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Research Article

Niphargus hegmatanensis sp. nov. (Crustacea, Amphipoda, Niphargidae), a new species from subterranean freshwaters of western Iran

Esmaeili-Rineh S.[1]*; Mirghaffari S.A.[1]

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Abstract

A new subterranean amphipod (*N. hegmatanensis* sp. nov.) belonging to the genus *Niphargus* Schiödte, 1849 is described and illustrated. It was collected from Boghati spring in Hamedan Province, Iran. Materials are examined based on the morphological and molecular analyses. Molecular data derived from the nuclear gene 28S rDNA recovered relationships new species. *N. hegmatanensis* sp. nov. is placed within Iranian clade and phylogenetically has most similarity to *N. alisadri*. A relatively equal length of distal to proximal article in uropod III, equal size of rami in uropod I, trapezoid shape of propodi in both gnathopods and the short palpus that does not reach the tip of the outer lobe in maxilla I, are the major characteristics which serve to distinguish the new species from its congeners.

Keywords: *Niphargus hegmatanensis* sp. nov., Taxonomy, 28S rDNA, Boghati spring, Hamedan Province, Iran

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1-Department of Biology, Faculty of Science, Razi University, Kermanshah, Iran.
*Corresponding author’s E-mail: sesmaeili@razi.ac.ir

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Introduction

Niphargus Schiödte, 1849 inhabits in all types of subterranean freshwater habitats and contributes a significant role in the Western Palearctic groundwater diversity. All members within this genus are eyeless and show depigmentation for adaptation to the life in permanent darkness (Trontelj *et al*., 2012). Compared to epigean species, they have poor dispersal ability (Culver and Sket, 2000; Trontelj *et al*., 2009) and most species show small range size and are known only from type localities. Therefore, there are high degrees of endemicity in subterranean regions (Eme *et al*., 2017; Copilaş-Ciocianu *et al*., 2018). Although environment properties including the habitat fragmentations and physical and chemical characteristics potentially influence on dispersal in groundwater resources (Lefébure *et al*., 2007).

Up to this time, Iran has been identified as the easternmost border of *Niphargus* genus (Esmaeili-Rineh *et al*., 2015). The distribution of Iranian niphargids is focused on permanent water reserves along of Zagros and Elburz Mountains. To date, 18 species belonging to this genus have been reported from Iranian freshwaters (Esmaeili-Rineh and Sari, 2013; Esmaeili-Rineh *et al*., 2017a, b); all species except *N. valachicus* are endemic in the country (Karaman, 1998).

In this paper, the results of a recent study on niphargid fauna from Hamedan Province in the west of Iran is presented, and one new species identified in this region is described using morphological and molecular analyses.

Materials and methods

Morphologic and morphometric studies

Three specimens were collected by hand net in Boghati Spring (35°34'22"N,48°36'10"E) in June 16, 2014 near to Damagh City in Hamedan Province (Fig. 1). The materials fixed in 70% and 90% ethanol for morphological and molecular studies, respectively. Then, samples were examined and dissected under a stereomicroscope (LABOMED Lx500). All dissected appendages were mounted on slides and drawn using Zeiss microscope (Primo Star). All materials were deposited at the Zoological Collection, Razi University (ZCRU).

Molecular studies

Genomic DNA was extracted from some appendages (2–3 pereopods) using Tissue Kits (GenNet Bio™) following the manufacturer’s instructions (Seoul, South Korea). We amplified the first fragment of 28S ribosomal DNA using the forward primer recommended 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 (Sinaclon, Iran), 0.2 µL of each primer (10 µM), and 50-100 ng of genomic DNA template.

