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Two New Species of Stygobiotic Amphipod *Niphargus* (Amphipoda: Niphargidae) and their Phylogenetic Relationship with Other Congeners from Iran

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Two new amphipod species from Iran, *Niphargus sahandensis* sp. nov. and *Niphargus chaldoranensis* sp. nov., are described based on their morphological characteristics and molecular analyses. Bayesian inference analyses of *COI* and 28s rDNA sequence data provided evidence for the validity of the two species and their placement in the *Niphargus* genus. *N. sahandensis* sp. nov. primarily differs from similar species by having more than two hook-like retinacles on the inner surface of pleopods I–III, the presence of two spines at the base of uropod I and rectangular-shaped propodi in both gnathopods. *N. chaldoranensis* sp. nov. is distinguished by the trapezoidal-shaped propodi in gnathopods I to II, the equal sizes of pereopods V and VI, and the proportional size of periopod VII in relation to the total body (60%). Morphological descriptions with illustrations of the new species, as well as a DNA-based phylogeny generated from analyses of a multigene dataset, are provided to better understand species relationships.

Key words: *Niphargus sahandensis* sp. nov., *Niphargus chaldoranensis* sp. nov., Morphological characters, *COI* and 28s rDNA, Iran

BACKGROUND

Groundwater ecosystems are physically heterogenous and complex systems recognized by low oxygen levels, constant temperature, limited space, and scarcity of food and energy sources (Macario-González et al. 2021; Danielopol et al. 2000). Resident species have been able to occupy groundwater environments through specific physiological, morphological and behavioral adaptations, such as low metabolic rates, vermiform body shape, reduced or complete loss of vision and pigments, and enhancement of certain sensory structures (Sket 1985; Gibert et al. 1994; Langecker 2000; Parzefall 2000; Culver and Sket 2000).

Amphipod crustaceans from the genus *Niphargus* are the largest genus of freshwater amphipods, most of which are found exclusively in groundwater (Petković et al. 2020). Their functional morphology and feeding habits likely played an important role in their evolution, leading to large morphological and ecological

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diversity within the genus (Borko et al. 2021). These characteristics make them an interesting ecological model system that can, at times, be used as a proxy for groundwater communities (Balázs et al. 2023).

The taxonomy of *Niphargus* is unresolved. New species are continuously being found, both morphologically indistinguishable (so-called 'cryptic species') as well as morphologically distinct. However, accurate reconstruction and resolution of their phylogenetic relationships as well as reconstructions of their ecology and biogeography require a complete taxonomic structure (Mammola et al. 2019; Esmaeili-Rineh et al. 2020; Petković et al. 2020). Due to numerous morphologically cryptic species, the taxonomy of *Niphargus* should rely on both molecular and morphological analyses. By combining these approaches, scientists can achieve a more accurate and comprehensive understanding of the taxonomy and evolutionary history of the *Niphargus* genus, as well as other groups of organisms found in groundwater ecosystems (Esmaeili et al. 2020; Balázs et al. 2023).

Niphargus is distributed between Ireland and Iran. Taxonomic research of this genus in Iran has begun relatively recently and so far relatively few species are known from Iran (Esmaeili et al. 2015). This is in stark contrast with the fact that the Alborz and Zagros mountain ranges in Iran have high biodiversity and are considered hotspots. New cave explorations, however, continuously unveil new findings of *Niphargus*. In this study, we present three populations of the *Niphargus* genus collected in northwest Iran and morphological and molecular evidence that suggests they should be treated as new species.

MATERIALS AND METHODS

Morphological and morphometric studies

The samples were provided from Hargalan Spring in East Azerbaijan Province, and the Salmas and Shoan Springs in West Azerbaijan Province, Iran (Fig. 1). The animals were collected using a hand net. Shoan and Salmas Springs are located in the proximity of Chaldoran and Salmas cities, respectively, in West Azerbaijan. The distance between the localities is about

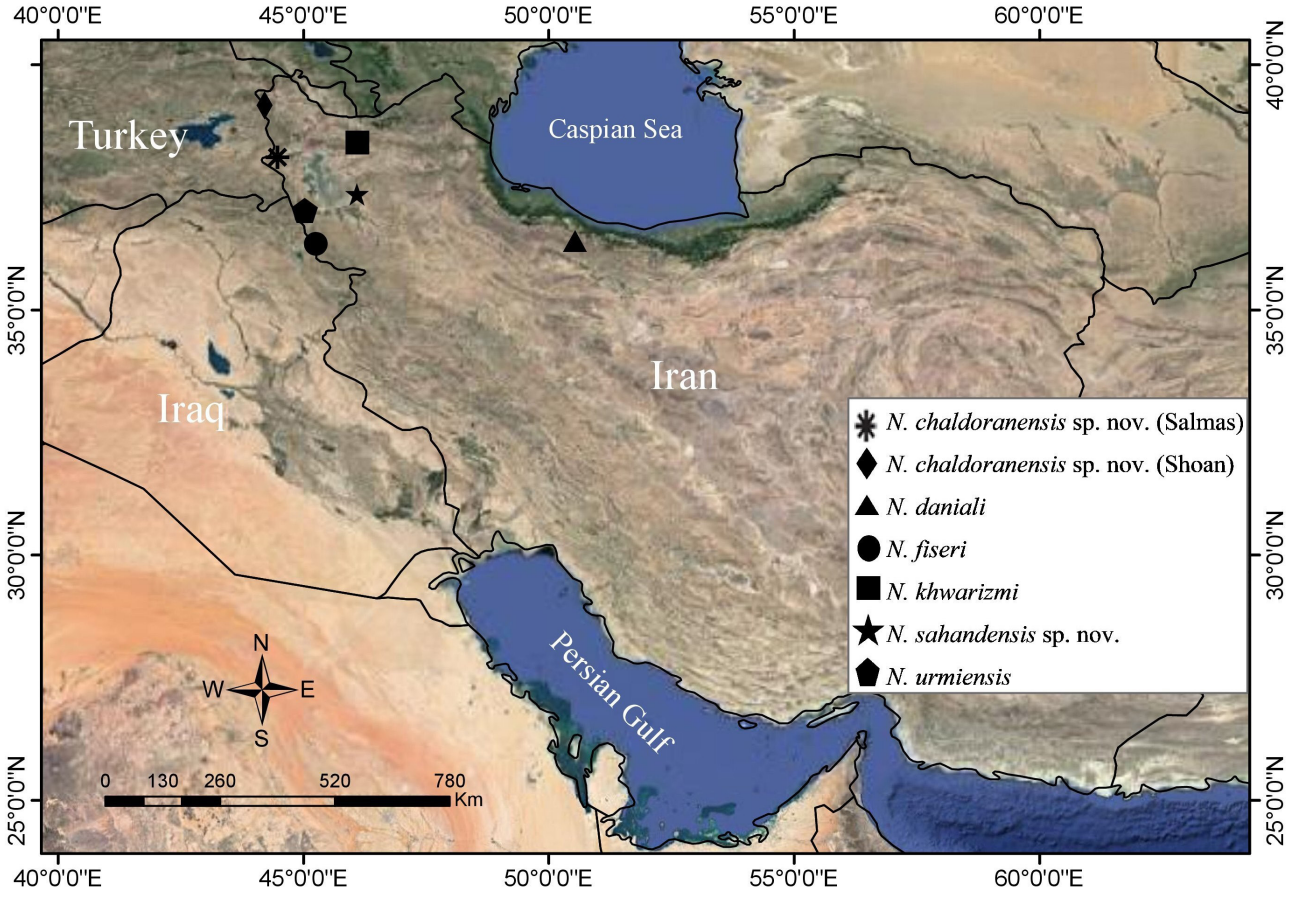

Fig. 1. Distribution map of the genus *Niphargus* Schiödte, 1849 in North and Northwest Iran.

120 km in a straight line. The materials were examined morphologically and mounted on slides in Euparal® medium. An Olympus LABOMED iVu7000 camera fitted on a LABOMED Lx500 stereo microscope was used to take the digital photos. The computer program ProgRes Capture Pro ver. 2.7 was used to perform the measurements and the counts. We measured and counted characters from six individuals. All materials were deposited in the Zoological Collection, Razi University (ZCRU).

Phylogenetic analyses and molecular divergence

For the molecular analyses, we extracted the total genomic DNA from part of an animal using Tissue Kits (GenNet Bio™) following the manufacturer's instructions (Seoul, South Korea). Mitochondrial *COI* gene was amplified using the modified primer pair LCO1490-JJ and HCO2198-JJ (Astrin and Stüben 2008). The amplification and sequencing of the first fragment of 28S ribosomal DNA (rDNA) were performed using the forward primer proposed by Verovnik et al. (2005) and the reverse primer used by Zakšek et al. (2007). Each 25 µl reaction consisted of optimized amounts of PCR water, 12.5 µl of Master Mix kit (Ampliqon), 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, 72°C for 1 minute, and a final extension of 72°C for 7 minutes. Purification of PCR products and sequencing were commercially performed by Microsynth AG (Swiss). Sequencing was performed with both primers mentioned above.

