

## Molecular systematics of *Haploginglymus*, a genus of subterranean amphipods endemic to the Iberian Peninsula (Amphipoda: Niphargidae)

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### Abstract

The molecular systematics of the subterranean amphipod genus *Haploginglymus* is addressed through the phylogenetic analysis of three DNA gene fragments (nuclear ribosomal 28S and protein-coding Histone 3, plus mitochondrial Cytochrome c Oxidase subunit I). We take advantage of the description of a new species from southern Spain (*Haploginglymus geos* sp. nov.) to assess the singularity of this genus endemic to the Iberian Peninsula and the inclusion of the morphologically aberrant *H. morenoi* within *Haploginglymus*. Our results corroborate the monophyly of the family Niphargidae but shows *Niphargus* to be paraphyletic as it currently stands, with *Haploginglymus* appearing nested within it. A strongly supported sister-group relationship between niphargids and the (thalassoid) pseudoniphargids is recovered as well, but we propose the Niphargidae should continue to be considered as a primary limnic group for biogeographic purposes despite its presumed marine derivation. Our findings are in agreement with previous studies that suggest the family Niphargidae originated in the late Cretaceous in the NE Atlantic, from where it eventually expanded across continental Europe.

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### Introduction

*Haploginglymus* Mateus and Oliveira-Mateus, 1958, is a genus of niphargid amphipod endemic to the fresh inland subterranean waters of the Iberian Peninsula (Spain and Portugal). It is found in caves, wells and the interstitial medium associated to riverbanks, but apparently avoids the brackish waters of coastal aquifers. First discovered in wells from the lower course of river Douro in NW Portugal, the genus displays a broad distribution across the entire Peninsula, where it is one of the most common subterranean crustaceans (Notenboom, 1990). The genus was originally distinguished from *Niphargus* Schiödte, 1849 – by far the largest genus among freshwater amphipods (408 valid species; Lowry, 2010) – based on the display of an unsegmented (vs. 2-segmented) exopod on uropod III. Nevertheless, two additional niphargid genera described later on, both monotypic, viz. *Carinurella* Sket, 1971, and *Niphargobates* Sket, 1981, share with *Haploginglymus* the same condition of uropod III, although both can be readily told apart from *Haploginglymus* based on other features (Sket, 1971; 1981).

Despite its almost generalised distribution across Iberia, only five species of *Haploginglymus* have been

formally described thus far (Mateus and Oliveira-Mateus, 1958; Stock, 1980; Karaman, 1986; Pretus and Sabater, 1990; Iannilli et al., 2009). Aside the morphologically aberrant *H. morenoi* Iannilli, Minelli and Ruffo, 2009, which displays a modified spatulate maxillipedal palp, slender gnathopods and strongly sexually-dimorphic gnathopod II, all show a rather uniform, ordinary *Niphargus*-like aspect. Anyway, molecular analyses under way suggest the genus mirrors the condition described elsewhere for *Niphargus*, where each species displays a very reduced distribution and the few presumed widespread species correspond in fact to complexes of stenochorous cryptic species (Trontelj et al., 2009; Meleg et al., 2013).

As a first step to explore the diversification of *Haploginglymus* in Iberia, we combine herein morphology and molecular traits to describe a new species from southern Spain. We use DNA sequences of three gene fragments (nuclear ribosomal 28S and protein-coding Histone 3, plus mitochondrial Cytochrome c Oxidase subunit I) to assess the distinctiveness of this Iberian genus in front of the rest of niphargids, and the placement of the morphologically aberrant *H. morenoi* within *Haploginglymus*. In addition, we have found the Pseudoniphargidae Karaman, 1993 to be the closest relative to niphargids, which gives a new perspective to the debate on the origin, diversification and biogeography of this extremely diversified family of continental water amphipods.

## Material and methods

### Study site

The new species is known only from phreatic waters found in two caves located at Cerro de Santiago, a Cambrian limestone outcrop within the municipality of Cazalla de la Sierra (Seville, southern Spain). The area is next to Riera de Benalija, a tributary brook to the Guadalquivir River that flows into El Pintado reservoir. The cave lakes are hydrographically connected to the reservoir so that their level follows the annual oscillation of the reservoir's water table. Specimens were found crawling in high numbers on the bottom of the cave lakes and were readily attracted by bait. Some specimens were observed venturing in open water, but most remained associated to the substratum. Direct observations did not suggest the species carried an interstitial life. Crustaceans found accompanying the new species at the time of sampling include *Metahadzia*

*uncispina* Notenboom, 1988; *Salentinella seviliensis* Platvoet, 1987; *Stenasellus escolai* Magniez, 1977; *Microcharon marinus* Chappuis and Delamare, 1954; *Megacyclops brachypus* Kiefer, 1954; *Diacyclops bicuspidatus odessanus* (Shmankevich, 1875); *Cypria ophthalmica* (Jurine, 1820) and a not yet determined harpacticoid copepod. In addition, the bathynellid syncarid *Hexabathynella sevillaensis* Camacho, 2005, was recently described from the same caves (Camacho, 2005).

### Material examined

Specimens were collected directly with a hand-held plankton net in the cave lakes and fixed *in situ* in 95% ethanol. Once in the laboratory, they were dissected in lactic acid under the stereomicroscope, and appendages figured with a Leica DM2500 microscope equipped with Nomarski differential interference contrast and a drawing tube. Body measurements were derived from the sum of the maximum dorsal dimensions of body somites and exclude telson length. Type material is deposited in the invertebrate collection of Naturalis Biodiversity Center, Leiden (RMNH). Abbreviations and nomenclature used in morphological descriptions are as follows: A1 (antennule); A2 (antenna); G1-G2 (gnathopods I and II, respectively); P3-P7 (pereopods III to VII, respectively); and U1-U3 (uropods I to III, respectively).

### DNA isolation, PCR amplification and sequencing

Genomic DNA was purified from 13 specimens corresponding to nine different *Haploginglymus* taxa, viz. the new species described herein, the aberrant *H. morenoi*, and seven not yet formally described species coming from several Iberian locations (v. Table 1 & Fig. 1). In addition, sequences from 14 specimens of *Niphargus* from four different populations from the Iberian western edge of the Pyrenees (Basque Country), as well as eight specimens belonging to six different *Pseudoniphargus* species from the Iberian Peninsula, Portugal and the Canary Islands were also obtained and included in the dataset as potentially close outgroups. DNA extraction was performed using the DNeasy Tissue kit (Qiagen, West Sussex, UK) following the manufacturer's protocol. Elutions were done in 100 µL volume and 1 µL was used in PCR reactions. Three different molecular markers were selected for the study, namely: a partial sequence of the mitochondrial Cytochrome c Oxidase subunit 1 gene (*cox1*; primers LCO1490 and HCO2189; Folmer et al., 1994), a partial sequence of

Table 1. Material included in molecular analyses.

| Taxon   | Locality   |
|---|--|
| <i>Haploginglymus geos</i> sp. nov.                             | Cazalla de la Sierra (Seville; Spain): Cueva del Ocho                  |
| <i>Haploginglymus morenoi</i> Iannilli, Minelli and Ruffo, 2009 | Nonaspe (Zaragoza; Spain): resurgence under bridge at River Matarranya |
| <i>Haploginglymus</i> sp.                                       | Zagrilla la Alta (Córdoba; Spain): Manantial de Zagrilla               |
| <i>Haploginglymus</i> sp.                                       | Priego de Córdoba (Spain): well at C/ Noria 27                         |
| <i>Haploginglymus</i> sp.                                       | Córdoba City (Spain): Arab aqueduct at C/ Cardenal Portocarrero        |
| <i>Haploginglymus</i> sp.                                       | Villaluenga del Rosario (Cádiz; Spain): El Pozo Blanco                 |
| <i>Haploginglymus</i> sp.                                       | Estremenho Massif (Portugal): Gruta da Contenda                        |
| <i>Haploginglymus</i> sp. 1                                     | Nonaspe (Zaragoza; Spain): resurgence under bridge at River Matarranya |
| <i>Haploginglymus</i> sp. 2                                     | Nonaspe (Zaragoza; Spain): resurgence under bridge at River Matarranya |
| <i>Niphargus</i> sp. 1  | Zugarramurdi (Navarra; Spain): Cueva de las Brujas                     |
| <i>Niphargus</i> sp. 2  | Ilardua (Alava; Spain): Manantial de Porraipi                          |
| <i>Niphargus</i> sp. 3  | Axpe (Busturia; Vizcaya; Spain): Cueva de San Pedro                    |
| <i>Niphargus</i> sp. 4  | Andoain (Guipúzcoa; Spain): turbera (peatbog) de Usabeiartza           |
| <i>Pseudoniphargus branchiatus</i>                              | Complejo el Romeral (Antequera, Spain)                                 |
| <i>Pseudoniphargus daviui</i>                                   | Font d'Enciola (Cabrera Island, Spain)                                 |
| <i>Pseudoniphargus gorbeanus</i>                                | Artzegi'ko Koba (Alava, Spain)   |
| <i>Pseudoniphargus portosancti</i>                              | Fonte do Tanque (Madeira, Portugal)                                    |
| <i>Pseudoniphargus</i> sp.                                      | Cueva Udfas (Cantabria, Spain)   |
| <i>Pseudoniphargus</i> sp.                                      | Mina de los Llanetes (Gran Canaria, Spain)                             |