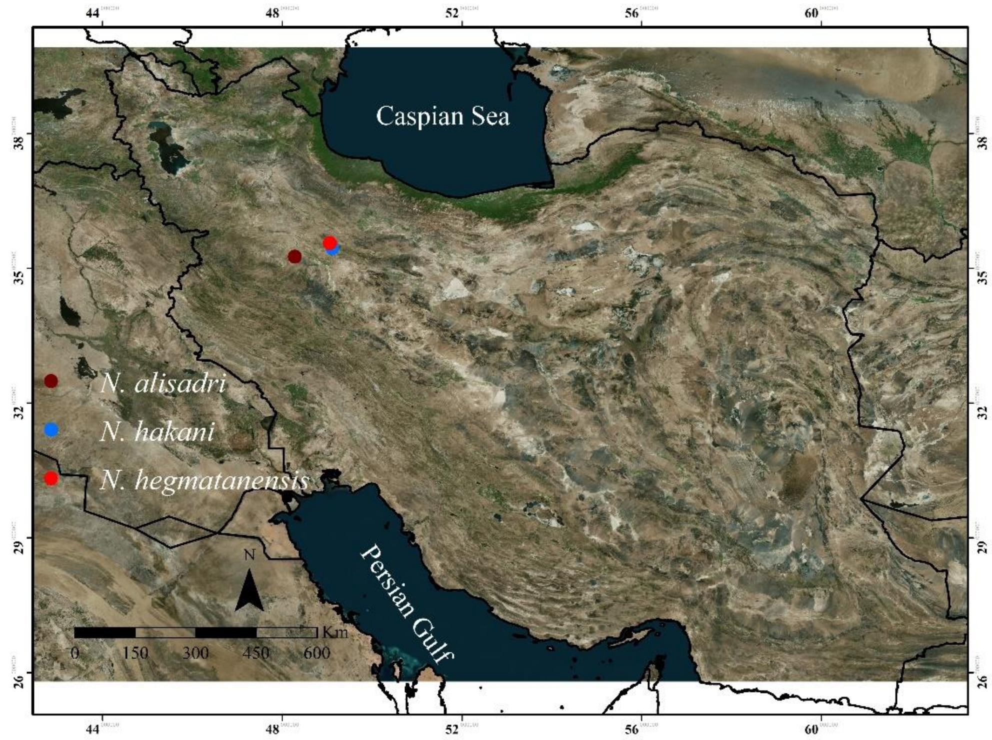

Figure 1: Distribution map of the genus *Niphargus* in Hamedan Province.

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 Macrogen Inc. (Korea). Sequencing was performed with both primers mentioned above. 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.

In order to identify the phylogenetic position of the newly discovered materials, the acquired sequences (with GenBank accession numbers MT446424, and MT446425) were analyzed within the data set of Mamaghani-Shishvan et al. (2017). The NCBI available sequences for three species including *Synurella ambulans*, *Obesogammarus crassus* and *Gammarus fossarum* were used as out-groups (accession numbers: KF719240, KF719242 and KF71924).

Phylogenetic analysis

We compiled a sequence dataset of 23 *Niphargus* species (44 individuals). 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 the trees were sampled every 1000 generations under TIM2+I+G models (jModelTest, version 0.1.1, Posada, 2008). 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. To assess divergence from the other, as already described Iranian species of *Niphargus*, we calculated the corrected genetic distances using Kimura two-parameter (K2P) model (Kimura, 1980) as implemented in MEGA ver. 5 (Tamura *et al*., 2011).

Results

Systematics

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

Niphargus hegmatanensis sp. nov.
urn:lsid:zoobank.org:pub:11269B2B-F062-46B6-8E80-F497B48281B3

Material examined and type locality

Holotype, male specimen (12.5 mm) from Boghati Spring in Orta Ghamish Village, near to Damagh City, Hamedan Province, Iran; (35° 34' 22"N, 48° 36'10" E). Specimens were collected by S. Mirghaffari. Holotype and two paratypes are stored under catalogue number ZCRU Amph.1012 in the Zoological Collection, Razi University, Iran (ZCRU).

Diagnosis

At the base of uropod I only one robust seta was observed. The propodus of gnathopods I and II each have three robust setae with lateral projections on outer surface in palmar corner. The propodi of both gnathopods are trapezoid in shape. The dactylus of gnathopod I cannot reach to the posterior margin of propodus. Inner ramus in uropod I is as long as outer ramus. The palpus of maxilla I is short and does not reach the tip of the outer lobe. The proximal and distal articles in outer ramus of uropod III have equal length. Epimeral plates I–III are not pointed.

Description of holotype

The total length of specimen is 12.5 mm. Body is strong and stout. Head length is 8% of body length (Fig. 2). Antennae I length is almost 0.45 of total body length (Fig. 2A). Peduncular articles 1–3 are progressively shorter; peduncular articles 2:3 in ratio 1.77:1; main flagellum has more than 20 articles; accessory flagellum biarticulated, reaching 1/4 of article 4 of main flagellum, with three simple setae on each articles (Fig. 2A). Peduncular article 4 of antennae II is slightly longer than the article 5, each with nine groups of simple setae; flagellum has 11 articles. Length of flagellum: length of peduncle articles 4 + 5 as 0.72:1(Fig. 2B).

Labium (Fig. 3D) has inner lobes and bears setae on the tip of the lobes. Inner plate of maxilla I has two long

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simple setae; outer plate has seven robust setae with 1-2-2-1-1-3-1 lateral projections; palp biarticulated, shorter than the outer lobe, with three long distal simple set ae (Fig. 2D-E). Both plates of maxilla II bear numerous distal simple setae and four lateral simple setae (Fig. 3E).