In order to identify the phylogenetic position of the newly discovered materials, the acquired sequences (with GenBank accession numbers PP492996–PP493003, and PP492709–PP492716 for *COI* and 28S rDNA gent respectively) were analyzed within the data set of Esmaeili-Rineh et al. (2015 2017a) and Bargrizaneh et al. (2021). The NCBI sequences of *Synurella ambulans*, *Pontogammarus crassus* and *Gammarus fossarum* were used as out-groups (accession numbers: KF719240, KF719242 and KF71924). 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 MrBayes, version 3.1.2 (Ronquist and Huelsenbeck 2003). Bayesian analyses were run for 20 million generations, under GTR+G and TIM3+I+G models (jModelTest, version 0.1.1, Posada 2008) for 28S and *COI* genes, respectively. We run four chains, and the trees were sampled every 1000 generations. The first 5000 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. The data on the analyzed species can be found in the Electronic Supplement of Esmaeili-Rineh et al. (2015 2020) and Bargrizaneh et al. (2021). To evaluate the divergence from other previously described Iranian species of *Niphargus*, we calculated the genetic distances using the Kimura two-parameter (K2P) model (Kimura 1980), which was implemented in MEGA ver. 5 (Tamura et al. 2011).

RESULTS

Phylogenetic position of the new species and their genetic distinctness

We sequenced and analyzed eight new individuals; namely, three from Salmas, three from Shoan, and two from Hargalan springs. The two specimens from the Hargalan population showed a unique haplotype for a 902 base pairs long fragment of 28S ribosomal DNA gene, and two haplotypes for 513 base pairs of *COI* gene. However, the six specimens from the Salmas and Shoan populations showed a unique haplotype for the 28s gene, while two haplotypes for the *COI* gene, one was found in Salmas and one in Shoan. The phylogenetic analyses of 62 specimens consistently placed the two new species into two different clades. One of the species was nested in a clade sister to the Middle East clade, while the other species was placed in a European clade. The phylogenetic relationships of this clade and other Middle Eastern clades remained mainly unresolved based on the combined data of 28S and *COI* gene fragments (Fig. 2), and therefore the accurate phylogenetic position of the Salmas and Shoan clade cannot be determined.

The two new species are genetically distinct from all other Iranian species. *N. sahandensis* sp. nov. is most genetically similar to *N. daniali* (3.59% based on 28s rDNA genes fragment) and the most divergent from *N. sarii* (11.82%) and *N. alisadri* (23.07%) based on both 28 rDNA and *COI* genes. *N. chaldoranensis* sp. nov. is most genetically similar to *N. alisadri* (2.31%) and

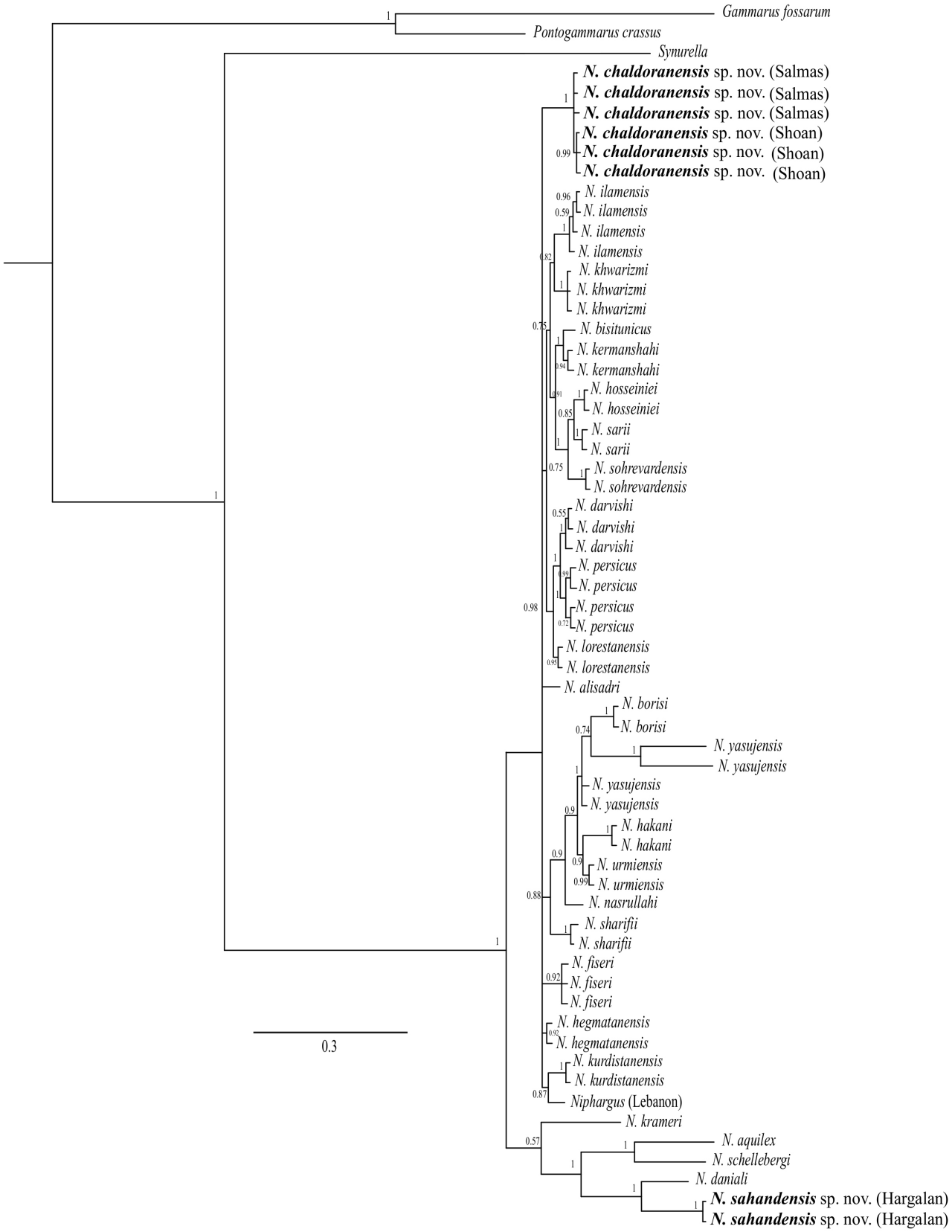

Fig. 2. Bayesian consensus tree of 59 *Niphargus* specimens, based on the 28S ribosomal DNA and *COI* gene sequences. Species are identified and named according to available taxonomic descriptions. Posterior probabilities are indicated on branches.

N. fiseri (11.37%) for 28srDNA and *COI*, respectively. Also, *N. chaldoranensis* sp. nov. is the most genetically divergent species from *N. daniali* (22.96% and 11.93%) for the studied *COI* and 28s rDNA genes fragments, respectively. All pairwise Kimura two parameter genetic distances of the Iranian taxa are shown in table 1.

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

Niphargus sahandensis sp. nov.

(Figs. 3–6)

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Type locality and Material examined: Holotype, Male specimen (7 mm) from Hargalan Spring, Ajabshir City, East Azerbaijan Province, Iran, coordinates (N 37°37'35", E46°09'50"). Specimens collected by M. Mamaghani-Shishvan; 11 Aug 2022. Holotype with two paratypes are stored under catalogue number ZCRU Amph.1604.

Etymology: The name "sahandensis" refers to Sahand Mountain in East Azerbaijan (Iran). Hargalan Spring is located on its slope.

Diagnosis: Peduncle of pleopods I to III with 3–4 hooked retinacles at distal part of inner margin. At the base of uropod I peduncle with two spines. Maxilla I palp long, reaching beyond the tip of the outer lobe. A relatively equal size of coxae of gnathopods I–II. The propodi of gnathopod I with two supporting spines in palmar corner. Ventro-posterial corner in epimeral plates I to III not produced. Rectangular shape of propodi in both gnathopods. Outer ramus of uropod I slightly shorter than inner ramus.

Description of holotype: Measurements: The total length of the holotype is 7 mm. Head represents 12% of the total body length (Fig. 3A).

Antennae: Antenna I is 0.40 times body length. Peduncular articles 1–3 progressively shorter; length of peduncular article 3 exceeds half of peduncular article 2 (ratio 1.00 : 1.32). Main flagellum with 14 articles (most with short setae). Accessory flagellum bi-articulated and reaching 0.5 of article 4 of main flagellum; both articles with two and three setae, respectively (Fig. 3B). Antenna II with flagellum formed of 9 articles, approximately 0.80 times as long as antenna I. Flagellum length is 0.84 times the length of peduncle articles 4 + 5. Peduncular article 4 of antenna II is longer than article 5 (1.12 : 1.00), peduncle articles 4 and 5 with seven and eight groups of setae, respectively (Fig. 3C).

