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Hedyselmis opis: Description of the larva and its phylogenetic relation to *Graphelmis* (Coleoptera: Elmidae: Elminae)

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Abstract. The riffle beetle genus *Hedyselmis* Hinton, 1976 includes two species from the Malay Peninsula, with adults with a highly deviating morphology. Its phylogenetic relationships are unclear, although it has been hypothesized to be related to *Graphelmis* Delève, 1968, a large genus widely distributed in the Oriental and East Palaearctic regions. In this paper the larva of *H. opis* Hinton, 1976 is described based on material collected in the Cameron Highlands (Malaysia) and the conspecificity with co-existing adults tested using sequences of one nuclear (5' end of 18S rRNA) and three mitochondrial gene fragments (5' end of the large ribosomal unit + tRNAleu + 5' end of the NADH dehydrogenase subunit 1; 5' end of cytochrome c oxidase subunit I; and a fragment of cytochrome b) with a total of ca. 2,600 bp. This is the first example of the use of molecular data to match different life stages within the family Elmidae. The larva of *H. opis* has a subcylindrical body typical of many other elmid genera; abdominal segments 1–7 with preserved pleura; and ninth segment with oval operculum. The last instar larvae have clearly visible prominent spiracles on mesothorax and abdominal segments 1–8. The phylogenetic position of *Hedyselmis* in relation to *Graphelmis* was investigated using molecular data for three species of *Graphelmis* plus a selection of other Elmidae genera. *Hedyselmis opis* is nested within *Graphelmis*, confirming their close relationship and suggesting that their status requires taxonomic revision.

INTRODUCTION

Other than for several well known genera, descriptions of elmid larvae, especially from tropical regions, are rarely found in the literature. The major problem lies in the high diversity of the material collected and the difficulty of assigning larvae to conspecific adults. The most reliable method of determining elmid larvae to species is to rear them under laboratory conditions, which is usually very difficult due to the habitat requirements and the length of larval development (Brown, 1987). Thus, the description of larvae is normally confined to species collected with only one representative of the family or with clearly unrelated species. An alternative way of matching adults with larvae is by using DNA sequences with the appropriate level of variation (i.e., with enough variability for there to be differences among closely related species but not enough to impede recognition). There are surprisingly few examples of this use of DNA sequences (e.g., Balke et al., 2005; Jeon & Ahn, 2005; Miller et al., 2005), despite the increasing availability of molecular facilities and their wide use in forensic science (e.g., Wells & Sperling, 2001). Previous descriptions of Elmidae larvae are based exclusively on morphological characters (e.g., Manzo & Archangelsky, 2001; Springer & Acosta, 2003) and this is the first study to use molecular data to assign larvae to specific adults within the Elmidae.

Hinton (1976) described the genus *Hedyselmis* based on six females of one species, *H. opis*, from the Malay Peninsula. Only recently the males of *H. opis* and one additional congeneric species were described, also from the Malay Peninsula (Jäch & Boukal, 1997). Adults of both species of *Hedyselmis* have unusual morphology and seem to be most closely related to the genus *Graphelmis* (Jäch & Boukal, 1997; Čiampor Jr., unpubl.). In 2001 some additional material of *H. opis*, together with unknown elmid larvae, was collected by the junior author in the Malay Peninsula. The lack of any other species of Elmidae at that locality made it likely that the larvae belonged to the same species.

The aim of the current study was to describe the unknown Elmidae larva and using four molecular markers to test the hypothesis that it belongs to the species *Hedyselmis opis*. The molecular data was also used to outline possible relationship between *Hedyselmis* and *Graphelmis*, as suggested most closely related genus.

MATERIAL AND METHODS

Taxon sampling

Eight specimens of putative *Hedyselmis opis* larvae were examined from the following locality data as follows: " Malaysia, Pahang, Cameron Highlands, Tanah Rata env.; small, shaded stream crossing path Nr. 9 surrounded by primary forest; 9. VI. 2001. Shaded, very shallow stream, 0.5–1.0 m wide, flowing in dense primary forest on steep slope, with small waterfalls; bottom littered with submerged wood". At this locality, the larvae were collected in association with adults of *H. opis* and some species of Dryopidae.