Left mandible has pars incisiva with five teeth, lacinia mobilis with four teeth and five setae with lateral projections between lacinia and triturative molar (Fig. 2F). Right mandible with pars incisiva has four teeth, lacinia mobilis with two teeth and six setae with lateral projections between lacinia and triturative molar (Fig. 2G). Mandibular palp articles 1:2:3 ratios as 1:1.45:1.85. The proximal article has no setae, the second article bears eight setae along inner margin and the third article with one group of three A-setae, four groups (two single and two multiple groups) of B-setae, no C-setae, 17 D-setae and five E-setae (Fig. 2H).

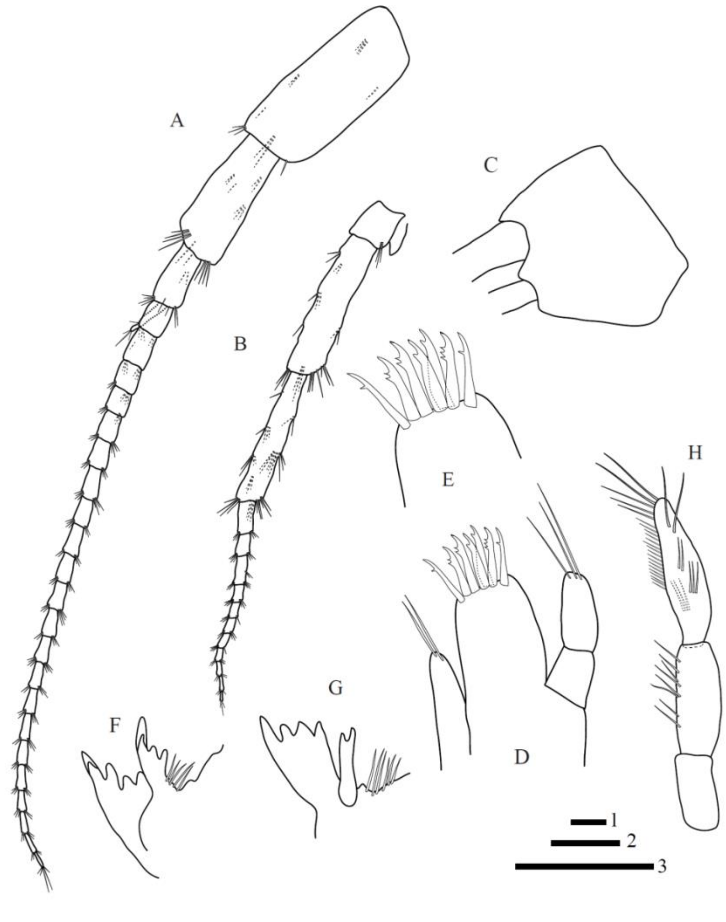

Figure 2: ***Niphargus hegmatanensis*** **sp. nov., male 12.5 mm (holotype, ZCRU Amph.1012). (A) Antenna I; (B) Antenna II; (C) Head; (D-E) Maxilla I; (F) Left mandible; (G) Right mandible; (H) Mandibular palp. Scale bars: 1=0.25 mm (F-G); 2=0.5 mm (C–E, H); 3=1 mm (A-B).**

Maxilliped with short inner plate bears four distal robust setae intermixed with five distal simple setae and three lateral simple setae; outer plate exceeds half of the palp article 2, with 11 robust setae along inner margin and five simple setae distally. Maxilliped palp article 3 bears two groups of 9-11 setae on inner margin and one apical group with five setae; palp terminal article has one simple seta at outer margin and two setae at the base of nail, nail is shorter than the pedestal (Fig. 3C).