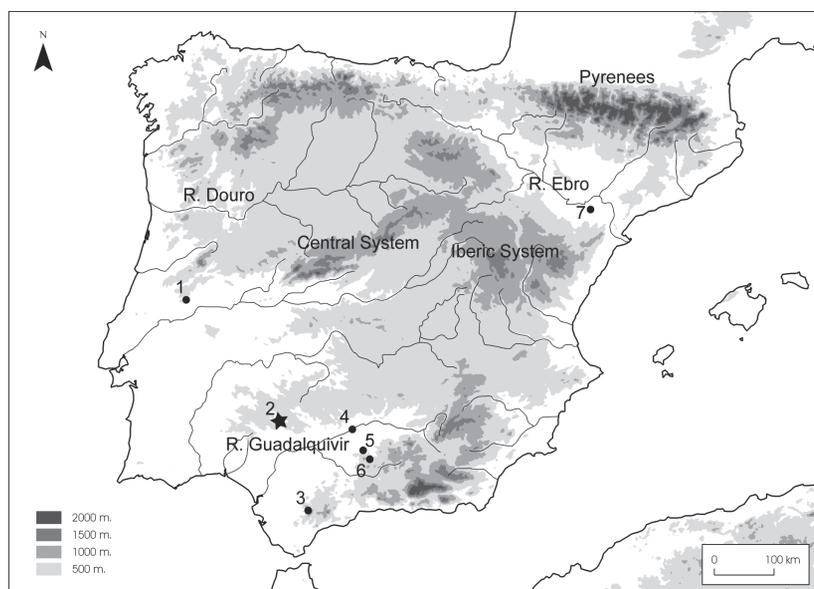


Fig. 1. Map of the Iberian Peninsula showing the location of the *Haploginglymus* samples included in the phylogenetic analysis. The type locality of the new *Haploginglymus* species described herein is marked with a star symbol. 1: Estremenho Massif; 2: Cerro de Santiago; 3: Villaluenga del Rosario; 4: Córdoba; 5: Zagrilla la Alta; 6: Priego de Córdoba; 7: Nonaspe.

the nuclear ribosomal 28S (*LSU*; primers 28S lev2; Verovnik *et al.*, 2005 Zakšek *et al.*, 2007), and a fragment of the nuclear Histone 3 gene (H3; primers H3aF and H3aR; Colgan *et al.*, 1998). PCR conditions included 0.2  $\mu$ M of each primer and 3.5 mM  $MgCl_2$  using a standard protocol of 35 cycles, with annealing temperature ranging from 50 to 45 °C (60s) depending on the sample. Denaturation (94 °C) and elongation (72 °C)

lasted 30 and 60s, respectively. PCR products were inspected by electrophoresis in 1% agarose gel and purified using MSB Spin PCRapace (Invitex, Berlin, Germany). Sanger sequencing was performed with the same primers using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). Sequences were edited and contigs were assembled using CodonCode Aligner (CodonCode Corporation,

Dedham, MA, USA), and deposited at GenBank under the accession numbers referred in Table S1.

### Phylogenetic analyses

The molecular dataset was complemented with sequences retrieved from GenBank corresponding to a selection of 134 *Niphargus* species/populations covering the entire geographical distribution and morphological disparity of the genus, plus four of the nine additional recognized niphargid genera (v. Horton and Lowry, 2013), viz. *Carinurella* Sket, 1971, *Pontoni-phargus* Dancău, 1968, *Niphargobates* Sket, 1981 and *Niphargellus* Schellenberg, 1938. We completed the dataset with a series of more distant outgroups in the families Gammaridae (6 genera), Gammaracanthidae (1), Eulimnogammaridae (1), Acanthogammaridae (1), Pallaseidae (1), Pontogammaridae (1), Anisogammaridae (1) and Crangonyctidae (3). We rooted the tree with a phoxocephalid and an urothoid, in accord with the most recent hypothesis on amphipod supra-family-level relationships (Lowry and Myers, 2013). Data on all sequences and taxa used are shown in Table S1.

Multiple sequence alignment was performed using MAFFT 7 online version (<http://mafft.cbrc.jp/alignment/server/>; Katoh and Standley, 2013), using the default FFT-NS-1 algorithm for the *cox1* and H3 datasets, and the Q-INS-i option for the LSU sequences since it considers the secondary structure of the RNA sequences. In order to evaluate the effect on the topology of the indels in the LSU alignment, an additional LSU matrix was generated by removing ambiguously aligned regions with Gblocks v. 0.91b (Talavera and Castresana, 2007), and specifying a minimum length of two positions for a block and selecting only positions with a gap in less than 50% of the sequences if they are within an appropriate block. Nucleotide substitution saturation was assessed with the Xia's test (Xia *et al.*, 2003) implemented in DAMBE v. 5.2.64 (Xia, 2013). Since evidence of substitution saturation was detected on third coding positions of the *cox1* alignment (see Results), we also performed the phylogenetic analyses by excluding these characters from the dataset. Genetic divergence among *Haploginglymus* taxa was estimated through the calculation of the uncorrected pairwise *cox1* distances (p-distance) in MEGA7 (Kumar *et al.*, 2016). In order to test the phylogenetic congruence among the three molecular markers, single-gene trees were inferred and the resulting topologies were compared. Optimal partitioning strategy and evolutionary models for each alignment were assessed with Parti-

tionFinder (Lanfear *et al.*, 2012) under the Bayesian Information Criterion (BIC), whereas the phylogenetic inference was conducted using IQTREE multicore v. 1.3.12 (Nguyen *et al.*, 2015) performing 1000 ultrafast bootstrap approximation replicates. Finally, a phylogenetic analysis of the three molecular markers combined was independently carried out under both a maximum likelihood and a Bayesian framework using IQTREE and MrBayes 3.2 (Ronquist *et al.*, 2012), respectively. For the latter, two independent analyses consisting of four chains each were run for  $5 \cdot 10^7$  generations specifying a sampling frequency every 1000 generations, and setting up a burn-in fraction of 35%. MCMC convergence and effective sample size (ESS) estimates were checked with TRACER v. 1.6 (Rambaut *et al.*, 2013).

### Results

Sequence statistics of the for the studied DNA datasets and their best partitioning and model selection schemes are shown in Tables 2 and 3, respectively. The topologies derived from the analyses performed both separately on each individual marker (Figs S2-S4) and on their concatenation (Figs 2; 3; v. Figs S5 & S6 for a more detailed version of these trees) were consistent in showing the same evolutionary relationship between *Haploginglymus* and the rest of niphargid taxa. In all cases, all members of *Haploginglymus* were recovered as a monophyletic lineage nested within a clade that also included *Niphargus*, *Pontoni-phargus*, *Carinurella* and *Niphargobates*. In turn, this paraphyletic clade appeared as sister to the members of the family Pseudoniphargidae. Although Xia's test showed low levels of substitution saturation except for third coding positions of the *cox1* alignment (ISS=1.01, ISSc=0.689, P two-tailed < 0.00001), the exclusion of the latter from the alignment did not affect the above-mentioned phylogenetic relationships, although nodal support was generally lower (Fig S7), suggesting third positions contributed phylogenetic signal rather than noise. Seemingly, the exclusion of ambiguous positions in the LSU alignment (58% of the alignment positions were removed using Gblocks) did not affect the relationships among the main lineages (Fig S8).

As regard the main phylogenetic relationships among the nine *Haploginglymus* samples (Figs 2; 3; node A), two main lineages could be established. One of them (node B) corresponded to the three species from North Iberia, while the other clade (node C) embraced the species from the southern half of the Peninsula. The

latter includes three monophyletic lineages corresponding to the Portuguese taxon (node D), the new species from Cazalla de la Sierra described herein (see Appendix section; Node E), and the three species collected at the Subbaetic System (node F), respectively. The genetic divergence among *Haploginglymus* lineages – measured as uncorrected pairwise *cox1* p-distance – is shown in Table 4.

## Discussion

### On the genus *Haploginglymus*

Our phylogenetic analysis corroborates the monophyly of the family Niphargidae but shows *Niphargus* to be paraphyletic as it currently stands (Figs 2; 3). The paraphyly of *Niphargus* was already suggested by Sket (1981) and implicitly recognized by Cene Fišer and co-workers, who in different molecular phylogenetic papers (Fišer *et al.*, 2008; Hekmatara *et al.*, 2013; Švara *et al.*, 2015; Esmacili-Rineh *et al.*, 2015; Brad *et al.*, 2015) showed *Carinurella*, *Pontoniphargus*, *Niphargobates* and *Niphargellus* nested within that genus. In our analysis, *Haploginglymus* appears also nested within *Niphargus* conforming a robustly supported monophyletic group. This clade is sister to another one conformed by the English *Niphargellus glenniei* (Spooner, 1952) and the Irish *Niphargus irlandicus* Schellenberg, 1932. These two taxa were already recovered as the most basal niphargids in the analyses performed by McInerney *et al.* (2014) and Brad *et al.* (2015). The retention of *Haploginglymus* as a valid genus in this context is thus implausible, especially taking into consideration that its presumed more remarkable diagnostic trait (the unsegmented U3 exopod) is shared also by the monotypic genera *Niphargobates* and *Carinurella*. Nevertheless, the former two genera differ remarkably from *Haploginglymus* in many relevant features, in accord with their peculiar life-styles.

In any event, our analysis recovers all *Haploginglymus* species conforming a clade geographically restricted to the Iberian Peninsula, where it replaces the rest of niphargids except for two small disjunct areas on both edges of the Pyrenees (Notenboom, 1990). As in *Niphargus s.l.*, this monophylum includes some morphologically aberrant members such as *H. morenoi* that, if it were not because it displays an unsegmented U3, it would deserve that a new genus was erected to accommodate it. The potential for morphological disparity in *Haploginglymus* is thus as high as in *Niphar-*

*gus s. l.*, and would suggest a long, separate evolutionary history between both lineages, enabling *Haploginglymus* to colonise and adapt to even the most demanding subterranean niches in Iberia. Considering all these pieces of evidence and that *Niphargus* already accounts with more than 400 valid species, we opt here for the retention of *Haploginglymus* as a valid genus. As regard *Haploginglymus morenoi* and its aberrant morphology, our results fully agree with Iannilli *et al.* (2009) in considering it as a highly modified member of the genus with a peculiar, interstitial lifestyle.