For the phylogenetic analysis of *Hedyselmis opis* three species of the genus *Graphelmis* were used as the likely sister group (Jäch & Boukal, 1997), plus some other genera of Elmidae (Table 1). The tree was rooted in a genus of a related family (Dryopidae), *Pomatinus* Sturm, 1806.

TABLE 1. Geographical origin, collectors and Genbank accession numbers of the taxa studied.

Species	Locality	Collector	18S	*Cox1*	*cob*	16S
Hedyselmis opis Hinton, 1976 larva	Malaysia	Čiampor Jr.	DQ005516	DQ005514	DQ005512	DQ005518
Hedyselmis opis Hinton, 1976 adult	Malaysia	Čiampor Jr.	DQ005517	DQ005515	DQ005513	DQ005519
Graphelmis obesa Čiampor Jr., 2005	Malaysia	Čiampor Jr.	DQ266481	DQ266492	DQ266503	DQ266471
Graphelmis picea Čiampor Jr. & Kodada, 2005	Papua New Guinea	Balke	DQ266482	DQ266493	DQ266504	DQ266472
Graphelmis clermonti (Pic, 1923)	Laos	Jendek & Šauša	DQ266483	DQ266494	DQ266505	DQ266473
Stenelmis sp.	Malaysia	Čiampor Jr.	DQ266484	DQ266495	DQ266506	DQ266474
Limnius intermedius Fairmaire, 1881	Spain	Ribera	DQ266485	DQ266496	DQ266507	DQ266475
Oulimnius tuberculatus (Müller, 1806)	Spain	Ribera	DQ266486	DQ266497	DQ266508	DQ266476
Dubiraphia sp.	USA	Ribera & Cieslak	DQ266487	DQ266498	DQ266509	DQ267445
Rhopalonychus levatorponderis Jäch & Kodada, 1996	Malaysia	Čiampor Jr.	DQ266488	DQ266499	DQ266510	DQ266477
Ancyronyx procerus Jäch, 1994	Malaysia	Čiampor Jr.	DQ266489	DQ266500	DQ266511	DQ266478
Potamophilinus sp.	Malaysia	Čiampor Jr.	DQ266490	DQ266501	DQ266512	DQ266479
Pomatinus substriatus (Müller, 1806)	Spain	Ribera	DQ266491	DQ266502	DQ266513	DQ266480

Morphological analyses

Specimens prepared for morphological study were cleaned and examined under a Nikon SMZ-1B stereo-microscope under diffuse lighting at magnifications up to 140×. Mouth parts were dissected and placed in lactic acid for a few days before examination using a Carl Zeiss transmitted light microscope at magnifications up to 600×. Drawings were made using a drawing tube.

For scanning electron microscopy, specimens were dehydrated in a graded ethanol series, air-dried from absolute ethanol, mounted on stubs using double-sided tape, sputter coated with gold and then viewed in a Hitachi S800 at 10kV.

Metric characters were measured to the nearest 0.05 mm using a Nikon SMZ-1B with an ocular grid. Morphological terms follow Lawrence (1991).

Molecular methods

One adult of each species and one unknown larva were used for non-destructive DNA extraction. These individuals are kept as voucher specimens in the collection of the junior author and DNA aliquotes are kept in the MNCN Madrid, ref. No. FC-C17 (larva), and adults FC-A11 (*H. opis*), FC-D16 (*G. obesa*), FC-D14 (*G. clermonti*), FC-D02 (*Stenelmis* sp.), FC-B10 (*Limnius intermedius*), FC-D05 (*Oulimnius tuberculatus*), FC-E10 (*Dubiraphia* sp.), FC-B01 (*Rhopalonychus levatorponderis*), FC-B05 (*Ancyronyx procerus*), FC-A16 (*Potamophilinus* sp.), FC-B11 (*Pomatinus substriatus*).

DNA was extracted from whole specimens using the Qiagen DNeasy tissue kit. Four fragments were amplified using PCR; three mitochondrial [826 bp from the 3' end of the cytochrome oxidase subunit I (*cox1*), 358 bp of the cytochrome b apoenzyme (*cob*), and 835 bp comprising the 3' end of the rrnL (16S rRNA), the adjacent transfer RNA leucine 2 (tRNAleu), and part of NADH dehydrogenase subunit 1 (*nad1*)] and one nuclear (602 bp of the 5' end of the ribosomal 18S rRNA gene) (see Table 2 for the primers used). Amplification products were purified using Qiagen Qiaquick PCR purification columns and sequenced in both directions. Sequences were sent to GenBank and have accession numbers DQ005512–DQ005519, DQ266471–DQ266513 and DQ267445 (Table 1).