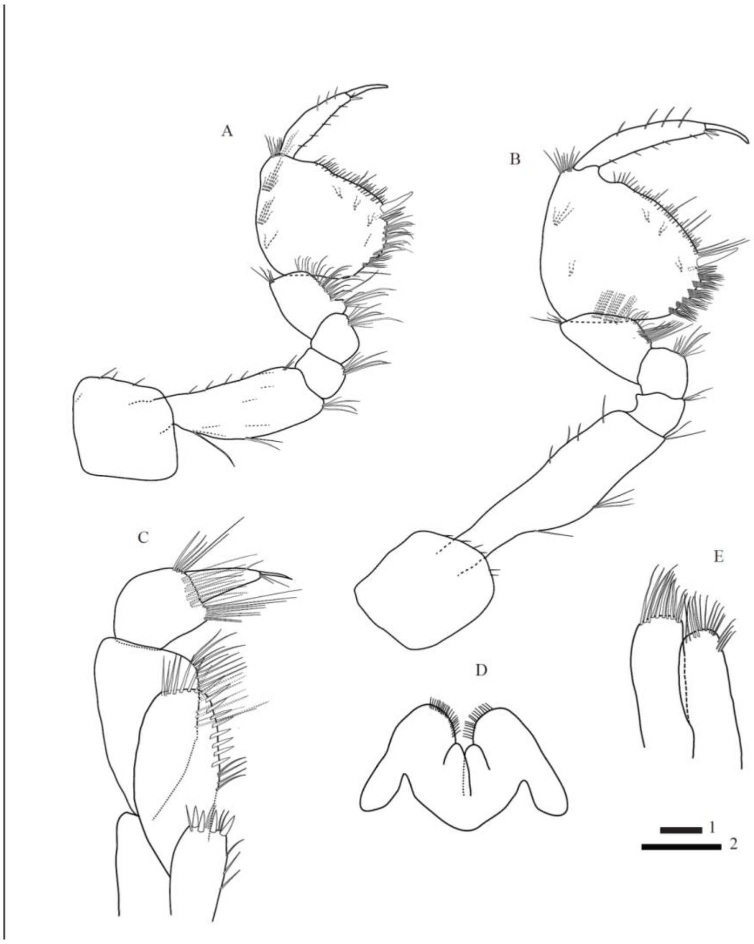

Figure 3: *Niphargus hegmatanensis* sp. nov., male 12.5 mm (holotype, ZCRU Amph.1012). (A) Gnathopod I; (B) Gnathopod II; (C) Maxilliped; (D) Labium; (E) Maxilla II. Scale bars: 1=0.5 mm (C-E); 2=1 mm (A-B).

Coxa of gnathopod I is quadratic, with three setae along ventral margin. Basis bears setae in groups and single setae along anterior and posterior margins; posterior margins of ischium and merus have one posterior group of setae each. Carpus length is 0.4 of basis length and 0.56 propodus length. Carpus has one group of four setae antero-distally, and rows of setae on the posterior bulk. Propodus of gnathopod I is trapezoid shape and broader than long; anterior margin with 12 setae in three groups in addition to antero-distal group of nine simple setae. Palm is convex, with one strong palmar robust seta, one supporting robust seta without lateral projections on inner surface, and three

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robust setae with lateral projections on outer surface; two setae under supporting robust seta in palmar corner. Dactylus does not reach posterior margin of propodus, outer and inner margins of dactylus bear three and five simple setae, respectively. Nail length is 0.45 of total dactylus length (Fig. 3A).

Coxa of gnathopod II is rectangular, longer than broad, ventral margin with six simple setae. Basis bears setae on anterior and posterior margins; ischium and merus bear posterior group of setae. Carpus has one group of three setae antero-distally, a bulge with long simple setae; carpus length is 0.43 of basis length and 0.71 of propodus length. Propodus is broader than long; anterior margin with six setae in two groups in addition to antero-distal group of seven simple setae. Palm is slightly convex, with one strong long palmar robust seta, one short supporting robust seta on inner surface and three robust setae with lateral projections on outer surface. Dactylus reachs posterior margin of propodus, outer and inner dactylar margins bear four and five simple setae, respectively; nail is short, 0.25 of total dactylus length (Fig. 3B). Coxa III is quadrate; anterior, ventral, and posterior margins with a total of 11 simple setae. Coxa IV is quadrate, anterior and ventral margins with a total of eight simple setae, posterior concavity is shallow and approximately 0.1 of coxa width (Fig. 4A–B).

Coxa V has anterior lobe, with five simple setae on anterior lobe. Coxa VI has anterior lobe, with two simple setae on posterior lobe. Coxa VII is half-ovoid, with one simple posterior seta (Fig. 4C–E).

Pereopod III: IV lengths in ratio as 1.05:1 (Fig. 4A- B). Dactylus IV is short, length of dactylus is 0.33 of propodus length, nail is shorter than pedestal (Fig. 4B). Pereopods V: VI: VII length ratios are 1 : 1.32 : 1.37. Pereopod VII length is 0.4 of body length. Pereopod bases V-VII have 7, 10 and 6 groups of robust setae along anterior margins and 11, 14 and 11 simple setae along posterior margins, respectively (Fig. 4C–E). Postero-ventral lobe of ischium in pereopods V–VII is weakly developed. Ischium, merus and carpus in pereopods V–VII have several groups of robust and simple setae along anterior and posterior margins; propodus of pereopod VII is longer than those in V–VI, dactyli of pereopods V–VII bear one robust and one short simple seta at the base of nail on inner margin, nail length of pereopod VII is 0.29 of total dactylus length (Fig. 4C–E).