### Origin, intra-generic relationships and biogeography

*Haploginglymus* is endemic to the Iberian Peninsula, where it displays an almost generalised distribution in fresh subterranean waters and is the only niphargid known unless for two disjunct areas on both edges of the Pyrenees, where *Niphargus* also occurs (Notenboom, 1990). In those areas, the orography is less pronounced than at the central Pyrenean sector, to the point of enabling the local trans-Pyrenean colonization by *Niphargus*. The latter genus is broadly distributed across the rest of Europe and the Middle East (Esmacili-Rineh *et al.*, 2015), and seems to have colonised the two foregoing Spanish regions quite recently, apparently after the establishment of *Haploginglymus* in Iberia. Thus, at least three of the four *Niphargus* species recorded in Catalonia display a trans-Pyrenean distribution (Karaman, 2015a; b), whereas those from the Basque Country conform a derived monophyletic group within the niphargid tree that appears to be unrelated to *Haploginglymus* (v. Fig. 2). Thus, the rise of the Pyrenees with its current structural organisation already at the Paleocene/Eocene boundary, 55–47 Myr ago (Vergés *et al.*, 2002) might have played a major role in the differentiation of *Haploginglymus* from the rest of niphargids, with this age representing a *terminus post quem* for its origin (Fig. 1).

The nine *Haploginglymus* samples included in our analysis conform two main monophyletic groups (Figs 2; 3; nodes B and C). One (node B) appears associated to the River Ebro and its tributaries, and comprises the three species recorded at River Matarranya, of which only *H. morenoi* has been formally described thus far. The second group (node C) is distributed across the southern half of the Iberian Peninsula and comprises the rest of taxa. The genetic divergence among these *Haploginglymus* lineages is high, as deduced from the observed values of uncorrected pairwise *cox1* p-distances (Table 4). With the caveat that DNA sequences

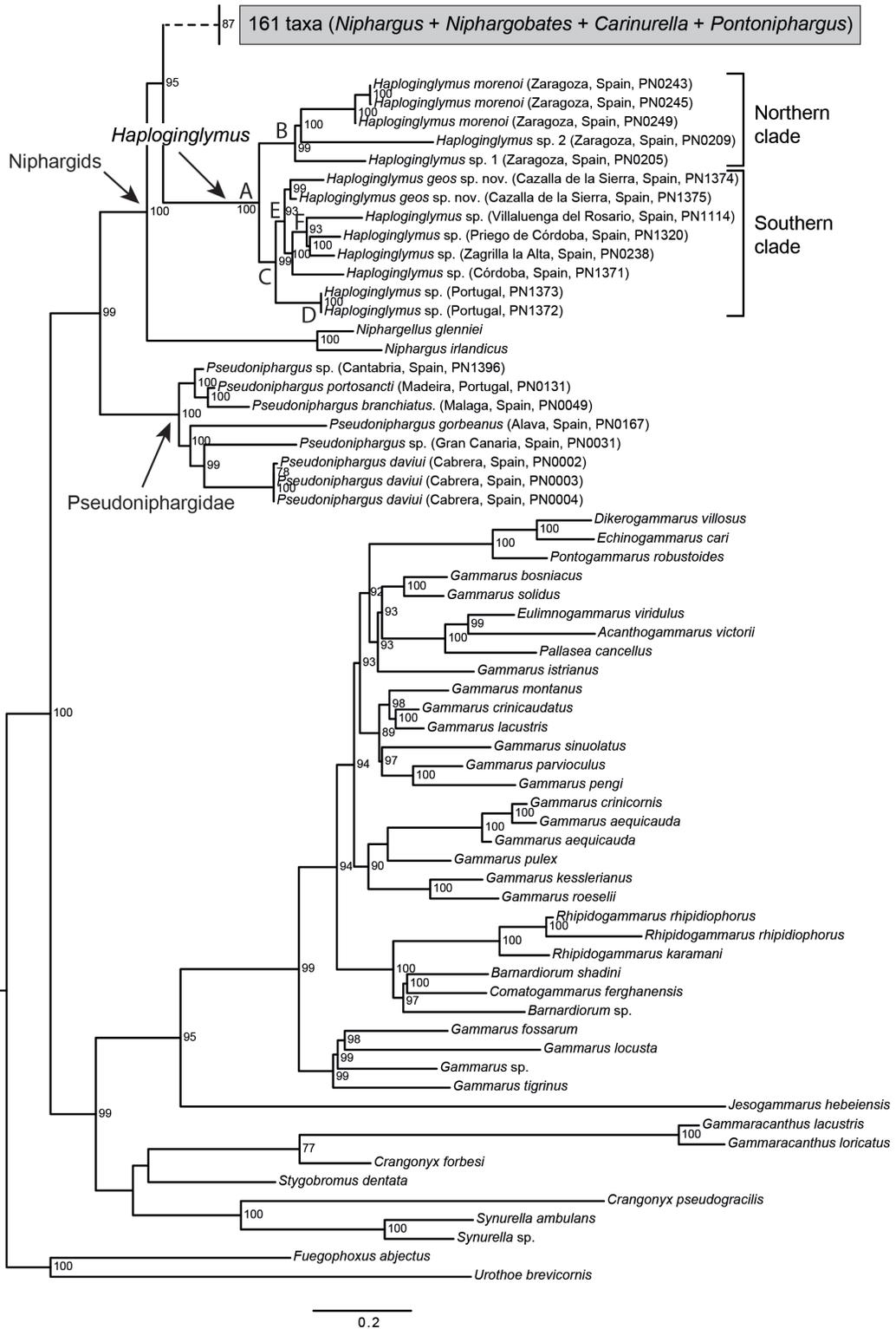


Fig. 2. Maximum likelihood phylogenetic tree based on the combined analysis of *cox1*, H3 and LSU. Node numbers represent statistical bootstrap support (only values  $\geq 70$  are shown). Nodes in the *Haploginglymus* clade have been labelled for discussion in the text. See Supplementary Figure S5 for a more detailed version of this tree.

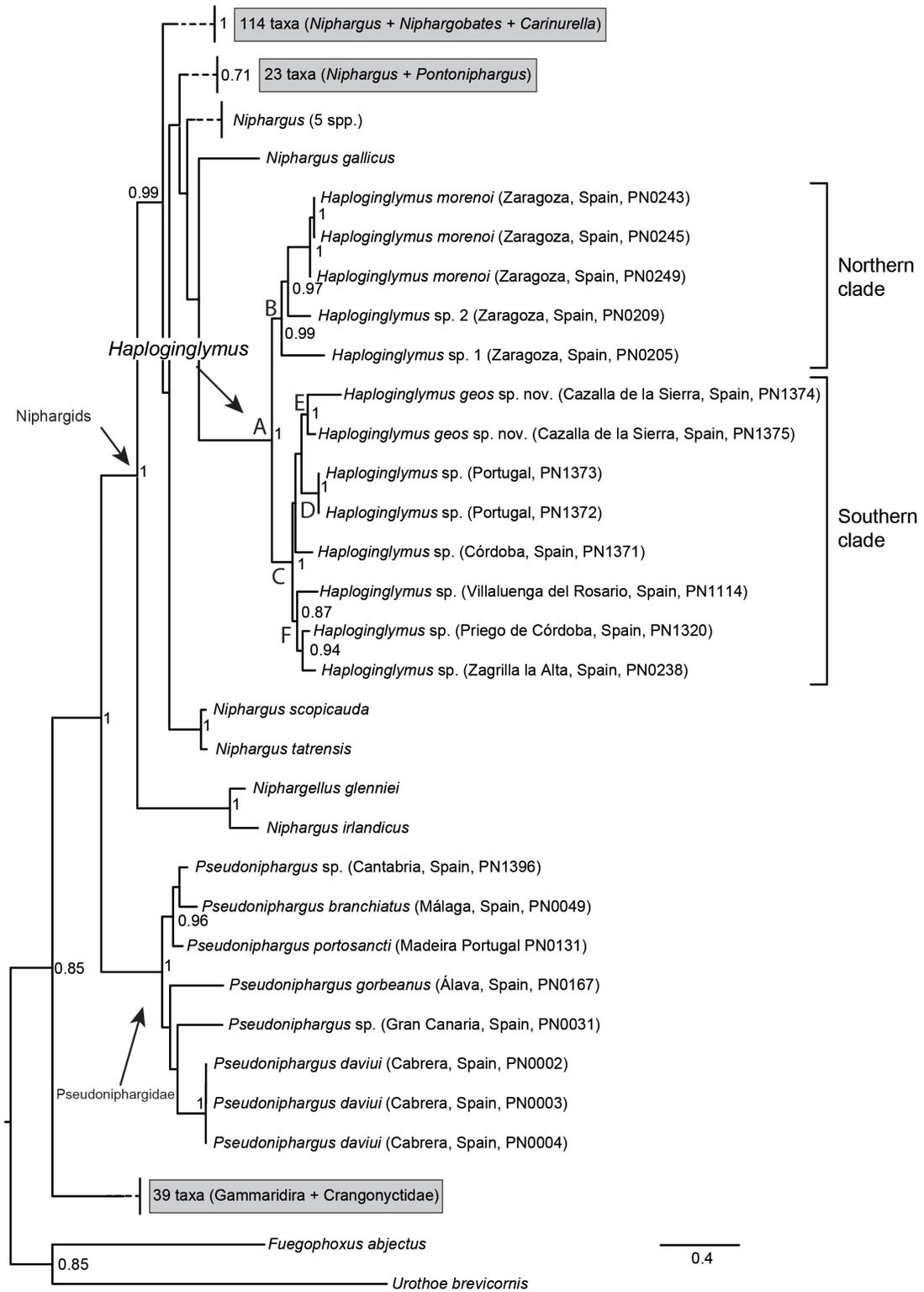


Fig. 3. Bayesian phylogenetic tree based on the combined analysis of *cox1*, H3 and LSU. Node numbers represent posterior probabilities (only values  $\geq 0.70$  are shown). Nodes in the *Haploginglymus* clade have been labelled for discussion in the text. See Supplementary Figure S6 for a more detailed version of this tree.