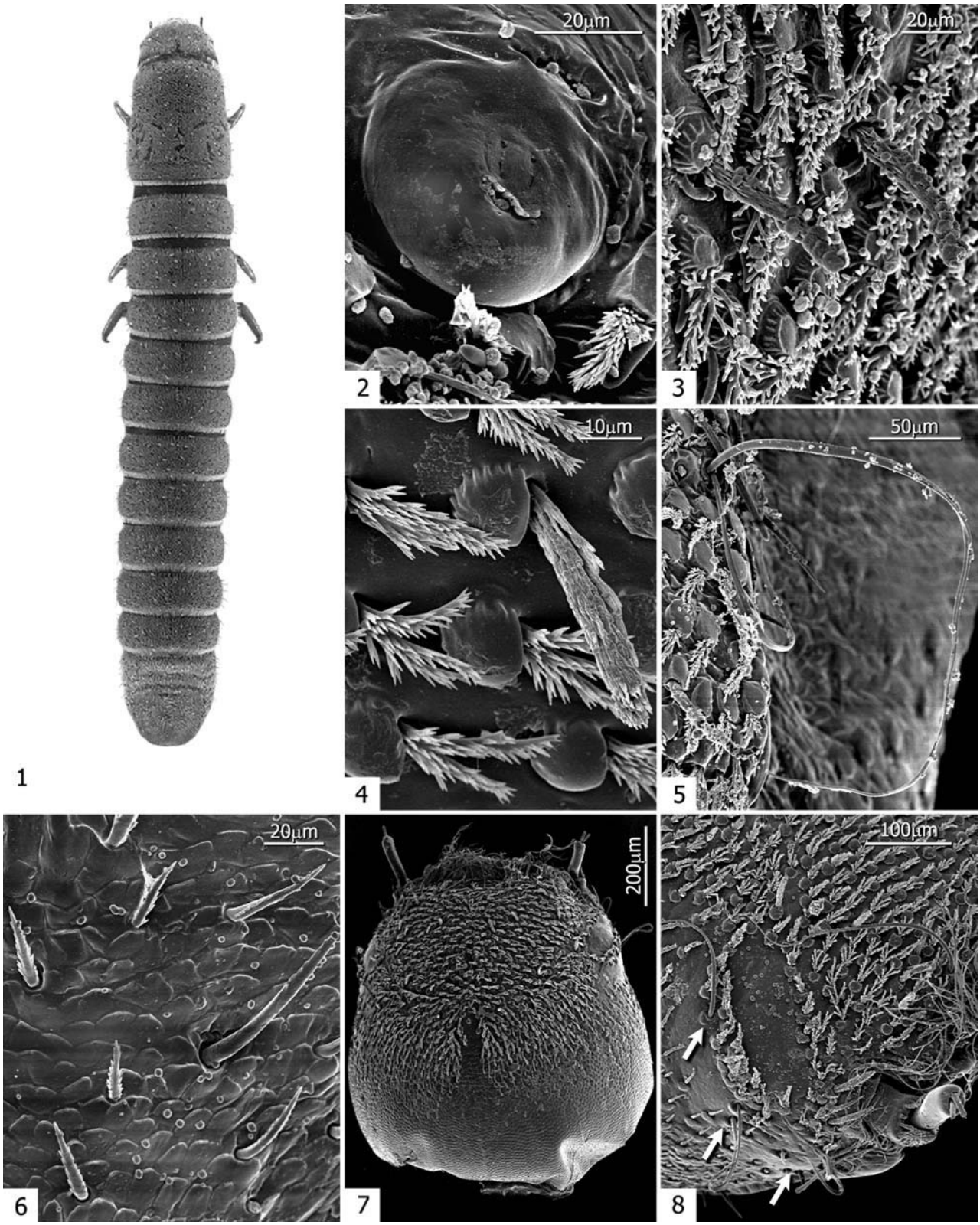
Phylogenetic analyses

Protein-coding genes were not length variable and there was little variation in the ribosomal genes within Elmidae (length of the 18S rRNA fragment was between 626 and 628 bp; of the 16S rRNA fragment between 817 and 827 bp). The sequences were aligned manually. The combined data matrix (all four genes) was analysed using parsimony in PAUP* software version 4.0b10 (Swofford, 2002), with a TBR heuristic search of 10,000 replicates and the option 'save multiple trees' activated. For comparison with the Bayesian probability results (see below), gaps in all searches were coded as missing characters, although treating them as a 5th character state did not change the topology of the trees (not shown). Node support was measured using Bremer Support (PBS) values (Bremer, 1994) on constraint trees generated by means of TreeRot.v2 (Sorenson, 1996) and non-parametric bootstrapping (Felsenstein, 1985) using 1,000 pseudoreplicates of 50 random additions each.