Pereonites I–VI are without setae. Pereonite VII has one simple seta along dorsal surface. Pleonites I–III are without setae. Epimeral plates I–III (Fig. 5G) include angular postero-ventral corners, anterior and ventral margins convex; postero-ventral margins of plates I–III each have three, four and four robust and simple setae posteriorly, respectively. Epimeral plates II–III each bear three robust setae along the ventral margins. Peduncle of pleopods I–III bear two-hooked

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retinacles. Peduncle of pleopod I has one simple seta along the median margin (Fig. 5A–C); rami of pleopods I–III each bear 10 to 12 articles (Fig. 5A–C).

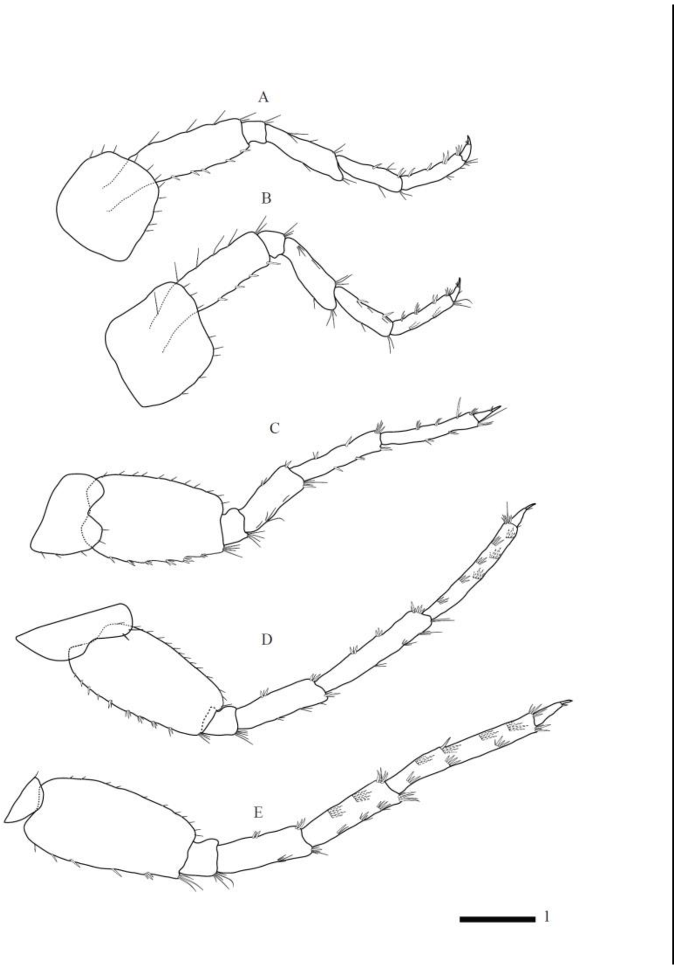

Figure 4: *Niphargus hegmatanensis* sp. nov., male 12.5 mm (holotype, ZCRU Amph.1012). (A) Pereopod III; (B) Pereopod IV; (C) Pereopod V; (D) Pereopod VI; (E) Pereopod VII. Scale bars: 1 mm (A-E).

Urosomites I and III are without setae. Urosomite II bears two robust setae dorso-laterally. Urosomite I bears one robust seta at the base of uropod I. Peduncle of uropod I has five and two large robust setae along dorso-lateral and dorso-medial margins, respectively. Inner ramus of uropod I is slightly shorter than outer ramus (ratio 1: 1.05); inner ramus has five groups of robust setae laterally and five robust setae distally; outer ramus has four groups of seven robust setae laterally and five robust setae distally (Fig. 5D). Inner ramus in uropod II is longer than outer, both rami bear lateral and distal long robust setae (Fig. 5E). Uropod III is long, almost 0.50 of body length. Peduncle of uropod III has six robust setae. Outer ramus bi-articulated, distal article measures slightly as long as proximal article. The proximal article of outer ramus bearing six groups of robust setae along both of the margins (Fig. 5F); distal article bears simple setae laterally and many simple setae

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distally. Inner ramus is short, with two robust distal setae. Telson is two times longer than the broad, lobes are slightly narrow; each lobe has three robust setae distally, with one long robust and two plumose setae laterally (Fig. 5H).