Table 2. DNA datasets sequence statistics (cod. pos. = coding position; seq = sequence; bp = base pairs; stdev. = standard deviation).

| Dataset                               | N. of seqs. | Seq. length (bp)<br>(mean, stdev.) | Alignment<br>length (bp) | Informative<br>positions (%) | A+T (%) |
|---------------------------------------|-------------|------------------------------------|--------------------------|------------------------------|---------|
| <i>cox1</i>                           | 170         | 155-621 (572.4, 95.7)              | 621                      | 53.3                         | 64.3    |
| <i>cox1</i> (no cod. pos. 3)          | 170         | 104-414 (381.7, 63.7)              | 414                      | 30.2                         | 56.5    |
| LSU                                   | 191         | 436-921 (816.9, 89.8)              | 1721                     | 43.9                         | 42.8    |
| LSU (reduced with Gblocks)            | 191         | 359-724                            | 724                      | 60.5                         | 42.1    |
| H3                                    | 143         | 209-330 (323.4, 21.1)              | 330                      | 31.5                         | 42.8    |
| Combined                              | 210         | –                                  | 2672                     | 44.57                        | 49.8    |
| Combined (no <i>cox1</i> cod. pos. 3) | 210         | –                                  | 2465                     | 39.96                        | 46.1    |

Table 3. Results of partition and model selection analyses carried out in PartitionFinder (cod. pos. = coding position).

| Dataset                                      | Number of subsets | Partitioning scheme   | Substitution model |
|--|-------------------|---|--------------------|
| <i>cox1</i>                                  | 1                 | unpartitioned   | GTR+I+G            |
| <i>cox1</i> (excluding cod. pos. 3)          | 1                 | unpartitioned   | SYM+I+G            |
| LSU  | 1                 | unpartitioned   | GTR+I+G            |
| LSU (reduced with Gblocks)                   | 1                 | unpartitioned   | GTR+I+G            |
| H3   | 1                 | unpartitioned   | K80+I+G            |
| Combined                                     | 2                 | LSU+H3+ <i>cox1</i> cod. pos. 1+ <i>cox1</i> cod. pos. 2<br><i>cox1</i> cod. pos. 3 | GTR+I+G<br>GTR+I+G |
| Combined (excluding <i>cox1</i> cod. pos. 3) | 1                 | unpartitioned   | GTR+I+G            |

from representatives of other parts of the northern sector of the Peninsula are lacking, it is feasible to relate this primary subdivision of the genus to the uplift during the Tertiary Alpine orogeny of two intra-Peninsular mountain ranges: The Central System (that separates the Duero and Tajo river basins) and the Iberian System (that separates the Ebro river basin from the rest of the Peninsula) (v. Fig. 1).

#### Age and origin of the *Niphargidae*

*Niphargids* are almost completely bound to subterranean waters except for a few species recorded from surface habitats or the bottom of deep lakes (Karaman and Ruffo, 1986). The family occurs only in the Palaearctic west of the Caspian Sea and currently comprises ten valid genera, of which *Niphargus*, with 408 species, is by far the most species-rich. The rest embraces only 16 species (Horton and Lowry, 2013). Only *Haploginglymus* is allopatric with respect to the rest of *niphargids*, although it overlaps with *Niphargus* (exceptionally co-existing with it) in two small disjunct areas on both edges of the Pyrenees (Pretus and Sabater, 1990; Notenboom, 1991; Karaman, 2015a; b).

Karaman and Ruffo (1986) suggested that the diversification of the family began in the basins of the Para-

tethys Sea during the Tertiary Period, from which European fresh waters were subsequently colonised. But the discovery of casts of *niphargids* in Late Eocene Baltic amber (*ca.* 45-50 Myr old; Coleman and Myers, 2001; Coleman and Ruffo, 2002; Jażdżewski and Kupryjanowicz, 2010) rules out this hypothesis since the birth of Parathethys as an enclosed basin with reduced salinity and endemic faunas took place only afterwards, at the early Oligocene at most (Rögl, 1997). McInerney *et al.* (2014) have attributed a much older, late Cretaceous age to the family, and an origin in NW Europe rather than in the Balkan area followed by a gradual range expansion across central Europe to reach its current distribution range. A third, alternative scenario where *niphargids* colonized freshwaters directly from the sea multiple times independently during their evolutionary history, and where the lineage of *Haploginglymus* settled in Iberia when it was an island during the late Cretaceous is not favored here since, if this were the case: (1) it is hardly conceivable that *Niphargus* was not established throughout the entire Peninsula when it is present in French territories adjacent to the Atlantic and the Mediterranean, and *vice-versa*, that *Haploginglymus* is not present in France; (2) there are no *niphargids* on North Atlantic oceanic islands, nor on Mediterranean islands such as the Balearics, discon-

Table 4. Uncorrected pairwise *cox1* distances (p-distance) among *Haploginglymus* taxa.

|    | 1     | 2     | 3     | 4     | 5     | 6     | 7     | 8     | 9     | 10    | 11 |
|----|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|----|
| 2  | 0.231 |       |       |       |       |       |       |       |       |       |    |
| 3  | 0.209 | 0.209 |       |       |       |       |       |       |       |       |    |
| 4  | 0.209 | 0.209 | 0.004 |       |       |       |       |       |       |       |    |
| 5  | 0.212 | 0.212 | 0.004 | 0.007 |       |       |       |       |       |       |    |
| 6  | 0.208 | 0.227 | 0.201 | 0.201 | 0.195 |       |       |       |       |       |    |
| 7  | 0.194 | 0.198 | 0.179 | 0.179 | 0.183 | 0.110 |       |       |       |       |    |
| 8  | 0.211 | 0.194 | 0.212 | 0.212 | 0.212 | 0.182 | 0.161 |       |       |       |    |
| 9  | 0.213 | 0.198 | 0.209 | 0.209 | 0.209 | 0.149 | 0.144 | 0.167 |       |       |    |
| 10 | 0.233 | 0.223 | 0.201 | 0.201 | 0.196 | 0.162 | 0.151 | 0.189 | 0.164 |       |    |
| 11 | 0.231 | 0.218 | 0.199 | 0.199 | 0.199 | 0.148 | 0.135 | 0.212 | 0.179 | 0.000 |    |

1: *Haploginglymus* sp. 1 (PN0205, Zaragoza, Spain)

2: *Haploginglymus* sp. 2 (PN0209, Zaragoza, Spain)

3: *Haploginglymus morenoi* (PN0243, Zaragoza, Spain)

4: *Haploginglymus morenoi* (PN0245, Zaragoza, Spain)

5: *Haploginglymus morenoi* (PN0249, Zaragoza, Spain)

6: *Haploginglymus* sp. (PN0238, Zagrilla la Alta, Spain)

7: *Haploginglymus* sp. (PN1320, Priego de Córdoba, Spain)

8: *Haploginglymus* sp. (PN1371, Córdoba, Spain)

9: *Haploginglymus* sp. (PN1114, Villaluenga del Rosario, Spain)

10: *Haploginglymus* sp. (PN1372, Estremenho Massif, Portugal)

11: *Haploginglymus* sp. (PN1373, Estremenho Massif, Portugal)

nected from the continents at least since the end of the Oligocene, but where ulterior marine transgressive pulses have constrained severely the extension of emerged land; (3) the distribution of the family is not limited to areas covered by epicontinental seas in the geological past (Notenboom, 1991); and (4) brackish water species are exceptional among members of the family (Notenboom, 1991).

Our study reveals a strongly supported sister relationship between niphargids and pseudoniphargids that deserves a comment since it is relevant for the origin and biogeography of the Niphargidae. Contrary to niphargids, which are mainly limnic and are present on territories not formerly occupied by the sea, the Pseudoniphargidae are undeniably thalassoid, being present even on oceanic islands that have never been connected to the continents (Bermuda, Canaries, Madeira, Azores; Stock *et al.*, 1986; Stock, 1988; Stock and Abreu, 1992; Stock, 1980). The study of the phylogenetic relationships among members of the family based on both morphological (Notenboom, 1988) and molecular features (analyses currently under way in our lab) has led to the identification of a monophyletic cluster of species of *Pseudoniphargus* Chevreux, 1901 cantoned on the western edge of the Pyrennees – Basque Country, bordering the Gulf of Biscay – as the most primitive lineage within the family. Remarkably, the most primitive niphargids are also found in an area adjacent to the Gulf of Biscay (Great Britain; Mcinerney *et al.*, 2014). Accordingly, we suggest that niphargids – contrary to pseudoniphargids – have colonized continental waters only once, and from a

marine ancestor common to both families, and that this common ancestor most probably lived on the NE Atlantic coasts at the end of the Cretaceous. Once established in continental waters, niphargids proceeded to spread across Europe, with the colonisation of Iberia by the ancestor of *Haploginglymus* taking place before the rise of the Pyrenees 55–47 Myr ago. Niphargids should continue to be considered as a primary limnic group for biogeographic purposes, despite its presumed relatively recent (late Cretaceous) marine origin and sister relationship with the (thalassoid) Pseudoniphargidae.

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## Online Supplementary Information

S1. List of all taxa included in the analyses with their respective geographical origin, voucher ID and GenBank accession codes.

S2. Maximum likelihood phylogenetic tree based on the analysis of the *cox1* alignment. Node numbers represent statistical bootstrap support (only values  $\geq 70$  are shown). Tip labels refer to the species names and their respective GenBank accession codes.

S3. Maximum likelihood phylogenetic tree based on the analysis of the H3 alignment. Node numbers represent statistical bootstrap support (only values  $\geq 70$  are shown). Tip labels refer to the species names and their respective GenBank accession codes.

S4. Maximum likelihood phylogenetic tree based on the analysis of the LSU alignment. Node numbers represent statistical bootstrap support (only values  $\geq 70$  are shown). Tip labels refer to the species names and their respective GenBank accession codes.