Mouth parts: Labium (Fig. 4D) bi-lobate; with fine setae on tip of outer lobes. Inner plate of maxilla I

Table 1. Kimura 2-parameter-distances (K2P) between Iranian species and new collected populations of the genus *Niphargus* Schiödte, 1849 (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	17	18	19	20	21	22	23	24
1: N. sahandensis **(Hargalan)**		20.80	20.80	23.07	18.29	20.32	20.64	19.02	19.78	20.86	17.77	20.12	17.76	18.28	20.62	19.27	19.52	21.16	20.34	20.88	*	*	*	*
2: N. chaldoranensis **(Salmas)**	11.79		0.39	13.36	11.90	12.53	22.96	12.28	11.37	14.67	12.79	13.24	12.08	13.06	13.50	13.71	13.72	16.67	12.08	12.82	*	*	*	*
3: N. chaldoranensis **(Shoan)**	11.79	0.00	-	13.36	11.90	13.01	23.51	12.75	11.37	14.67	13.27	13.24	12.08	13.54	13.50	14.19	14.21	17.18	12.08	13.30	*	*	*	*
4: *N. alisadri*	11.06	2.31	2.31		11.03	16.88	20.91	12.35	12.16	11.90	13.08	12.38	12.10	12.36	15.41	12.36	15.76	14.15	12.15	15.93	*	*	*	*
5: *N. bisitunicus*	11.21	3.37	3.37	1.15		14.70	21.67	10.49	10.72	10.51	11.16	11.16	12.07	12.80	14.90	8.70	14.71	13.60	9.80	13.50	*	*	*	*
6: *N. borisi*	11.78	4.29	4.29	2.17	2.70		23.23	13.19	14.12	17.07	15.34	15.82	14.88	14.37	10.04	13.90	12.76	17.86	13.91	7.38	*	*	*	*
7: *N. daniali*	3.59	11.93	11.93	10.89	11.35	11.33		17.12	19.62	22.18	21.96	19.89	19.87	17.06	23.05	17.82	22.18	21.70	18.59	21.93	*	*	*	*
8: *N. darvishi*	11.37	3.10	3.10	0.76	1.40	2.69	11.20		9.58	12.08	10.27	9.82	12.27	10.01	13.65	3.82	14.86	13.33	8.69	11.80	*	*	*	*
9: *N. fiseri*	11.02	2.56	2.56	2.57	2.96	4.29	11.16	3.09		12.52	12.98	11.37	11.59	11.37	12.98	10.02	13.95	13.78	7.62	15.14	*	*	*	*
10: *N. hosseiniei*	11.82	3.36	3.36	1.40	1.27	2.82	11.96	1.40	2.83		13.99	12.10	13.22	14.18	13.44	12.09	15.65	9.65	13.70	14.16	*	*	*	*
11: *N. ilamensis*	11.06	3.23	3.23	1.27	1.15	2.83	11.51	1.27	2.83	0.89		8.98	12.52	11.87	15.61	10.07	14.67	13.31	10.02	14.42	*	*	*	*
12: *N. khwarizmi*	11.52	2.97	2.97	1.02	1.15	2.56	11.66	1.27	2.83	1.14	1.02		11.61	10.52	13.43	8.95	15.85	15.53	9.13	16.09	*	*	*	*
13: *N. kurdistanensis*	10.90	2.97	2.97	0.63	1.27	2.04	10.73	1.14	2.96	1.66	1.66	1.40		9.78	13.70	11.58	14.70	15.44	12.05	14.42	*	*	*	*
14: *Niphargus* sp. (Lebanon)	11.23	2.97	2.97	0.63	1.53	2.04	11.06	1.14	2.97	1.66	1.66	1.40	0.25		15.82	9.57	15.61	16.16	9.82	14.62	*	*	*	*
15: *N. nasrullahi*	11.21	3.23	3.23	0.89	1.53	1.78	11.04	1.40	3.23	1.79	1.66	1.40	1.27	1.28		13.90	14.99	16.94	13.22	8.98	*	*	*	*
16: *N. persicus*	11.52	3.23	3.23	0.89	1.53	2.82	11.35	0.25	3.23	1.53	1.40	1.40	1.27	1.27	1.53		14.87	14.33	8.90	12.98	*	*	*	*
17: *N. sharifii*	10.90	2.97	2.97	0.63	1.02	1.78	10.73	1.14	2.96	1.53	1.40	1.15	0.76	0.76	0.76	1.27		15.71	13.70	14.48	*	*	*	*
18: *N. sohrevardensis*	11.64	3.09	3.09	1.02	0.89	2.56	11.78	1.01	2.56	0.38	0.51	0.76	1.40	1.40	1.40	1.14	1.15		14.72	16.42	*	*	*	*
19: *N. urmiensis*	11.02	3.52	3.52	2.33	2.20	3.79	11.47	2.59	2.06	2.59	2.60	2.46	2.46	2.47	2.73	2.73	2.20	2.46		13.47	*	*	*	*
20: *N. yasujensis*	11.05	3.77	3.77	1.66	2.31	1.14	11.20	2.18	4.03	2.57	2.44	2.18	1.79	1.79	1.27	2.31	1.27	2.18	3.26		*	*	*	*
21: *N. hakani*	11.64	3.09	3.09	2.43	2.56	3.88	12.08	2.95	2.83	2.56	2.43	2.30	2.83	2.83	2.83	3.09	2.56	2.17	2.20	3.35		*	*	*
22: *N. hegmatanensis*	11.21	2.57	2.57	0.25	1.15	1.91	11.20	0.76	2.83	1.40	1.27	1.02	0.63	0.63	0.89	0.89	0.63	1.02	2.33	1.40	2.43		*	*
23: *N. kermanshahi*	11.21	3.10	3.10	0.89	0.51	2.43	11.35	1.14	2.96	1.01	0.89	0.63	1.02	1.28	1.27	1.27	1.02	0.63	2.46	2.05	2.30	0.89		*
24: *N. lorestanensis*	11.21	2.70	2.70	0.38	1.27	2.56	11.04	0.38	2.70	1.27	1.15	1.15	1.02	1.02	1.27	0.50	1.02	0.89	2.46	2.05	2.83	0.63	1.02	
25: *N. sarii*	11.82	3.63	3.63	1.40	1.27	2.96	11.96	1.40	3.23	0.76	1.14	1.14	1.79	1.79	1.79	1.53	1.53	0.63	2.73	2.57	2.82	1.40	1.01	1.27

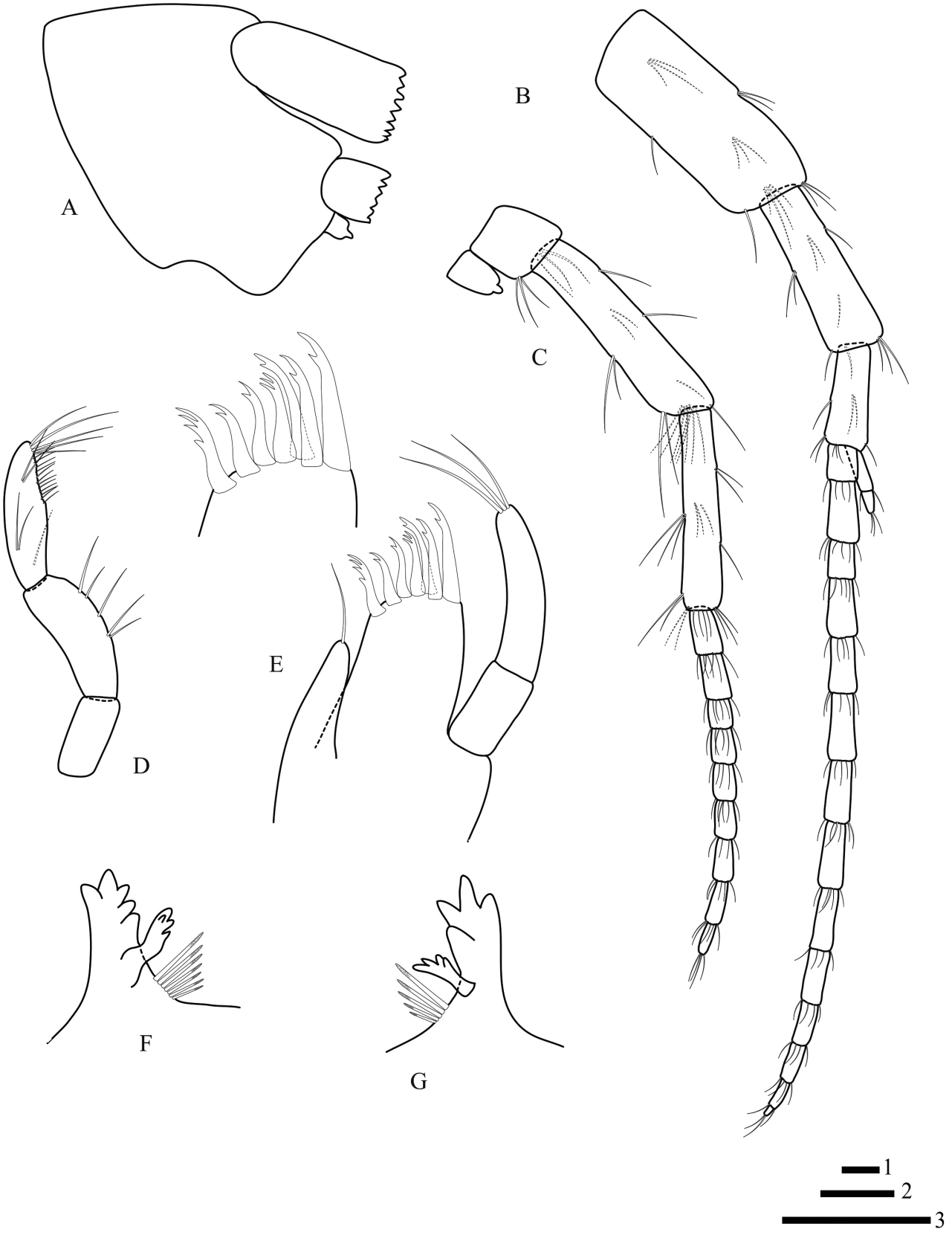

Fig. 3. *Niphargus sahandensis* sp. nov., Hargalan Spring, male 7 mm (holotype). A, Head; B, Antenna I; C, Antenna II; D, Mandibular palp; E, Maxilla I; F, Left mandible; G, Right mandible. Scale bars: 1 = 0.25 mm (F–G); 2 = 0.5 mm (A, D–E); 3 = 1 mm (B–C).