TABLE 2. Primers used in this study.

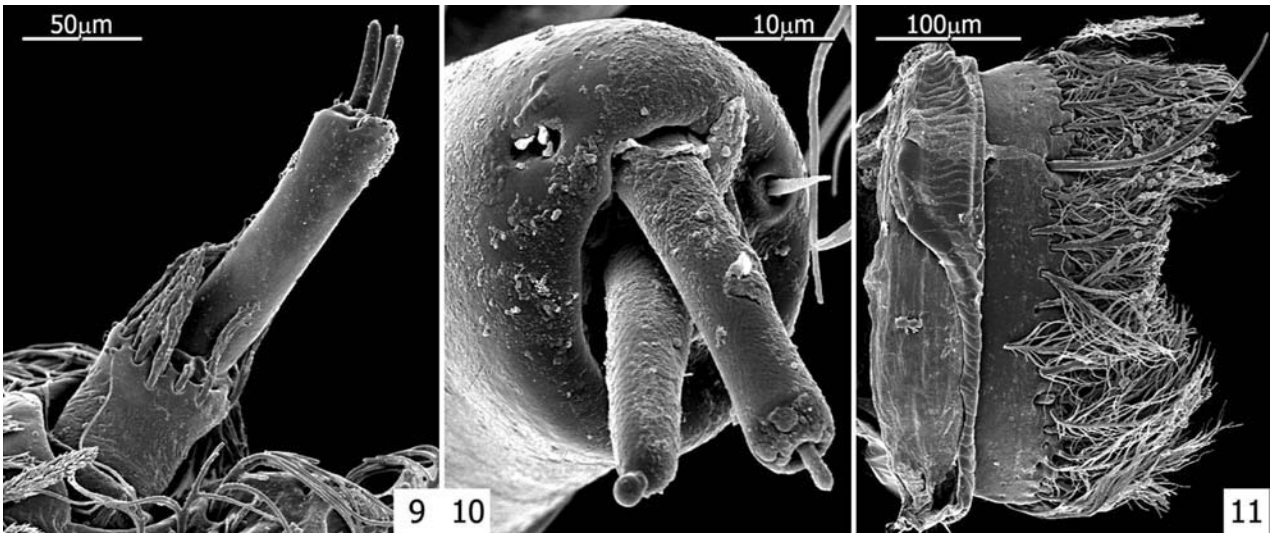
Gene	Name	Sense	Sequence	Reference
cox1	Jerry	F	CAACATTTATTTTGATTTTTTGG	Simon et al., 1994
	Pat	R	TCCAATGCACTAATCTGCCATATTA	Simon et al., 1994
Cob	CB3	F	GAGGAGCAACTGTAATTACTAA	Barraclough et al., 1999
	CB4	R	AAAAGAAA(AG)TATCATTCAGGTTGAAT	Barraclough et al., 1999
16S + nad1	16s aR	F	CGCCTGTTTAACAAAAACAT	Simon et al., 1994
	ND1A	R	GGTCCCTTACGAATTTGAATATATCCT	Simon et al., 1994
18S	18S 5'	F	GACAACCTGGTTGATCCTGCCAGT(1)	Shull et al., 2001
	18S b5.0	R	TAACCGCAACAACTTTAAT(1)	Shull et al., 2001

F – forward; R – reverse.



Figs 1–8. *Hedyselmis opis* larva: 1 – habitus; 2 – biforous spiracle; 3–6 – surface setae (3–5 – dorsal side, 6 – ventral side); 7 – head, dorsal view; 8 – stemmata, dorso-lateral view, arrows show elongate setae surrounding eyes.