S5. Maximum likelihood phylogenetic tree based on the combined analysis of *cox1*, H3 and LSU. Node numbers represent statistical bootstrap support (only values  $\geq 70$  are shown).

S6. Bayesian phylogenetic tree based on the combined analysis of *cox1*, H3 and LSU. Node numbers represent posterior probabilities (only values  $\geq 0.70$  are shown).

S7. Maximum likelihood phylogenetic tree based on the combined analysis of *cox1* (excluding third coding positions), H3 and LSU. Node numbers represent statistical bootstrap support (only values  $\geq 70$  are shown).

S8. Maximum likelihood phylogenetic tree based on the analysis of the reduced LSU alignment (ambiguously aligned positions removed with Gblocks). Node numbers represent statistical bootstrap support (only values  $\geq 70$  are shown).

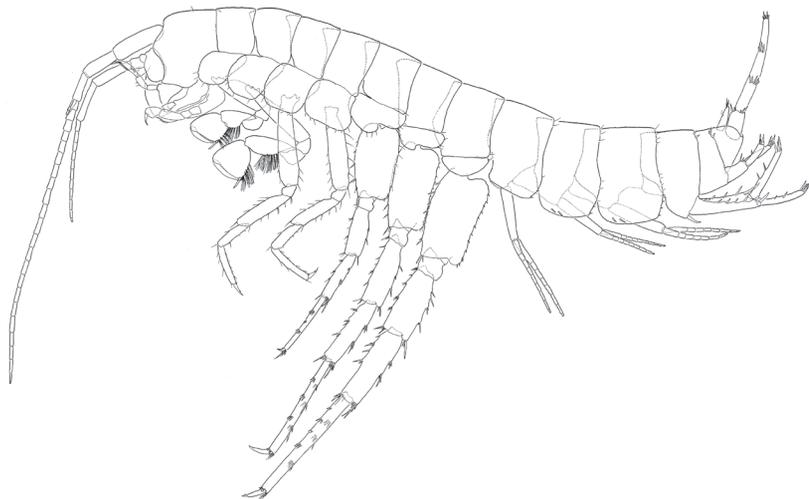


Fig. 4. *Haploginglymus geos* sp. nov., brooding female general aspect.

## Appendix

### Systematics

Order Amphipoda Latreille, 1816  
 Family Niphargidae Bousfield, 1977  
 Genus *Haploginglymus* Mateus and Oliveira-Mateus, 1958

### *Haploginglymus geos* sp. nov. Jaume and Alvarez (Figs 4–13)

**Material examined.** Cueva del Ocho, Cazalla de la Sierra, Seville, Spain. Geos Spelaeological Society Catalogue ref. no.: CLS-12-Geos. Coordinates: (Datum WGS 84) N 38° 2' 2.36"; W 5° 54' 58.15"; 346 m above sea level.. Phreatic lake occupying bottom of cave. Holotype: female 7.5 mm, preserved in single ethanol vial [RMNH.CRUS.A. 5072]. Paratypes: 9 females in single ethanol vial [RMNH.CRUS.A. 5073]. About 100 specimens in single ethanol vial deposited at IMEDEA. Collected by G. Alvarez and D. Jaume. Additional material examined: Cueva de Santiago Grande (CLS-2-Geos; cave placed adjacent to Cueva del Ocho). Numerous specimens. Collected by Alberto Tinaut, Virginia Salabert and M<sup>a</sup> Dolores Lara. Deposited at IMEDEA.

**Diagnosis.** Fourth and fifth peduncular segment of antenna about equal in length. Spines on basal endite (=outer lobe) of maxillule coarsely denticulate. Palp of maxilliped ordinary, unguiform, non-spatulate. Carpus of G1-G2 each longer than corresponding propodus. Basis of P5-P7 not expanded, slender, each with over-

hanging posterodistal lobe. Telson cleft to only one-third of its total length.

**Etymology.** Species name refers to Geos Spelaeological Society (Seville), whose members explored for the first time the caves where the new species was discovered and collected the first specimens.

**Distribution.** Known only from phreatic subterranean waters of Cerro de Santiago (Cazalla de la Sierra, Seville; southern Spain). The area is connected hydrographically to the Guadalquivir River basin.

**Description of brooding female.** Body (Fig. 4) up to 7.6 mm long, anophthalmous, colourless. **Head** (Fig. 5A) rostrum absent; lateral lobes evenly rounded, slightly produced, each with submarginal row of short sensillae along dorsal margin; antennal sinus hardly excavated. **Epimeral plates I-III** (Fig. 12A) each with evenly rounded posterior margin and straight distal (= ventral) margin; posterodistal angle not produced, marked with tiny flagellate spine; armature (strong flagellate spines) of distal margin of plates as follows: plate I, naked; plate II, 1 or 2 spines; plate III, 2 or 3 spines. **Urosomites I-III** dorsolateral armature (Fig. 13A, C) consisting of one (urosome I) or 2-3 (urosome II) short, relatively slender spines at each side; urosome III naked; urosome I with one (exceptionally two on one side) stout curved strong ("ecdysial") spine implanted adjacent to insertion of U1, just in front of it (Fig. 13A).

**Labrum** (Fig. 6A) ordinary, globose. **Paragnaths** (Fig. 6B) with well-developed inner lobes.

**Antennule** (Fig. 5B) peduncle segments progressively shorter towards distal, relative lengths as 100: 73: 49;

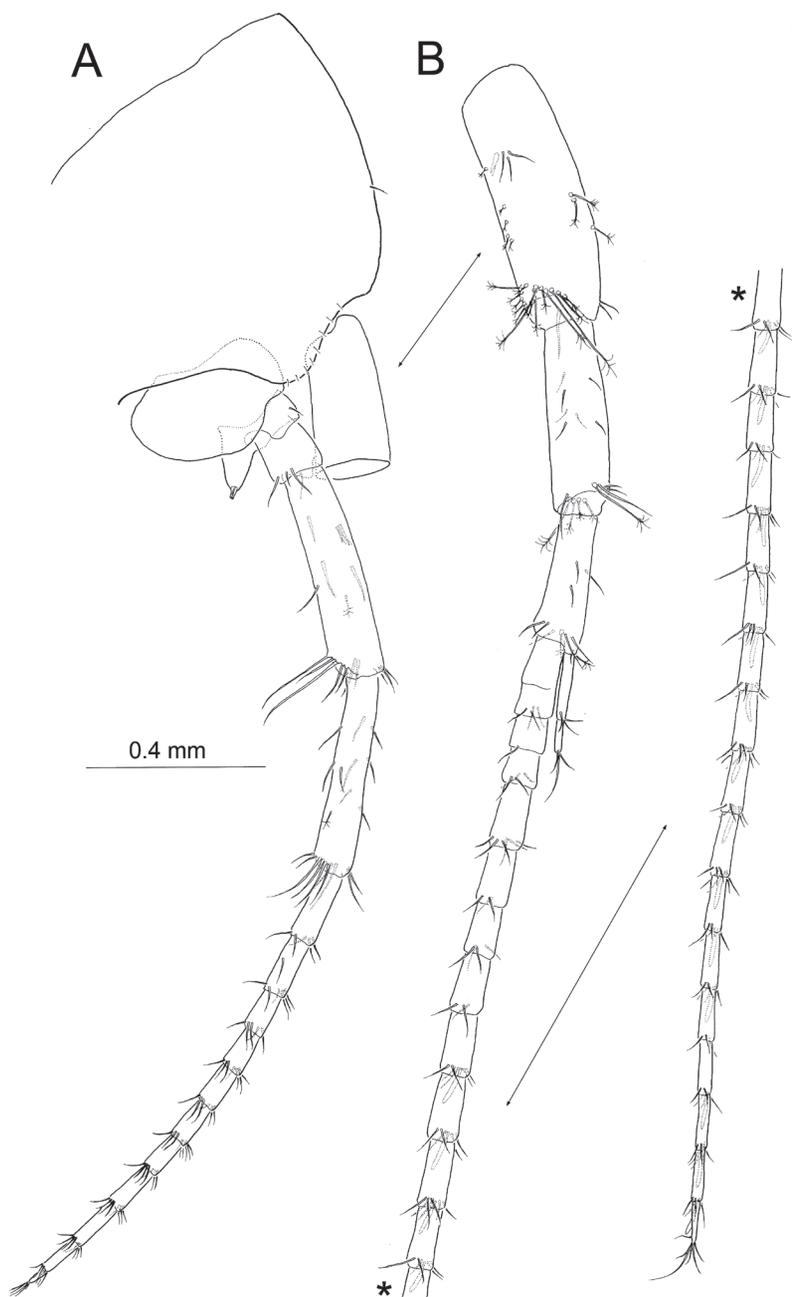


Fig. 5. *Haploginglymus geos* sp. nov., brooding female. A, inset of head with right A2 attached, lateral; B, right A1, lateral.

proximal segment provided with one (exceptionally two) short flagellate spines proximally on posteromedial surface. Main flagellum about 2.8 times longer than peduncle, distal articles each provided with short simple aesthetasc. Accessory flagellum 2-articulate, shorter than distal segment of peduncle.

*Antenna* (Fig. 5A) much shorter than antennule (at-

taining only 49% length of A1). Proximal segment of 5-segmented peduncle swollen; gland cone on second segment slender; third segment with short triangular process protruding on distomedial margin; fourth and fifth segments relative length as 1: 0.96, fourth segment with several spines on medial surface. Flagellum shorter than peduncle.