with one long apical seta, outer plate with seven long spines with 3-1-1-2-1-1-1 lateral projections; palp bi-articulated, long and reach the tip of outer lobe, with three apical setae (Fig. 3E–F). Both plates of maxilla II with numerous long distal setae (Fig. 4E). Mandibles: left mandible with five teeth on incisor process, lacinia mobilis with four teeth, between lacinia and molar a row of seven setae with lateral projections (Fig. 3F). Right mandible with four teeth on incisor process, lacinia mobilis pluritooth, between lacinia and molar a row of five setae with lateral projections (Fig. 3G). Mandibular palp articles 1:2:3 represent 21%, 38% and 41% of total palp length, respectively. Proximal article without setae; second article with five setae along inner margin and third article with one group of one A-seta, two groups of B-setae, no C-setae, 12 D-setae and five E-setae (Fig. 3D). Maxilliped with short inner plate bearing four distal spines intermixed with six distal setae; outer plate less than half of palp article 2, with 9 spines along inner margin and 4 setae distally; maxilliped palp article 3 at outer margin with one proximal and one distal group of long setae; palp terminal article with one seta at outer

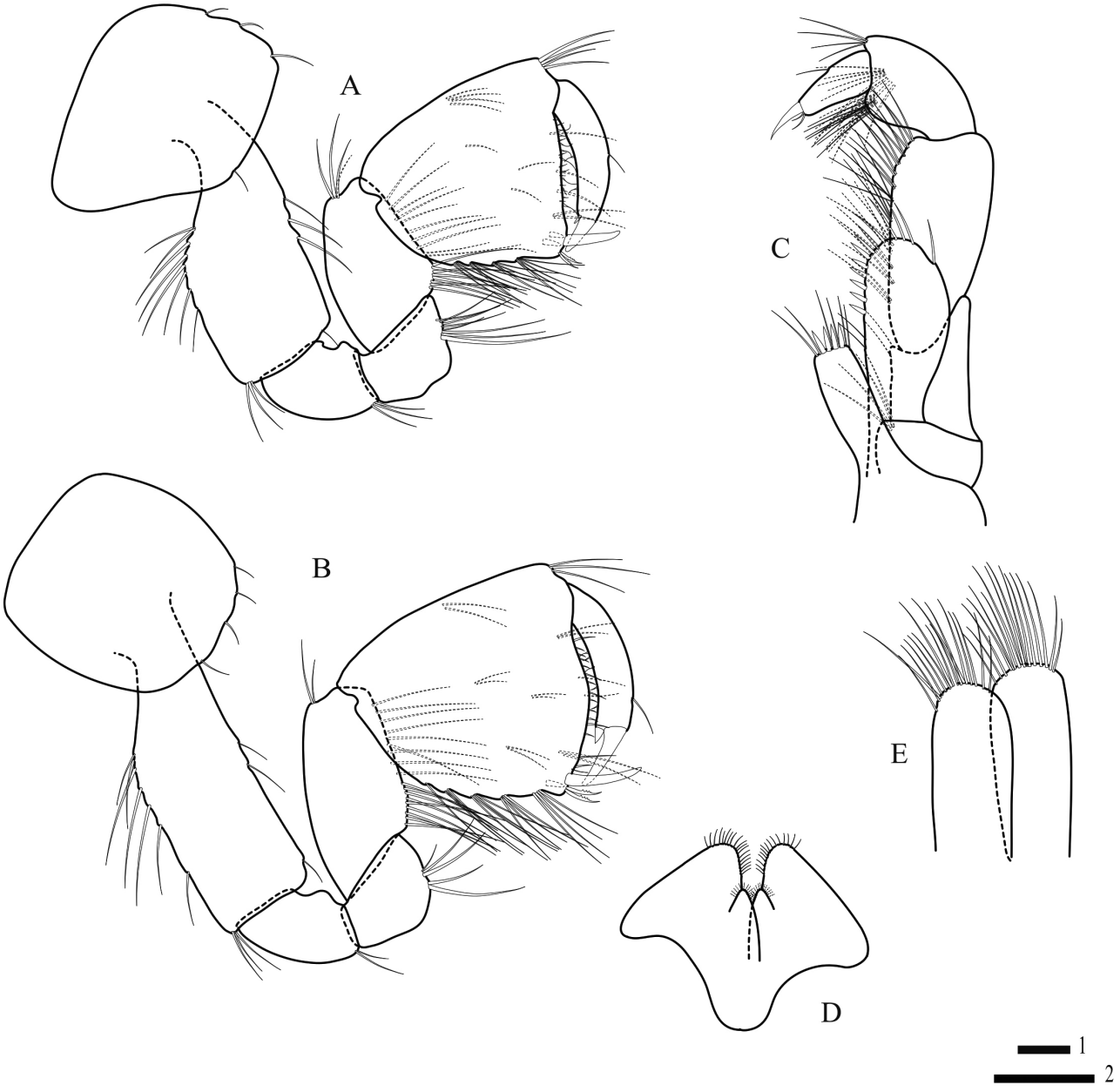

Fig. 4. *Niphargus sahandensis* sp. nov., Hargalan Spring, male 7 mm (holotype). A, Gnathopod I; B, Gnathopod II; C, Maxilliped; D, Labium; E, Maxilla II. Scale bars: 1 = 0.5 mm (D–E); 2 = 1 mm (A–C).

margin and two setae at base of nail, nail shorter than pedestal (Fig. 4C).

Gnathopods: Coxal plates of gnathopods I–II almost equal in size. Coxa of gnathopod I trapezoid, antero-ventral margins with four marginal setae. Basis with setae on anterior and posterior margins; ischium and merus with posterior group of setae. Carpus with one group of four setae antero-distally, bulge with long setae; carpus 0.61 times basis length and 0.65 times propodus length. Propodus of gnathopod I rectangular in shape and longer than broad; anterior margin with four setae in one group in addition to antero-distal group of four setae. Palm convex, defined on outer surface by one strong long corner S-seta accompanied laterally by two L-setae with lateral projections, on inner surface by two short sub-corner R-setae. Dactylus reaches posterior margin of propodus, outer and inner margins of dactylus with one and three simple setae, respectively. Nail length 0.25 times total dactylus length (Fig. 4A).

Coxal plate of gnathopod II with square, ventral margins with four 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, bulge with long setae; carpus 0.61 times basis length and 0.85 times propodus length. Propodus longer than broad; anterior margin with two setae in one group in addition to antero-distal group of three setae. Palm slightly convex, defined on outer surface by one strong, long corner S-seta accompanied laterally by two L-setae with lateral projections, on inner surface by one short sub-corner R-seta. Dactylus reaching posterior margin of propodus, outer and inner margins of dactylus with one and four simple setae, respectively; nail short, 0.28 times total dactylus length (Fig. 5B).

Pereopods: Coxal plate III rectangular, length to width ratio is 1.13: 1; anterio-ventral margin with four setae. Coxal plate IV quadrate, anterio-ventral margin with four setae (Fig. 5A–B). Coxal plate V with posterior lobe, with two setae each on anterior and posterior lobes. Coxal plate VI with anterior lobe, with two simple setae on anterior lobe and one simple seta on posterior lobe. Coxal plate VII with one simple seta on posterior lobe (Fig. 5E).

Pereopod III: IV length ratio is 1.06: 1. Dactylus IV short, dactylus length 0.32 times propodus length, nail shorter than pedestal (Fig. 5A–B). Pereopods V: VI: VII length ratios 1: 1.37: 1.49, respectively. Pereopod VII is 0.61 times the total body length. Pereopod bases V and VII each with four groups of spines along the anterior margins and five and six groups of setae along the posterior margins, respectively. Pereopod basis VI with five groups of spines and seven groups of setae along the posterior and anterior margins, respectively (Fig. 5C–E). Postero-ventral lobe of ischium in pereopods V–VII developed. Ischium, merus and carpus in pereopods V–VII with several groups of spines and setae along the anterior and posterior margins, dactyli of pereopods V–VII with one spine at base of nail on inner margin and one short seta on outer margin in pereopods V–VI. Nail length of pereopod VII 0.40 times the total dactylus length (Fig. 5C–E).

Epimeral Plates: With angular postero-ventral corner, postero-ventral corners of plates I–III posteriorly with three, four and three setae and spines, respectively. Epimeral plates II–III each have two spines along the ventral margins (Fig. 6H).

Pleopoda: Peduncle of pleopods I have three hooked retinacles at distal part of inner margins; peduncles of pleopods II-III each have four hooked retinacles at distal part of inner margins. Peduncle of pleopod III with one seta along outer margin. Rami of pleopods I-III with five to nine articles (Fig. 6A–C).

Urosomites: Urosomites I–II with two and three setae on dorso-lateral margins, respectively. Urosomite III lacks setae. Urosomite I with two spines at base of uropod I.

Uropods: Peduncle of uropod I with seven and one large spines along dorso-lateral and dorso-medial margins, respectively. Outer ramus of uropod I slightly shorter than inner ramus (ratio 1 : 1.02); inner ramus with two groups of two spines laterally and five spines distally; outer ramus with two groups of spines laterally and five spines distally (Fig. 6D). Inner ramus in uropod II longer than outer, both rami with lateral and distal long spines (Fig. 6E). Uropod III long, almost 0.31 times body length. Peduncle of uropod III with five spines, outer ramus bi-articulated, distal article 0.17 times proximal article. Proximal article of outer ramus bearing four and five groups of spines along outer and inner margins, respectively (Fig. 6F); distal article has lateral and distal setae. Inner ramus short, with one distal spine.

Telson: Longer than broad, lobes slightly narrowing; each lobe with three spines distally, with one plumose seta laterally (Fig. 6G).