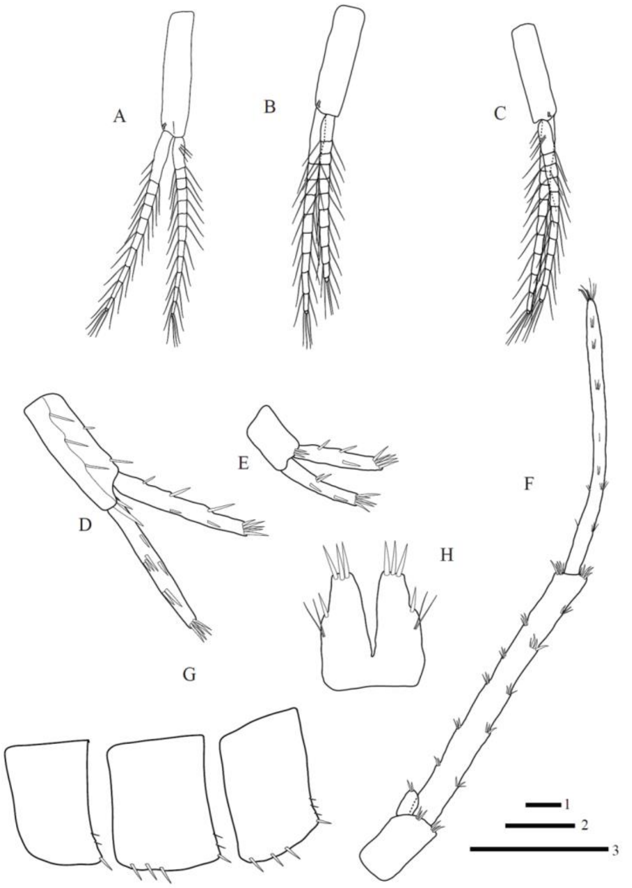

Figure 5: *Niphargus hegmatanensis* sp. nov., male 12.5 mm (holotype, ZCRU Amph.1012). (A) Pleopod I; (B) Pleopod II; (C) Pleopod III; (D) Uropod I; (E) Uropod II; (F) Uropod III; (G) Telson; (H) Epimeral Plates. Scale bars: 1=0.5 mm (G-H); 2=1 mm (A–E); 3=2 mm (F).

Etymology

The name “hegmatanensis” refers to Hegmataneh ancient city on the site of which stands the modern city of Hamedan (Iran). The city was founded in about 678 BC.

Phylogenetic position of materials

The unique haplotype was distinguished based on 810 base pairs of the first fragment of the 28S ribosomal DNA gene from Boghati spring. The topology resulting from Bayesian inference analysis of the dataset (44 individuals) is presented in Fig. 6. New species is nested within the main Iranian clade and shares a common ancestor with the other Iranian species except with *N. daniali.* Pairwise uncorrected Kimura 2-parameter genetic distances between

Niphargus hegmatanensis and all Iranian niphargid species ranged from 0.3–10.1% based on 28S gene (Table 1). New species is the most genetically similar to *N. alisadri* (0.30% K2P) and the most divergent species from *N. daniali*, (10.1% K2P).