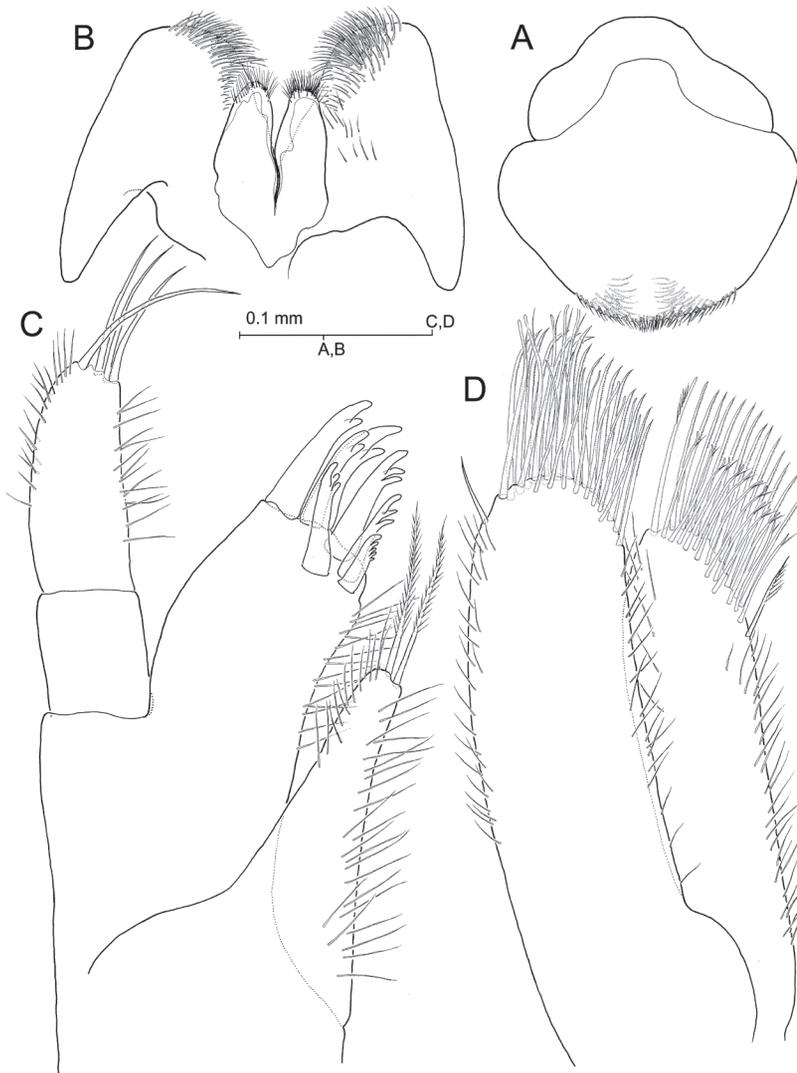


Fig. 6. *Haploginglymus geos* sp. nov., brooding female. A, labrum, anterior; B, paragnaths, dorsal; C, left maxillule; D, left maxilla.

*Left mandible* (Fig. 7A) incisor 5-denticulate; lacinia 4-denticulate; spine row comprising 5-6 pappose elements plus three additional shorter ones disposed as collateral row as figured; molar columnar with short, reduced pappose molar seta. Palp 3-segmented, segments progressively longer towards distal; proximal segment naked; middle segment with two (exceptionally three) setae on ventral margin; distal segment with row of 9 to 15 setae along distal half of ventral margin, three longer terminal setae – one of which much stouter than rest –, and two setae (exceptionally one) implanted adjacent to each other proximally on outer surface of segment; anteromedial surface of third segment setulose (not shown in figure, but see

Fig. 8A, corresponding to right counterpart).

*Right mandible* (Figs 7B; 8A) incisor 4-denticulate; lacinia bifid, each branch 5- to 9-denticulate; spine row consisting of 4-5 elements plus shorter intercalary elements; molar seta much longer than left mandible counterpart.

*Maxillules* (Fig. 6C) symmetrical. Coxal endite (= inner plate) finger-like, crowned with two setae; basal endite (= outer plate) with seven stout spines, of which innermost shortest and 4- to 6-denticulate, adjacent spine 3-denticulate, next two spines 2- or 3-denticulate (one exceptionally 4-denticulate), and three outermost spines 2-denticulate. Endopod (= palp) 2-segmented, distal segment with 3-4 terminal setae.



Fig. 7. *Haploginglymus geos* sp. nov., brooding female. A, left mandible, medial; B, inset of distal margin of coxal gnathobase of right mandible, lateral; C, left maxilliped, dorsal (= anterior).

*Maxilla* (Fig. 6D) ordinary, both endites subequal in length and with armature limited to distal margin; basal endite (= outer plate) with short simple seta implanted subdistally on outer margin, separated from terminal cluster of setae; coxal endite (= inner plate) devoid of oblique “facial” row of setae.

*Maxilliped* (Fig. 7C) basal endite (= inner plate) subrectangular, with 3-5 flattened, spatulate spines on distal margin; distal half of medial margin with submarginal row of pinnate setae. Ischial endite (= outer plate)

rhomboidal, with row of 9-11 spatulate spines along distomedial margin and row of pinnate setae on distolateral margin. Other armature present on endites as figured. Palp (= merus-to-dactylus) ordinary; unguis present, slender, pointed, claw-like, attaining 77 % length of dactylus.

*Coxal gills* (Figs 9A; 10A; 11A, C) present on G2 to P6, ordinary, sac-like, stalked. *Oöstegites* (Figs 9A; 11A) on G2 to P5, broad, with sparsely-set short simple setae on distal margin.

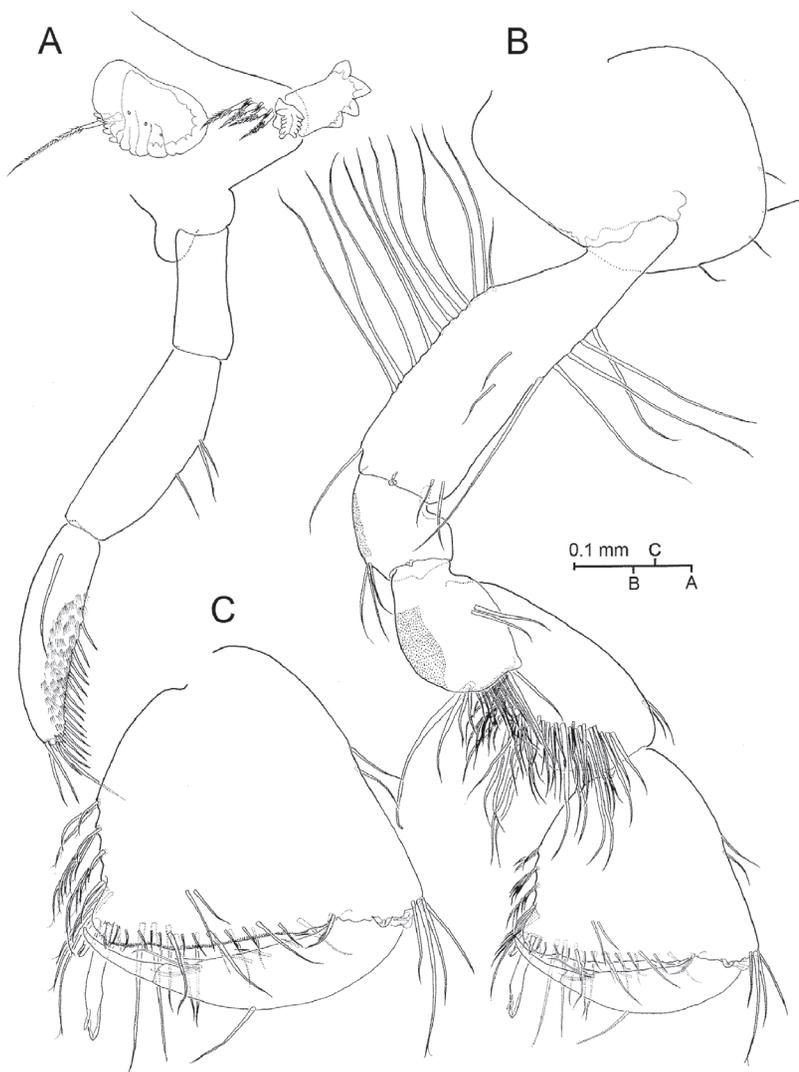


Fig. 8. *Haploginglymus geos* sp. nov., brooding female. A, right mandible; B, G1, medial; C, inset of hand (= propodus + dactylus).

*Gnathopod I* (Fig. 8B) integument of posterior margin on ischium and merus micro-spinulate; merus with strong, long, bended posterodistal seta. Carpus elongate, slightly longer than propodus, provided with oblique patch of setae on medial surface and with five clusters of setae on posterior margin. Propodus (Fig. 8C) sub-chelate, trapezoidal, about as long as broad; palm angle with three flagellate spines disposed as figured (1 on medial side of margin; 2 on lateral side); longest spine very strong, attaining 35 % length of propodus. Posterior margin of propodus with four clusters of setae. Dactylus with single seta on outer margin.

*Gnathopod II* (Fig. 9A) with integument on posterior margin of merus micro-spinulate. Carpus elongated,

about 1.2 times longer than propodus, with well-delimited row (vs. patch on G1) of setae on medial surface; posterior margin with seven clusters of setae. Propodus (Fig. 9B) subsimilar but larger than G1 counterpart, posterior margin with six clusters of setae (vs. 4 on G1); large spine on palm angle attaining 33 % length of propodus.

*Pereiopod III* (Fig. 10A) coxa subrectangular, about 1.2 times longer than broad. Nail (= dactylus+unguis) attaining 35 % length of propodus; unguis 58 % length of dactylus (Fig. 10B).

*Pereiopod IV* (Fig. 10C) shorter than P3 (92 % length of P3, excluding coxa); coxa subquadrate, slightly broader than long, with shallowly-excavated posterior margin. Nail attaining 36 % length of propodus; unguis 63 % length of dactylus (Fig. 10D).