Bayesian analyses were executed with MrBayes 3.04 (Huelsenbeck & Ronquist, 2001), using a GTR + I + G model as selected by Modeltest 3.6 (Posada & Crandall, 1998), with the parameters estimated for each partition (i.e., gene fragment). Searches were executed with default priors (uniform probabilities) starting with random trees with three heated and one cold Markov chains for 1,000,000 generations, sampled at intervals of 100 generations. To determine the point at which the Markov chains reached stationarity, the log-likelihood scores were plotted against generation time and the point when the likeli-



Figs 9–11. *Hedyselmis opis* larva: 9 – antenna, dorsal view; 10 – antennal flagellum and sensorium, apical view; 11 – labrum, dorsal view.

hood values reached a stable equilibrium visually determined. The parameter estimation (including tree topologies) obtained before reaching the stationary were discarded as a "burn-in" and only the trees sampled after that point were considered (Huelsenbeck & Ronquist, 2001).

RESULTS

Matching of adults and larvae

The sequences of the nuclear gene (fragment of 18S rRNA) of the elmid larva and adult *Hedyselmis opis* were identical. Among the mitochondrial genes, the only unambiguous difference was in the *cob* sequence, in which in position 352 the larva had a T and the adult a C. This was a synonymous change in the third codon position. In both the sequences of the *cox1* and 16S rRNA+nad1 of the adult there were some ambiguous positions (with double peaks in the chromatogram): in *cox1*, positions 157 and 166 had a T in the larva and T or C in the adult, positions 205 and 343 had A in the larva and A or G in the adult (all synonymous ambiguities in third codon positions); and in 16S rRNA, in positions 266 and 616, larva had G, adult A or G. All ambiguous positions were in sections of high quality sequence, without any ambiguous chromatogram in nearby positions. The total uncorrected genetic divergence between the two specimens was thus between 0.04% (if all ambiguities are resolved as matches) and 0.27% (when all ambiguities are resolved as mismatches). For the mitochondrial genes only, the divergence ranges from 0.05–0.35%.

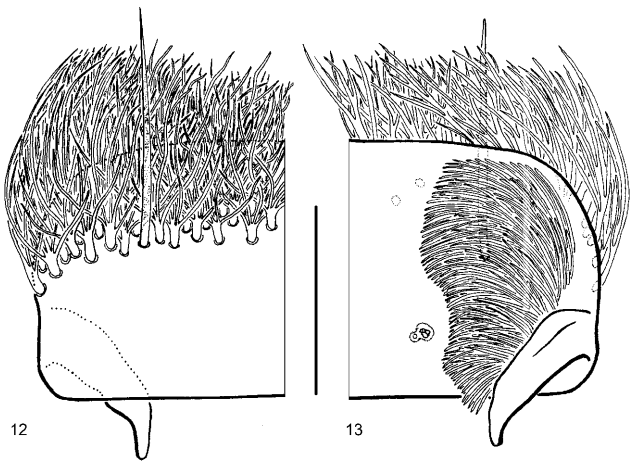
Phylogenetic analysis

The combined aligned matrix had 2,717 characters (*cox1* 826, *cob* 407, 16S 853, 18S 631). Within the whole dataset, there were 628 parsimony informative characters (658 when gaps were treated as a 5th character state). Heuristic searches resulted in a single most parsimonious tree (consistency index CI = 0.51, retention index RI = 0.37). The sequences of the larva and adult of *H. opis* were grouped together with high support (100% bootstrap) and included within *Graphelmis* in a well supported clade (100% bootstrap, 17 Bremer support, Fig. 44).

