar setae were found also in *N. borisi*, *N. sohrevardensis*, *N. ilamensis* and *N. hakani* (Esmaeili-Rineh et al. 2015a, 2017a b). Of these only one species, *N. ilamensis*, shares another rare diagnostic character with *N. kurdistanensis* sp. nov. that is robust setae along posterior margin of epimeral plates II-III. Posterior margins of epimeral plates are usually armed with thin and flexible setae and a single stout seta ventro-posteriorly (distal most). To our knowledge, stout and spiniform setae along posterior margins of epimeral plates II-III appear only in *N. balcanicus* Absolon 1927 (from Europe), *N. kurdistanensis* sp. nov., and *N. ilamensis* (both Iran). European *N. balcanicus* has body covered with spiniform setae and completely differs from all other *Niphargus* species (Karaman 1932).

The two species, *N. ilamensis* and *N. kurdistanensis* sp. nov., differ in the number of robust setae on telson apically, but also in the fourth diagnostic character, denticulation of robust setae on outer lobe of maxilla I. Most of *Niphargus* species, in this position, have seven robust setae, among which is the inner one densely denticulated, whereas the rest of these bear only few (1-3) denticles. Less frequent are maxillae bearing seven robust setae, all armed with numerous denticles

Table 1. K2P genetic distances (%) between all Iranian species and Lebanon sample of the genus *Niphargus* based on 28S ribosomal DNA gene (below diagonal) and mtDNA (COI) gene (above diagonal)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1: *N. kurdistanensis* sp. nov.	-	12.1	13.0	15.1	14.7	11.6	12.1	13.2	15.7	13.2	11.6	12.5	19.9	9.8	-	-
2: *N. alisadri*	0.6	-	13.3	17.1	15.8	12.4	11.0	12.4	14.4	11.9	12.4	13.1	20.9	12.4	-	-
3: *N. darvishi*	1.1	0.8	-	14.1	15.6	9.6	10.5	12.3	13.5	12.3	4.7	10.7	17.6	10.2	-	-
4: *N. borisi*	2.0	2.2	2.7	-	13.0	16.3	14.5	18.1	18.6	17.6	13.9	15.6	23.5	14.8	-	-
5: *N. sharifii*	0.8	0.6	1.1	1.8	-	15.8	14.7	16.1	16.0	15.6	14.9	14.7	22.2	15.6	-	-
6: *N. khwarizmi*	1.4	1.0	1.3	2.6	1.1	-	11.2	12.1	15.8	12.1	9.0	9.0	19.9	10.5	-	-
7: *N. bisitunicus*	1.3	1.1	1.4	2.7	1.0	1.1	-	11.0	13.8	10.5	8.7	11.2	21.7	12.8	-	-
8: *N. khayyami*	1.7	1.3	1.5	2.6	1.1	1.0	1.1	-	10.3	0.4	11.6	14.0	22.2	13.7	-	-
8: *N. sohrevardensis*	1.4	1.0	1.0	2.6	1.1	0.8	0.9	0.8	-	9.9	14.6	13.6	22.0	16.4	-	-
10: *N. hosseiniei*	1.7	1.4	1.4	2.8	1.5	1.1	1.3	1.1	0.4	-	12.1	14.0	22.2	14.2	-	-
11: *N. persicus*	1.3	0.9	0.3	2.8	1.3	1.4	1.5	1.7	1.1	1.5	-	10.1	17.8	9.6	-	-
12: *N. ilamensis*	1.7	1.3	1.3	2.8	1.4	1.0	1.1	1.3	0.5	0.9	1.4	-	22.0	11.9	-	-
13: *N. daniali*	9.7	9.7	10.0	10.0	9.6	10.4	10.4	10.4	10.4	10.8	10.2	10.4	-	17.1	-	-
14: NLebanon	0.3	0.6	1.1	2.0	0.8	1.4	1.5	1.7	1.4	1.7	1.3	1.7	9.9	-	-	-
15: *N. sarii*	1.8	1.4	1.4	3.0	1.5	1.1	1.3	1.0	0.6	0.8	1.5	1.1	10.8	1.7	-	-
16: *N. kermanshahi*	1.1	1.0	1.3	2.6	1.1	0.8	0.6	1.3	0.8	1.1	1.4	1.0	10.5	1.4	1.1	-
17: *N. hakani*	1.9	1.5	2.1	3.1	1.7	1.4	1.7	1.8	1.3	1.7	2.2	1.5	10.1	1.9	1.9	1.5

(*e.g.*, all more frequent than three denticles). By contrast, we are not aware of a species having these robust setae without denticles or with a single one, neither in Europe nor in the Middle East. This trait seems to be rare, perhaps even unique, and therefore strengthens the diagnosis *N. kurdistanensis* sp. nov.; unfortunately the trait can be visible only after dissection.

In order to ease identification of ever-growing list of *Niphargus* from Middle East, we revised the identification key by inclusion of all species described after 2015 (Esmaeil-Rineh et al. 2016, 2017a b). The identification key is available in the Appendix I.

CONCLUSION

We described another amphipod species from Iran, *N. kurdistanensis* sp. nov., using morphological, mitochondrial and nuclear genetic evidence. The number of species described from Iran has been raised to 16 and has almost reached the number of epigean amphipods from genus *Gammarus* (in total 18 species, see Zamanpoore et al. 2010). Of these only one species was found outside Iran (*N. valachicus*, see Karaman 1998), all other are endemic to the country. Noteworthy, all species endemic to Iran were described during the past four years, suggesting that the genus is heavily understudied and that additional species descriptions can be expected in a near future. Moreover, in analogy with Europe, we tentatively suggest that the same is true for all other aquatic troglobionts awaiting to be collected and taxonomically evaluated. For sure, subterranean environment of Iran warrants an exciting period of the forthcoming exploration.

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Authors' contributions: SE and CF designed the study. MM performed the field work and collected samples. MM and SE prepared the figures. CF provided Identification Key. SE and CF analyzed the data and wrote manuscript. All authors participated in revising the manuscript. All authors read and approved the final manuscript.

Competing interests: The authors have no competing interests to declare.

Availability of data and materials: Data are available from accession numbers (MG008301-MG008306) for DNA sequences deposited in GenBank.

Consent for publication: Not applicable.

Ethics approval consent to participate: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. No experiments were done on living animals in this study. The animal Ethics Committee of Razi University approved the experimental protocol.

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Appendix I. Identification key for *Niphargus* species from the Middle East. (download)
Table S1. K2P genetic distances (%) between all Iranian species and Lebanon sample of the genus *Niphargus* based on 28S ribosomal DNA gene (below diagonal) and mtDNA (COI) gene (above diagonal). (download)