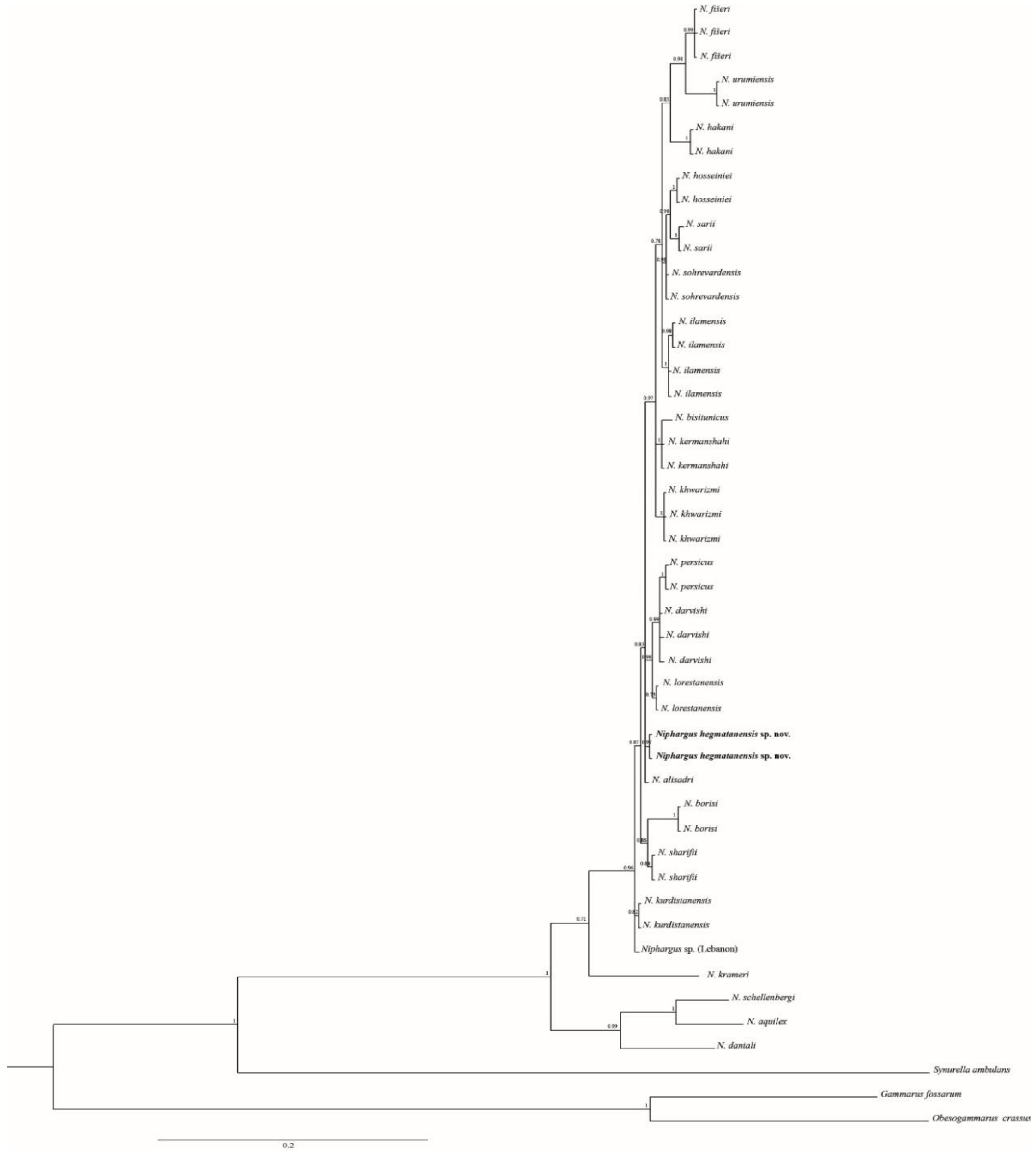

Figure 6: Bayesian consensus tree of 47 samples of *Niphargus* genus (from Esmaeili-Rineh *et al.*, 2015 and Mamaghani-Shishvan *et al.*, 2017), based on the 28S ribosomal DNA sequences. Species are identified and named according to the valid taxonomic description. Posterior probabilities are indicated on main branches.

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Table 1: K2P genetic distances (%) between all Iranian species and Lebanon samples of the genus *Niphargus* based on 28S ribosomal DNA gene.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1: *N. kurdistanensis*																				
2: *N. alisadri*	0.6																			
3: *N. darvishi*	1.1	0.8																		
4: *N. borisi*	2.0	2.2	2.7																	
5: *N. sharifii*	0.8	0.6	1.1	1.8																
6: *N. khwarizmi*	1.4	1.0	1.3	2.6	1.1															
7: *N. bisitunicus*	1.3	1.1	1.4	2.7	1.0	1.1														
8: *N. khayyami*	1.7	1.3	1.5	2.6	1.1	1.0	1.1													
8: *N. sohrevardensis*	1.4	1.0	1.0	2.6	1.1	0.8	0.9	0.8												
10: *N. hosseiniei*	1.7	1.4	1.4	2.8	1.5	1.1	1.3	1.1	0.4											
11: *N. persicus*	1.3	0.9	0.3	2.8	1.3	1.4	1.5	1.7	1.1	1.5										
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									
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								
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				
18: *N. urumiensis*	2.2	2.1	2.3	3.4	1.9	2.2	1.9	2.3	2.1	2.3	2.5	2.3	10.0	2.2	2.5	2.3	1.9			
19: *N. fišeri*	1.7	1.3	1.8	3.0	1.7	1.8	1.7	1.8	1.4	1.5	1.9	1.5	9.8	1.7	1.9	1.8	1.8	1.6		
20: *N. lorestanensis*	1.0	0.4	0.4	2.6	1.0	1.1	1.3	1.4	0.9	1.3	0.5	1.1	9.9	1.0	1.3	1.1	1.9	2.2	1.4	
21: *N. hegmatanensis* sp.nov.	0.6	0.3	0.8	1.9	0.6	1.0	1.1	1.3	1.0	1.4	0.9	1.3	10.1	0.6	1.4	1.0	1.5	2.1	1.5	0.6

Discussion

The materials collected from Boghati Spring proposed new species of the genus *Niphargus*. The DNA sequences confirmed the relative taxonomic position of *N. hegmatanensis*. The Bayesian analysis indicated that the newly described species is phylogenetically distinct from the other relative species. The species described here is distinguished on the basis of an exclusive combination of diagnostic characters.