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

Niphargus chaldoranensis sp. nov.

(Figs. 7–10)

urn:lsid:zoobank.org:act:6FC80139-410C-418D-B5C3-929A95A3F70C

Type locality and Material examined: Holotype, Male specimen (8 mm) from Shoan Spring, Chaldoran

City, West Azerbaijan Province, Iran, coordinates (N39°04'13", E44°09'28"). Specimens collected by M. Mamaghani-Shishvan; 6 Aug 2022. Holotype with three paratypes are stored under catalogue number ZCRU Amph.1602.

Material examined: One male specimen (holotype) and two paratypes from Shoan Spring. Three male specimens were examined from Salmas Spring, close

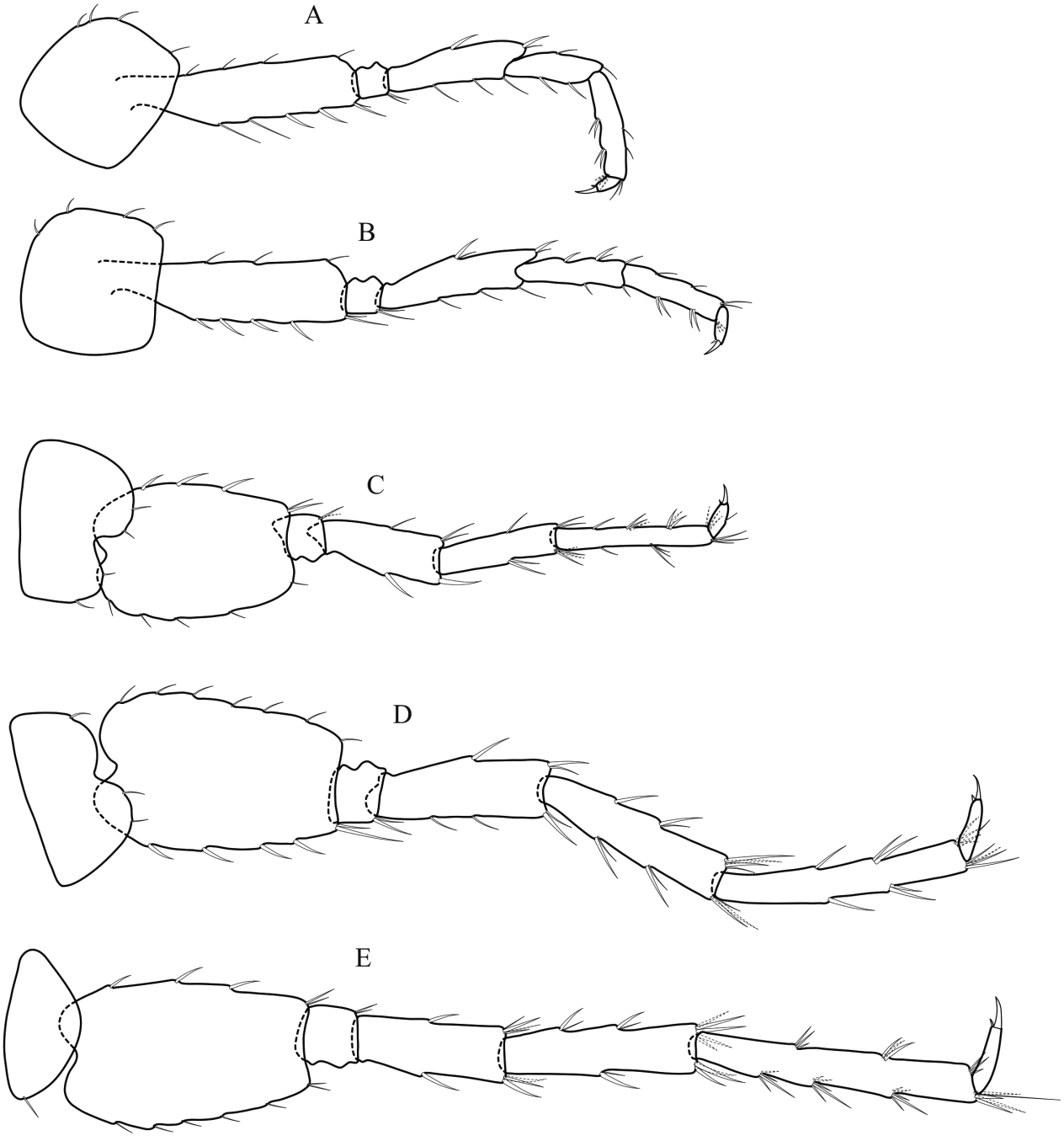

Fig. 5. *Niphargus sahandensis* sp. nov., Hargalan Spring, male 7 mm (holotype). A, Pereopod III; B, Pereopod IV; C, Pereopod V; D, Pereopod VI; E, Pereopod VII. Scale bars = 1 mm.

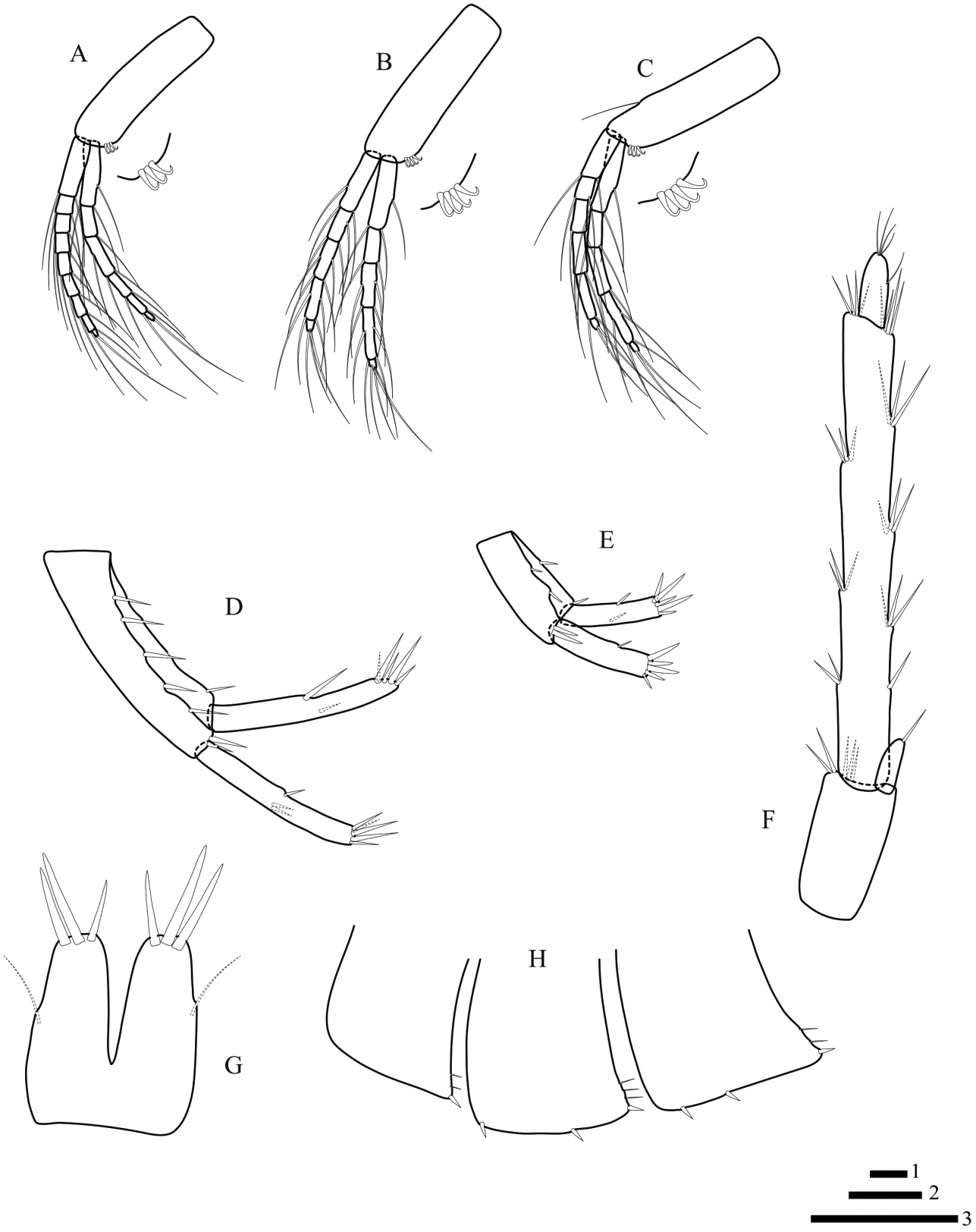

Fig. 6. *Niphargus sahandensis* sp. nov., Hargalan Spring, male 7 mm (holotype). A, Pleopod I; B, Pleopod II; C, Pleopod III; D, Uropod I; E, Uropod II; F, Uropod III; G, Telson; H, Epimeral plates I–III. Scale bars: 1 = 0.5 mm (G–H); 2 = 1 mm (A–E); 3 = 2 mm (F).

to Salmas City, West Azerbaijan Province, Iran, (38°12'48"N, 44°47'32"E); materials were collected by M. Mamaghani in 8 Aug 2022; Salmas materials are stored under catalogue number ZCRU Amph.1605.

Etymology: The name '*chaldoranensis*' refers to the type locality (Chaldoran City) where the species was found.