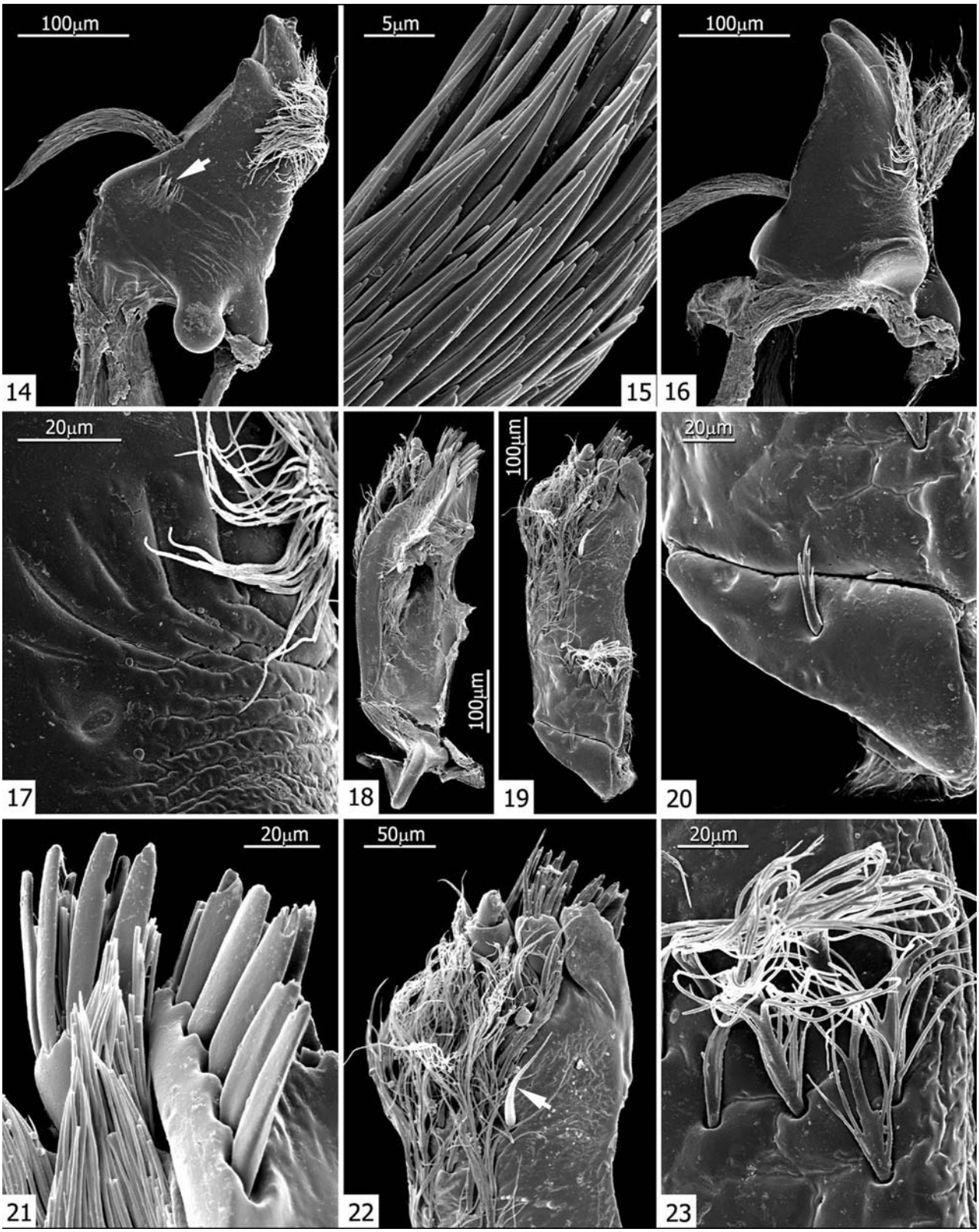
In the Bayesian analyses the sampled ML values reached stationarity at ca. 20,000 generations, but the first 100,000 (i.e., 1,000 trees) were discarded as a burn-in. The topology of the 50% majority rule consensus tree was almost identical to that of the parsimony tree, with the same highly supported nodes relating the larvae and adults of *H. opis* and the grouping of *Hedyselmis* within *Graphelmis.*

Description of the larva of *Hedyselmis opis*

Diagnosis. The larva of *H. opis* differs from the larvae presumably assigned to *Graphelmis* by: (1) dark brown colour; (2) shorter legs; (3) more flattened tufted scales of integument and (4) absence of paired dorsal spines on abdominal segment 9. Within Elmidae both, larva of *H. opis* and that assumed to belong to *Graphelmis*, differ by combination of the following characters: (1) body subcy-