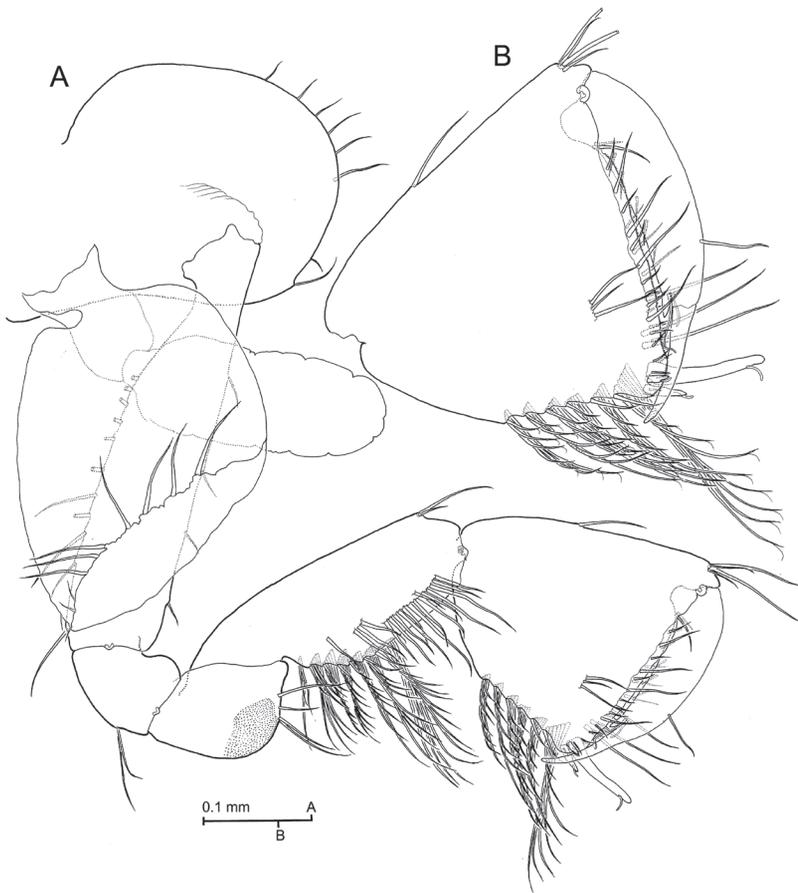


Fig. 9. *Haploginglymus geos* sp. nov., brooding female. A, G2 with oostegite partially folded, medial; B, inset of hand (= propodus + dactylus).

*Pereiopods V-VII* progressively longer towards posterior, each with sub-rectangular basis showing well-developed posterodistal lobe. *Pereiopod V* (Fig. 11A) longer than P4; coxa with broad, overhanging anteroventral lobe. Basis 1.8 times as long as broad. Nail attaining 28 % length of propodus; unguis 35 % length of dactylus (Fig. 11B).

*Pereiopod VI* (Fig. 11C) coxa with distinct, evenly rounded anteroventral lobe. Basis twice as long as broad. Nail attaining 33 % length of propodus; unguis 27 % length of dactylus (Fig. 11D).

*Pereiopod VII* (Fig. 11E) basis 1.8 times as long as broad. Nail attaining 27 % length of propodus; unguis 30 % length of dactylus (Fig. 11F).

*Pleopods I-III* (Fig. 12B), ordinary, biramous, rami multi-articulate and longer than respective protopod; latter with pair of 5-denticulate retinacles. Exopod slightly shorter than endopod. Endopod with modified, bifid seta proximally on inner margin of proximal article; rest of setae plumose.

*Uropod I* (Fig. 13B) almost as long as U3. Protopod longer than rami, lacking basofacial spine; 1 terminal strong flagellate spine at each side; posterolateral margin with 5-7 shorter flagellate spines, and 2-4 similar spines along posteromedial margin. Exopod slightly shorter than endopod, with five (exceptionally 6) terminal spines; 1-2 spines along posterolateral margin and 0-1 spine on posteromedial margin. Endopod with five terminal spines; 2-3 spines along posteromedial margin; posterolateral margin of segment unarmed.

*Uropod II* (Fig. 13C, D) protopod with two terminal flagellate spines on posterolateral angle and single flagellate spine on posteromedial angle; posterolateral margin of segment with 1-2 flagellate spines; posteromedial margin unarmed. Rami unequal in length, with exopod much shorter than endopod. Exopod about same length as protopod, with five terminal spines and one spine (exceptionally none) about midway on each margin. Endopod with five terminal spines and other 2-3 along medial margin; lateral margin with 0-1 spine about midway.

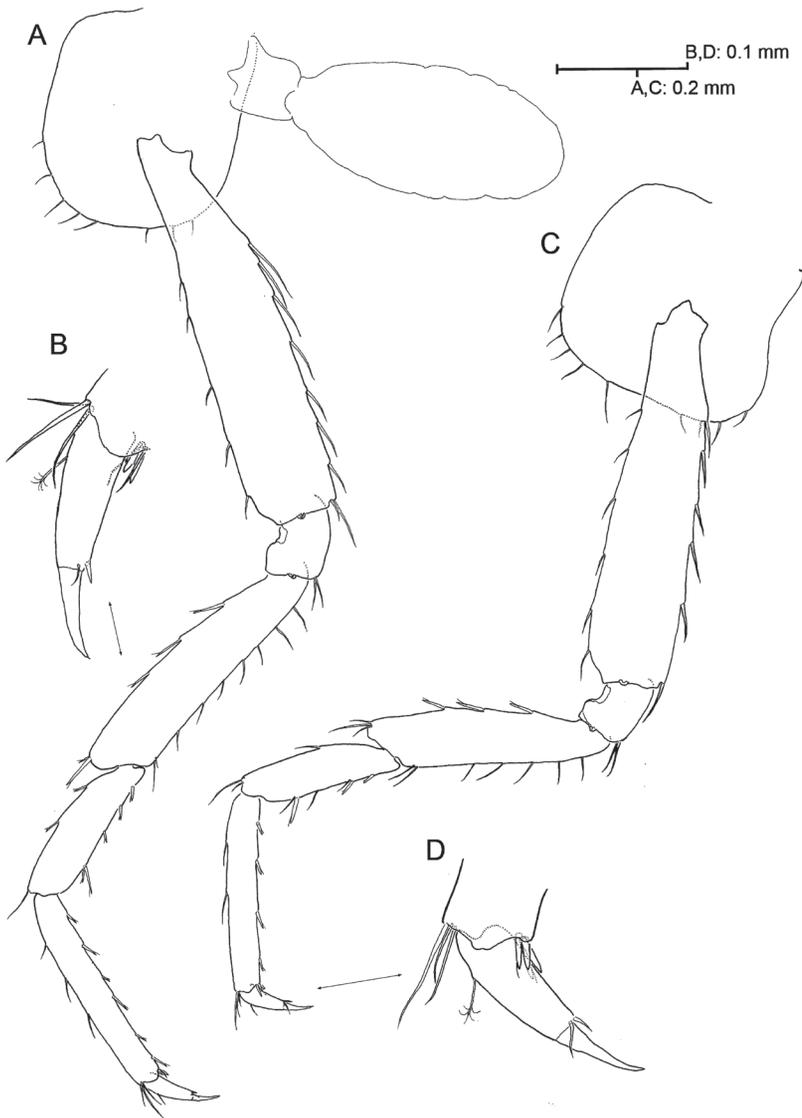


Fig. 10. *Haploginglymus geos* sp. nov., brooding female. A, right P3, medial (oostegite omitted); B, inset of dactylus + unguis, medial; C, left P4, medial (coxal gill and oostegite omitted); D, inset of dactylus + unguis, medial.

*Uropod III* (Fig. 13E) protopod about 1.5 times as long as broad, with 4-6 flagellate spines on distolateral angle and one short spine on distomedial angle; medial margin of segment with 0-2 short flagellate spines. Exopod about 6.4 times as long as broad, 3.7 times longer than protopod; armature consisting of 3-4 clusters of flagellate spines along lateral margin and 4-5 on medial margin distributed as figured. Endopod reduced, scale-like, with two (exceptionally 1 or 3) flagellate spines subdistally on medial margin.

*Telson* (Fig. 12C) slightly longer than broad, cleft to approx. one-third (34 %) of its maximum length. Armature consisting of 3-4 distal flagellate spines per

lobe (exceptionally 5 on one lobe; see Fig. 12C); lateral margins unarmed except in exceptional specimens displaying one flagellate spine on one or both margins (as in Fig. 12C).

#### Male

Unknown.

*Taxonomic remarks.* *Haploginglymus geos* sp. nov. is the only member of the genus whose telson is cleft only to less than half of its total length (Table 5). Of the five formally described *Haploginglymus* species, only *H. bergae* Pretus and Sabater, 1990, and *H. morenoi* share