Diagnosis: At the base of uropod I peduncle with only one spine. Trapezoid shape of propodi in both gnathopods. Maxilla I palp long, reaching beyond the tip of the outer lobe. Dactylus does not reach posterior margin of gnathopod I propodi. Pereopods V and VI with equal length. Pereopod VII propodi 1.5 times the pereopod VI propodi. Pereopod VII 60% of total body length.

Description of holotype: MEASUREMENTS. The total length of the holotype is 8 mm. Head represents 10% of the total body length (Fig. 7C).

Antennae: Antenna I (Fig. 7B) is 0.34 times body length. Peduncular articles 1–3 progressively shorter; length of peduncular article 3 exceeds half of peduncular article 2 (ratio 1.00: 1.58). Main flagellum with 12 articles (most with short setae). Accessory flagellum bi-articulated and 0.5 times the length of article 4 of main flagellum; both articles with two and one setae, respectively. Antenna II (Fig. 7C) with flagellum formed of 7 articles, approximately 0.63 times as long as antenna I. Flagellum length is 0.86 times length of peduncle articles 4+5. Peduncular article 5 of antenna II is longer than article 4 (1.13: 1.00), peduncle articles 4 and 5 each with five groups of setae.

Mouth parts: Labium (Fig. 8C) bi-lobate; with fine setae on tip of outer lobes. Inner plate of maxilla I with two apical setae, outer plate with seven long spines with 3-2-0-2-3-2-0 lateral projections; palp bi-articulated, long and reaching tip of outer lobe, with four apical setae (Fig. 7D). Both plates of maxilla II with numerous long distal and lateral setae (Fig. 8D). Mandible: Left mandible with five teeth on incisor process, between lacinia and molar a row of seven setae with lateral projections (Fig. 7F). Right mandible with five teeth on incisor process, between lacinia and molar a row of six setae with lateral projections (Fig. 7G). Mandibular palp articles 1:2:3 represent 24%, 36% and 39% of total palp length, respectively. Proximal article without setae; second article with five setae along inner margin and third article with one group of two A-setae, two groups of B-setae, no C-setae, 9 D-setae and five E-setae (Fig. 7E). Maxilliped with short inner plate bearing three distal spines intermixed with seven distal setae; outer plate exceeds half of palp article 2, with 8 spines along inner margin and 4 setae distally; maxilliped palp article 3 at outer margin with one proximal and one distal group of long setae; palp terminal article with one seta at outer margin and three setae at base of nail, nail shorter than pedestal (Fig. 8E).

Gnathopods: Coxal plates of gnathopod I slightly greater than those of gnathopod II. Coxa of gnathopod I trapezoid, antero-ventral margins with four marginal 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, bulge with long setae; carpus 0.45 times basis length and 0.70 times propodus length. Propodus of gnathopod I trapezoidal in shape and longer than broad; anterior margin with four setae in one group in addition to an antero-distal group of four setae. Palm convex, defined on outer surface by one strong long corner S-seta accompanied laterally by two L-setae with lateral projections, on inner surface by one short sub-corner R-seta. Dactylus does not reach posterior margin of propodus, outer and inner margins of dactylus with two and three simple setae, respectively. Nail length 0.44 times total dactylus length (Fig. 8A).

Coxal plate of gnathopod II with trapezoid; anterior margins with five setae. Basis with setae on anterior and posterior margins; ischium and merus with posterior groups of setae. Carpus with one group of three setae antero-distally, bulge with long setae; carpus 0.63 times basis length and 0.77 times propodus length. Propodus longer than broad; anterior margin with two setae in one group in addition to an antero-distal group of three setae. Palm slightly convex, defined on outer surface by one strong, long corner S-seta accompanied laterally by two L-setae with lateral projections, on inner surface by one short sub-corner R-seta. Dactylus reaches posterior margin of propodus, outer and inner margins of dactylus with two and four simple setae, respectively; nail long and 0.47 times total dactylus length (Fig. 8B).

Pereopods: Coxal plate III square, length to width equal; antero-ventral margin with four setae. Coxal plate IV rectangular, anterio-ventral margin with four setae (Fig. 9A–B). Coxal plate V with anterior lobe, with one and two setae on posterior and anterior lobes, respectively. Coxal plate VI with anterior lobe, with one simple seta on posterior lobe. Coxal plate VII with one simple seta on posterior lobe (Fig. 9C–E).

Pereopod III: IV length ratio is 1.04: 1 (Fig. 9A–B). Dactylus IV short, dactylus length 0.28 times propodus length, nail shorter than pedestal (Fig. 9B). Pereopods V: VI: VII length ratio 1: 1.007: 1.39. Pereopod VII is 60% total body length. Pereopod bases V–VII each with four groups of spines along anterior margins and six groups of setae along posterior margins, respectively (Fig. 9C–E). Postero-ventral lobe of ischium in pereopods V–VII developed; Ischium, merus and carpus in pereopods V–VII with several groups of spines and setae along

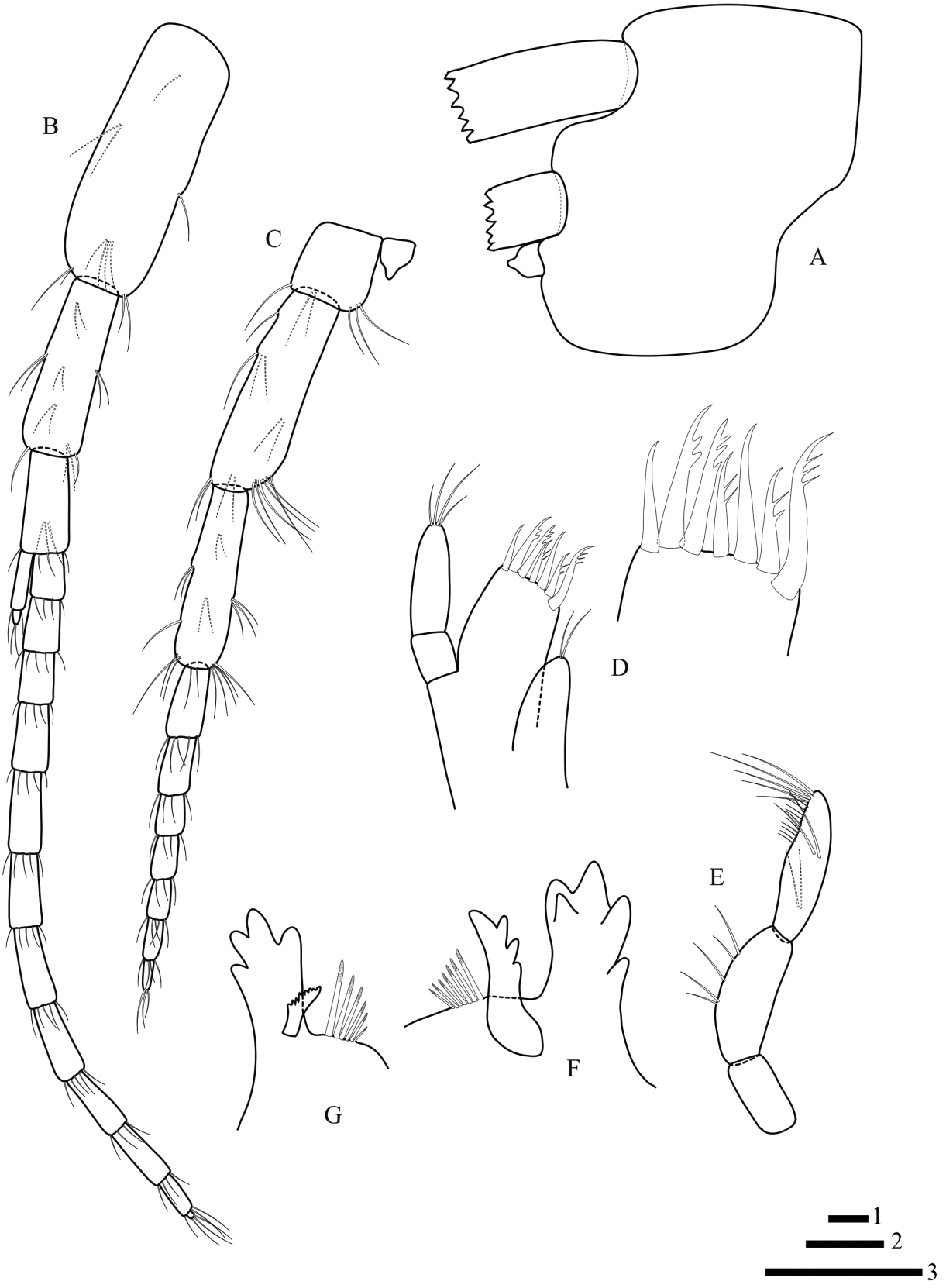

Fig. 7. *Niphargus chaldoranensis* sp. nov., Shoan Spring, male 8 mm (holotype). A, Head; B, Antenna I; C, Antenna II; D, Maxilla I; E, Mandibular palp; F, Left mandible; G, Right mandible. Scale bars: 1 = 0.25 mm (F–G); 2 = 0.5 mm (A, D–E); 3 = 1 mm (B–C).

anterior and posterior margins, dactyli of pereopods V–VII with one spine seta at base of nail on inner margin, and one short seta on outer margin. Nail length of pereopod VII 0.33 times total dactylus length (Fig. 9D–E).

Epimeral Plates I–III (Fig. 10H). Angular postero-ventral corner, postero-ventral corners of plates I–III posteriorly with two, three and three setae and spines, respectively; ventral margin in plates II–III each with two spines.

Pleopods: Peduncles of pleopods I–III with two-hooked retinacles at distal part of inner margins.