N. hegmatanensis sp. nov. phylogenetically shows close affinity to *N. alisadri* (Esmaeili-Rineh and Sari, 2013). The geographic distance between two species is about 78 km. Although, two species share several morphological traits including the number of supporting robust setae in the palmar corner of gnathopod I, the number of distal robust setae in telson lobes, the equal length of uropod I rami and the equal length of proximal and distal articles in the outer ramus of uropod III. However, *N. hegmatanensis* sp. nov. differs from *N. alisadri* in a number of features such as the dactylus is not reaching to the posterior margin of gnathopod I propodi, the number of supporting robust setae in inner surface of gnathopod II (1 versus 2 robust setae), the trapezoidal shapes of propodi in both gnathopods, the number of robust setae in outer surface of gnathopods I and II (3 versus 2 robust

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setae), and the outer plate being longer than the palpus in maxilla I.

N. hegmatanensis sp. nov. has the minimum geographic distance (13 km) from *N. hakani*. Genetic distance between two species is 1.5% based on 28srDNA. In addition to molecular divergence, morphological distinctness provides an additional support for separation of two species. New species is diagnosed from *N. hakani* by the shorter size of the palpus to outer plate in maxilla I, the shorter length of outer to inner ramus in uropod I, the equal size of distal to proximal article in uropod III, the absence of robust setae on dorso-lateral margin of urosomite III, and the more number of robust setae with lateral projections on the outer surface of the palmar corners in both gnathopods propodi.

N. hegmatanensis sp. nov., *N. hakani* and *N. alisadri* are present in Namak Lagoon sub basin. Two first species collected from springs and *N. alisadri* found from the lake part of Alisadr Cave (Esmaeili-Rineh and Sari, 2013; Esmaeili-Rineh *et al.*, 2017).

N. kermanshahi has some features in common with *N. hegmatanensis* sp. nov. like length of inner ramus of uropod 1, which is shorter than the outer ramus of uropod 1 and length of palpus of maxilla 1, which is shorter than the outer plate of maxilla 1; but differs from new species by gnathopods propodi much less robust setae with lateral projections on the outer surface of the palmar corners, shorter length of distal to proximal article in uropod III and the more number of supporting robust setae on palmar corner of gnathopod II.

N. sarii shows several morphological similarities to new species in the propodus shape of gnathopods I-II (trapezoidal-shaped), the number of supporting robust setae on the inner surface of the palmar corner in gnathopods I–II propodi (each with one robust seta) and inclined epimeral plates I–III. However, *N. sarii* differs from *N. hegmatanensis* sp. nov. in a number of features such as the absence of lateral robust seta in the each telson lobe, the longer size of the palpus to outer plate in maxilla I, the ornamentation of urosomite I and short dactylus.

N. bisitunicus is diagnosed from new species by the longer size of the palpus to outer plate in maxilla I, the lower number of robust setae with lateral projection on the outer surface of the palmar corner in gnathopods I–II propodi (1 versus 3 robust setae), rectangular-shaped of propodi in gnathopods I–II, the more number of supporting robust setae on the inner surface of the palmar corner in gnathopods I–II propodi (2 versus 1 robust setae) and pointed epimeral plates I–III.

N. daniali is separated from *N. hegmatanensis* sp. nov. by having more distal and lateral robust setae in each telson lobe (4 versus 3), longer size of the palpus to the outer plate in maxilla I, rectangular to quadrate-shaped of gnathopods I–II propodi, and the longer size of the inner to outer ramus in uropod I.

N. loristanensis and *N. hegmatanensis* sp. nov. show some similar morphological features regarding the both gnathopods bear rectangular-shaped propodi. However, *N. loristanensis* is separated from new species by the equal length of the palpus to the outer plate in maxilla I, the absence of lateral robust seta on the telson, the more number of supporting robust setae on the inner surface of the palmar corner in gnathopods I–II propodi, and the ratio of distal to proximal articles of the outer ramus of uropod III.

N. darvishi resembles to new species in the number of supporting robust setae on the inner surface of the palmar corner in gnathopods I–II propodi (each with one robust seta), and the trapezoidal-shaped of propodi in both gnathopods. However, *N. darvishi* is recognizable by longer size of the inner to outer ramus in uropod I, the ratio of distal to proximal article of the outer ramus of uropod

III, the longer size of the palpus to the outer plate in maxilla I, slightly pointed epimeral plates I–III, and lack of lateral robust setae on telson lobes.

N. khwarizmi and *N. hegmatanensis* sp. nov. share several morphological traits in the number of robust seta with lateral projections on the outer surface of the palmar corner in gnathopod I propodi (each with three robust setae), reaching the dactyli at the posterior margin of gnathopods II propodi, and trapezoidal-shaped of propodi in both gnathopods. However, *N. khwarizmi* is distinguished from new species by having two lateral robust setae in each telson lobes, the ratio of distal to proximal articles of the outer ramus of uropod III, longer size of the palpus to the outer plate in maxilla I, and rounded epimeral plates I–III.

In conclusion, our results indicate the presence of the new subterranean amphipod from this karstic spring in Iran based on morphological and molecular characters.

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