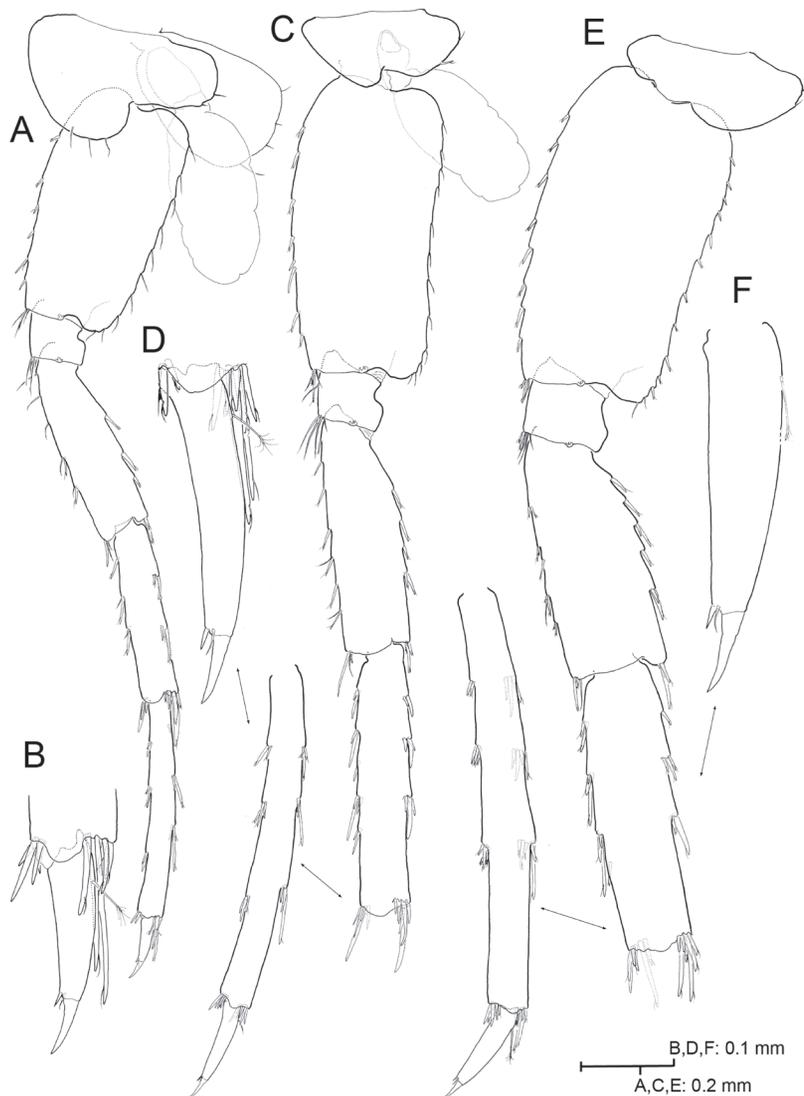


Fig. 11. *Haploginglymus geos* sp. nov., brooding female. A, left P5, lateral; B, inset of dactylus + unguis; C, left P6, lateral; D, inset of dactylus + unguis; E, left P7, lateral; F, inset of dactylus + unguis.

Table 5. Some features enabling distinction between *Haploginglymus* species.

| Species                 | Gnathopods, (carpus/propodus) length |         | Maxillule, denticulation of spines on basal endite | Telson excavation (vs. telson length) length | A2 peduncle, (seg. 4/seg. 5) lobe of basis | P5-P7, posterodistal |
|-------------------------|--------------------------------------|---------|--|--|--|----------------------|
|                         | G1                                   | G2      |  |  |  |                      |
| <i>H. bergae</i>        | 1.2                                  | 1.3     | fine; many denticles                               | > 50%  | 45%  | +                    |
| <i>H. bragai</i>        | 0.8                                  | ?       | coarse; few denticles                              | = 50% ?                                      | 51%  | -                    |
| <i>H. lobatus</i>       | 0.8                                  | 1.1     | coarse; few denticles                              | > 50%  | 47%  | +                    |
| <i>H. mateusi</i>       | 0.8                                  | 0.9     | coarse; few denticles                              | > 50%  | 51%  | -                    |
| <i>H. morenoi</i>       | 1.4                                  | 1.3     | fine; many denticles                               | > 50%  | 56%  | -                    |
| <i>H. geos</i> sp. nov. | 1.1-1.3                              | 1.2-1.3 | coarse; few denticles                              | < 50%  | 49%  | +                    |

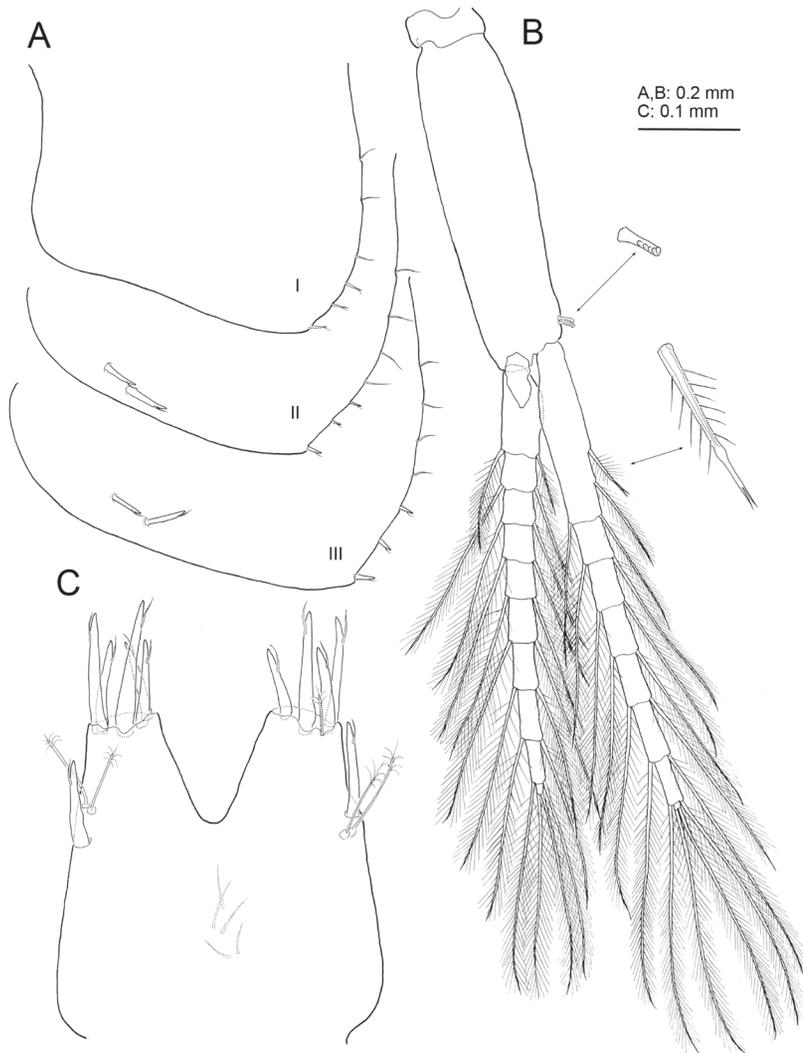
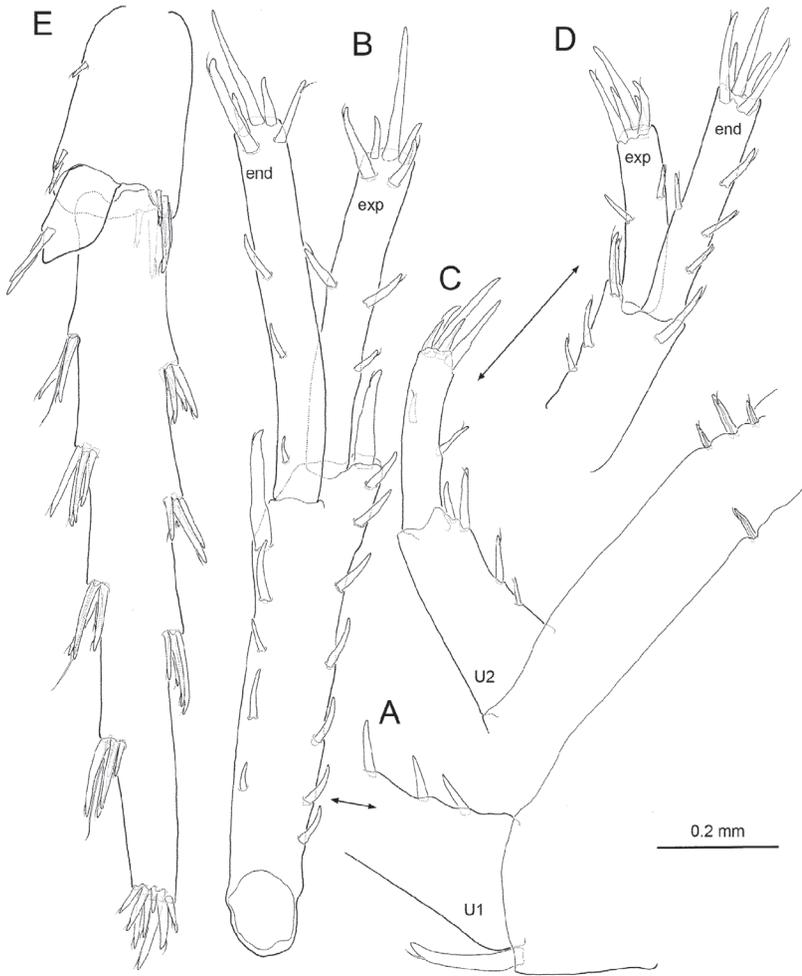


Fig. 12. *Haploginglymus geos* sp. nov., brooding female. A, left pleopod II, posterior; B, left epimeral plates I-III, lateral; C, telson, dorsal.

with the new taxon the display of a G1 carpus that is longer than the corresponding propodus (Table 5). These species share also the relative elongation of carpus of G2 (Table 5). Whereas *H. morenoi* differs from the new species in the display of an aberrant, spatulate maxillipedal palp, an elongate, slender propodus in both gnathopods, and in the multi-denticulate condition of the spines of the basal endite (= outer plate) of maxillule, differences with *H. bergae* are subtler, but also consistent. Thus, the spines present on the basal endite of the maxillule aside the innermost one are multi-denticulate in *H. bergae* whereas only coarsely 2- or 3-denticulate at most in the new species; the

epimeral plates are almost unarmed vs. armed in the new species; the telson is cleft to 60 % of its length vs. only cleft to only 34 % in the new species; the oöste-gites are hypertrofied vs. of ordinary size in the new species; the U1 protopod displays a single spine on the posteromedial margin vs. 2-4 spines in the new species; the outer margin of the U1 endopod bears one spine vs. none in the new species; and the distal segment of the mandibular palp displays only one proximolateral seta vs. two setae in the new species.

Additional relevant differences between the new taxon and the rest of *Haploginglymus* species are shown in Table 5.



*Fig. 13. Haploginglymus geos* sp. nov., brooding female. A, first urosomite with proximal portion of right U1 attached, lateral; B, left U1, posterior; C, second urosomite with right U2 attached, lateral; D, right U2, posterior; E, right U3, dorsal (= posterior).