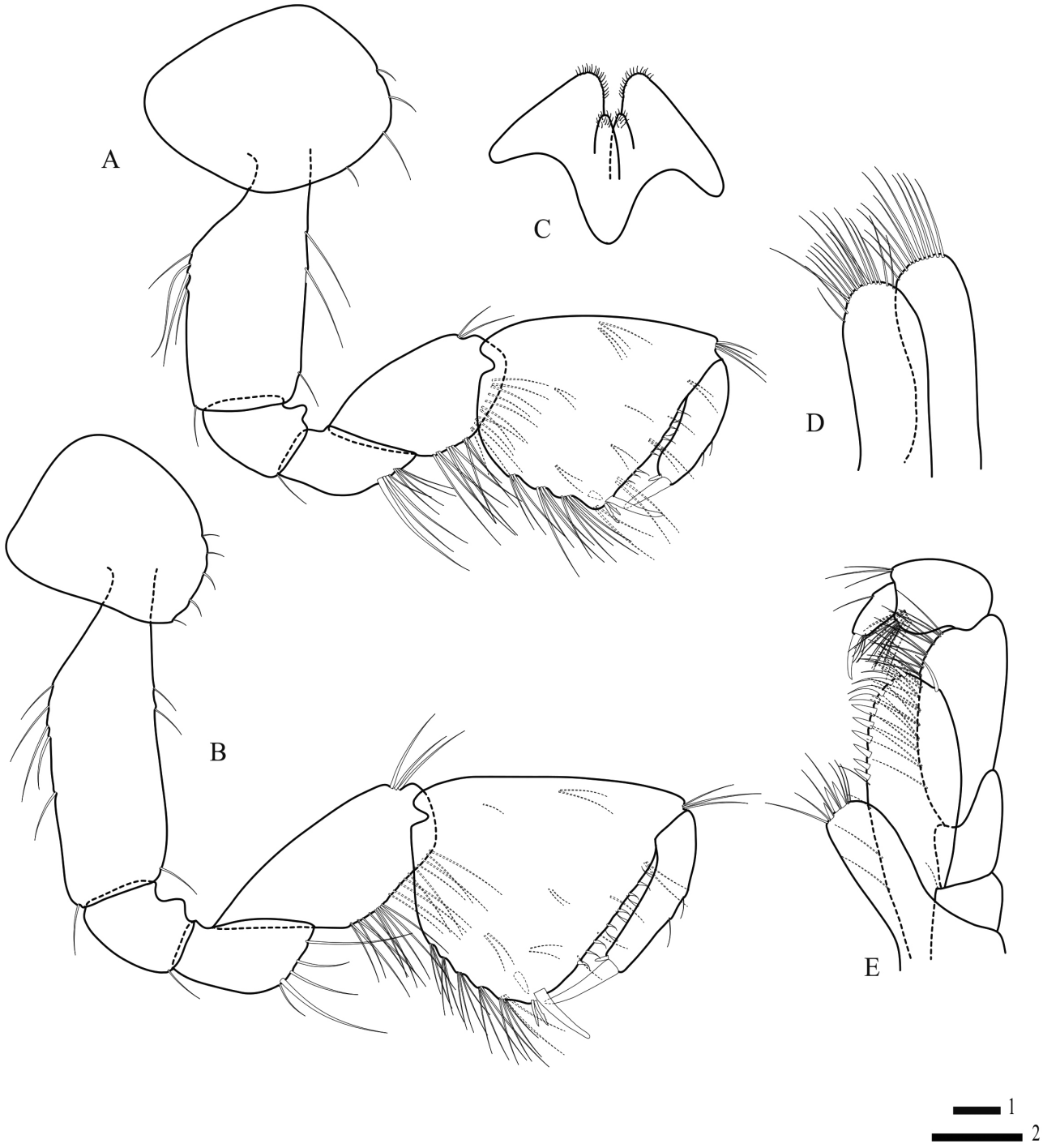

Fig. 8. *Niphargus chaldoranensis* sp. nov., Shoan Spring, male 8 mm (holotype). A, Gnathopod I; B, Gnathopod II; C, Labium; D, Maxilla II; E, Maxilliped. Scale bars: 1 = 0.5 mm (C–D); 2 = 1 mm (A–B, E).

Peduncle of pleopod III with one seta along outer margin. Rami of pleopods I–III with five to nine articles (Fig. 10A–B).

Uropods: Urosomite I with one spine at base of uropod I. Peduncle of uropod I with seven and one large spines along dorso-lateral and dorso-medial margins, respectively. Outer ramus of uropod I shorter than inner ramus; inner ramus with one group of spines laterally and five spines distally; outer ramus with two groups of three spines laterally and five spines distally (Fig. 10D). Inner ramus in uropod II longer than outer, both rami with lateral and distal long spines (Fig. 10E). Uropod

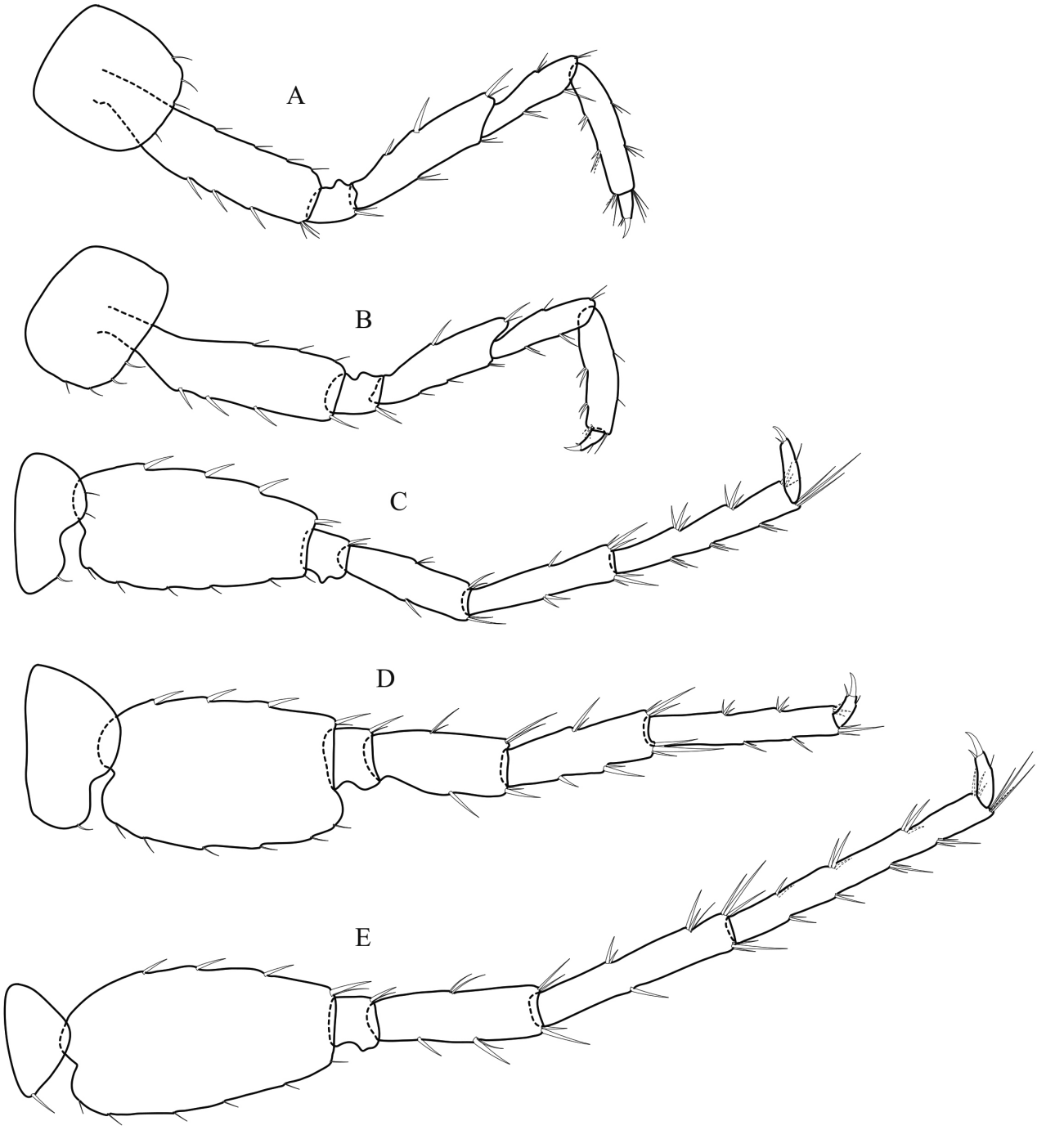

Fig. 9. *Niphargus chaldoranensis* sp. nov., Shoan Spring, male 8 mm (holotype). A, Pereopod III; B, Pereopod IV; C, Pereopod V; D, Pereopod VI; E, Pereopod VII. Scale bars: 1 mm.

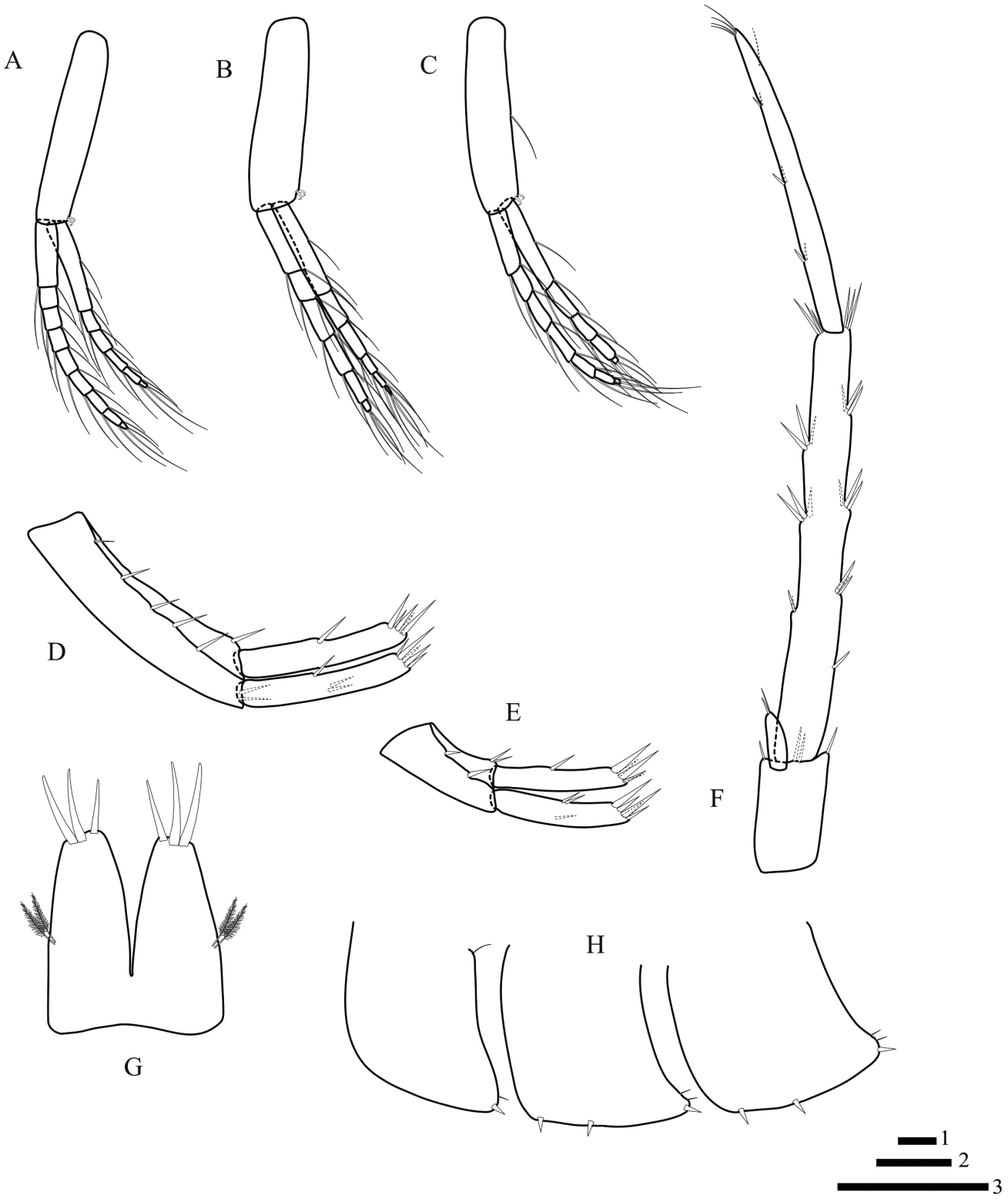

Fig. 10. *Niphargus chaldoranensis* sp. nov., Shoan Spring, male 8 mm (holotype). A, Pleopod I; B, Pleopod II; C, Pleopod III; D, Uropod I; E, Uropod II; F, Uropod III; G, Telson; H, Epimeral plates I–III. Scale bars: 1 = 0.5 mm (G–H); 2 = 1 mm (A–E); 3 = 2 mm (F).

III long, almost 0.36 times body length. Peduncle of uropod III with four spines, outer ramus bi-articulated, distal article 0.76 times proximal article. Proximal article of outer ramus bear four and five groups of setae and spines along inner and outer margins, respectively (Fig. 10F); distal article with four setae distally. Inner ramus short, with one distal spine and one distal seta.

Telson: Longer than broad, lobes slightly narrowing; each lobe with three spines distally and two plumose setae laterally (Fig. 10G).

Interpopulational variation

Although differences between species, however small, can be important, differences between populations of the same species are also significant. A total of six individuals from the two populations of Shoan and Salmas were examined and compared. Many taxonomic traits seem to be stable; however, we observed some differences that may be taxonomically important. In particular, there are notable differences in the number of supporting spines in the palmar corner of gnathopod propodi II (between 1–2), the number of spines with lateral projections on the outer surface in the palmar corner of gnathopod propodi II (between 1–2), the shapes of gnathopod II propodi (rectangular to trapezoid), and the ratio of inner to outer ramus of uropod I (similar size to longer).

DISCUSSION

In this study, three populations of the genus *Niphargus* were collected from northwest Iran and examined using morphological and molecular characteristics. DNA sequences support the species status of two new species, *N. sahandensis* sp. nov. and *N. chaldoranensis* sp. nov. Furthermore, the Bayesian analysis showed that the two new species are phylogenetically distinct, independent and different from all other related species.

N. sahandensis has the most dissimilar *COI* sequence with *N. alisadri* (distance after K2P correction = 23%) and the most similar *COI* sequence with *N. ilamensis* and *N. kurdistanensis* (distance after K2P correction = 17.7%) (Table 1) (Esmaeili-Rineh et al. 2017b; Mamaghani-Shishvan et al. 2017). Differences in nuclear 28S are smaller; *N. sahandensis* sp. nov. is the most dissimilar to *N. hosseiniei* and *N. sarii* (11.12%) and the most similar to *N. daniali* (3.5%) (Esmaeili-Rineh and Sari 2013; Esmaeili-Rineh et al. 2017b; Esmaeili-Rineh et al. 2018).

Up to this study, *N. daniali* was the only species from the Middle East that was nested within the clade with many species from Europe. Our study suggests that *N. sahandensis* sp. nov. is a member of the European clade. Quite expectedly, the newly described species from Hargalan is genetically the most similar to *N. daniali* (Esmaeili-Rineh and Sari 2013).

Morphological examination shows that although both species have similarities in the propodi shape of gnathopods I to II, the ratio of palpus to outer plate in maxilla I, the size of the inner to outer ramus in uropod I, and the ratio of proximal to distal of the outer ramus in uropod III, the new species can be distinguished from *N. daniali* by the absence of a lateral spine and the presence of only three distal spines on the telson (two lateral and four distal spines in *N. daniali*), the absence of setae in the first article of the mandibular palp (two setae in *N daniali*), the presence of more than two hook-like retinacles in the pleopods I–III, and a greater number of supporting spines on the outer surface of the palpus (one spine in *N daniali*) (Esmaeili-Rineh and Sari 2013).

In addition, *N. sahandensis* sp. nov. can be distinguished from *N. ilamensis* by a dactylus that extends to the outer corner of the gnathopod palps, a longer palp compared to the outer plate in maxilla I, and a lower number of supporting spines in gnathopods I and II. Also, *N. sahandensis* sp. nov. is distinguished from *N. kurdistanensis* by having a longer size of the inner to outer ramus ratio in uropod I, the presence of three distal spines, the shape of the propodus of gnathopod I, and the ratio of palp to outer plate in maxilla I (Esmaeili-Rineh et al. 2017b; Mamaghani-Shishvan et al. 2017).

Further results of the analysis of genetic distances showed that *N. chaldoranensis* sp. nov. is the most dissimilar from *N. daniali*, with 22% and 11.93% K2P distances in *COI* and 28S genes, respectively. The lowest genetic distances between *N. chaldoranensis* and any other Iranian species is 11.37% (*COI*) with *N. fiseri* and 2.31% (28S) with *N. alisadri*.

N. fiseri can be distinguished from *N. chaldoranensis* sp. nov. by the maxilla I palp, not reaching the tip of the outer plate in maxilla I, the smaller size of the inner to outer ramus in uropod I (the longer size of inner to outer ramus of uropod I in *N. chaldoranensis*), the presence of one lateral spine on the telson (absence of lateral spines in *N. chaldoranensis*), the presence of two supporting spines in gnathopod I (one supporting spine in *N. chaldoranensis*), and inclined angles in epimeral plates (slightly produced in *N. chaldoranensis*). Additionally, *N. alisadri* is distinguished from the new species by having equal size of the proximal to distal part of the outer ramus in uropod III (distal article 70% proximal article in *N. chaldoranensis*), equal lengths of the palp and outer

plate in maxilla I (The palpus is long and reaches beyond the tip of the outer lobe in *N. chaldoranensis*), and the presence of two lateral spines on the telson (Esmaeili-Rineh and Sari 2013; Mamaghani-Shishvan and Esmaeili-Rineh 2019).

In conclusion, it should be noted that molecular and morphological data indicate that populations of Hargalan and Salmas-Shoan belong to two separate species. The grouping of *N. sahandensis* sp. nov. with *N. daniali* in the European clade suggests a closer evolutionary relationship between these two species and their European counterparts. This finding expands our understanding of the European clade and its diversity by including a new species from the Middle East. It also implies that there may have been historical migrations between the Middle East and Europe, leading to the grouping of these two species in the same clade. Overall, this discovery highlights the importance and need for continuation of research and exploration in understanding the evolution and diversification of species across different regions.

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Authors' contributions: VA, SE and CF designed the study. MM performed the field work and collected samples. MM and SE prepared the figures. VA and SE analyzed the data. VA, SE and CF wrote the 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 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 permit for this study was issued with the approval of the Razi University Ethics Committee under the code number IR.RAZI.REC.1400.018.

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