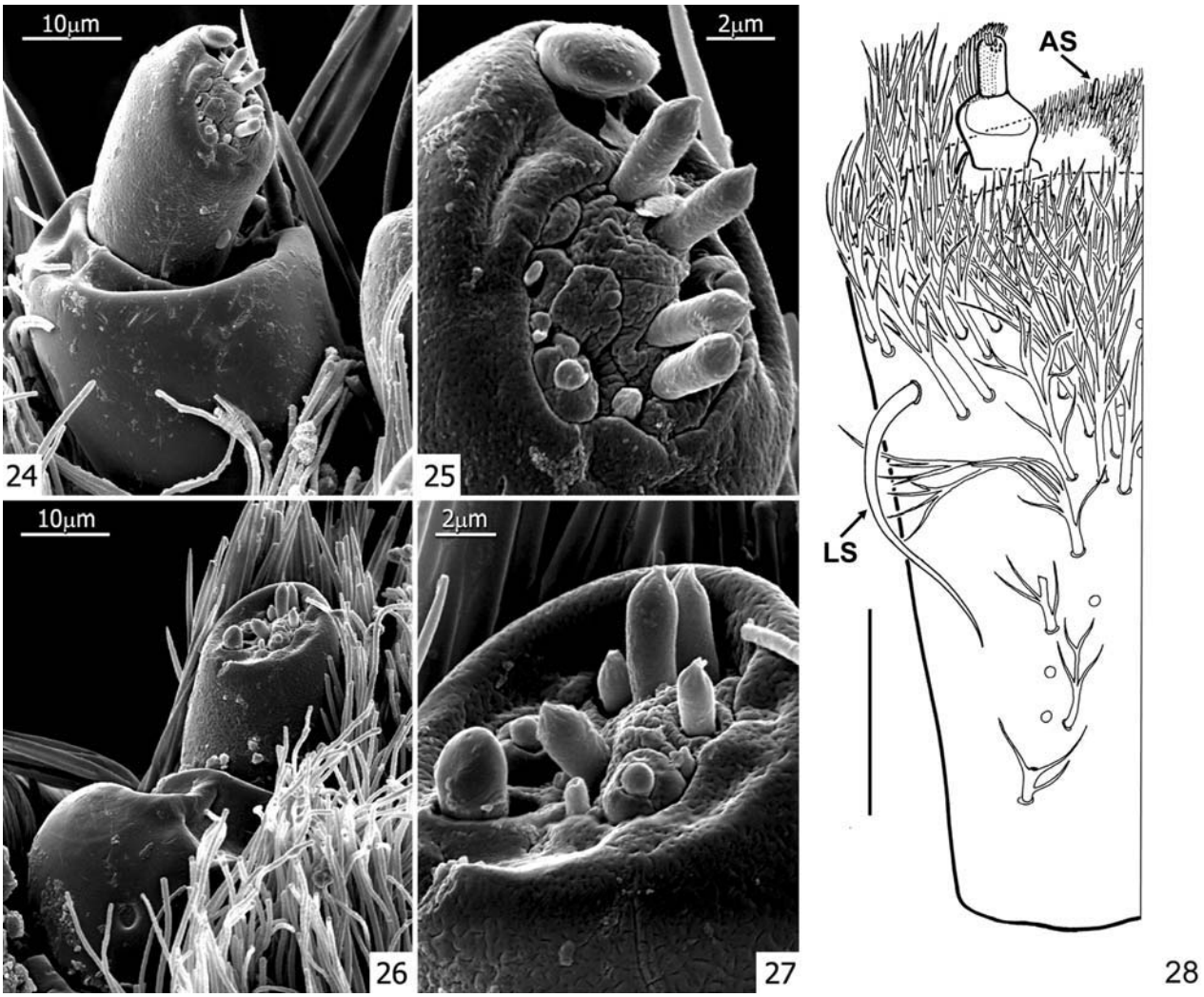


Figs 12–13. *Hedyselmis opis* larva: 12 – labrum, dorsal view; 13 – labrum, ventral view. Scale: 0.1 mm.



Figs 14–23. *Hedyselmis opis* larva: 14 – left mandible, ventral view; 15 – detail of articulated setose process of mandible, same view; 16 – right mandible, dorsal view; 17 – same, detail of oblique grooves; 18 – left maxilla, dorsal view; 19 – right maxilla, ventral view; 20 – right cardo, dorsal view; 21 – detail of maxillary apex, dorsal view; 22 – galea, lacinia and maxillary palpus, ventral view; 23 – detail of setal tuft on stipes, ventral view.

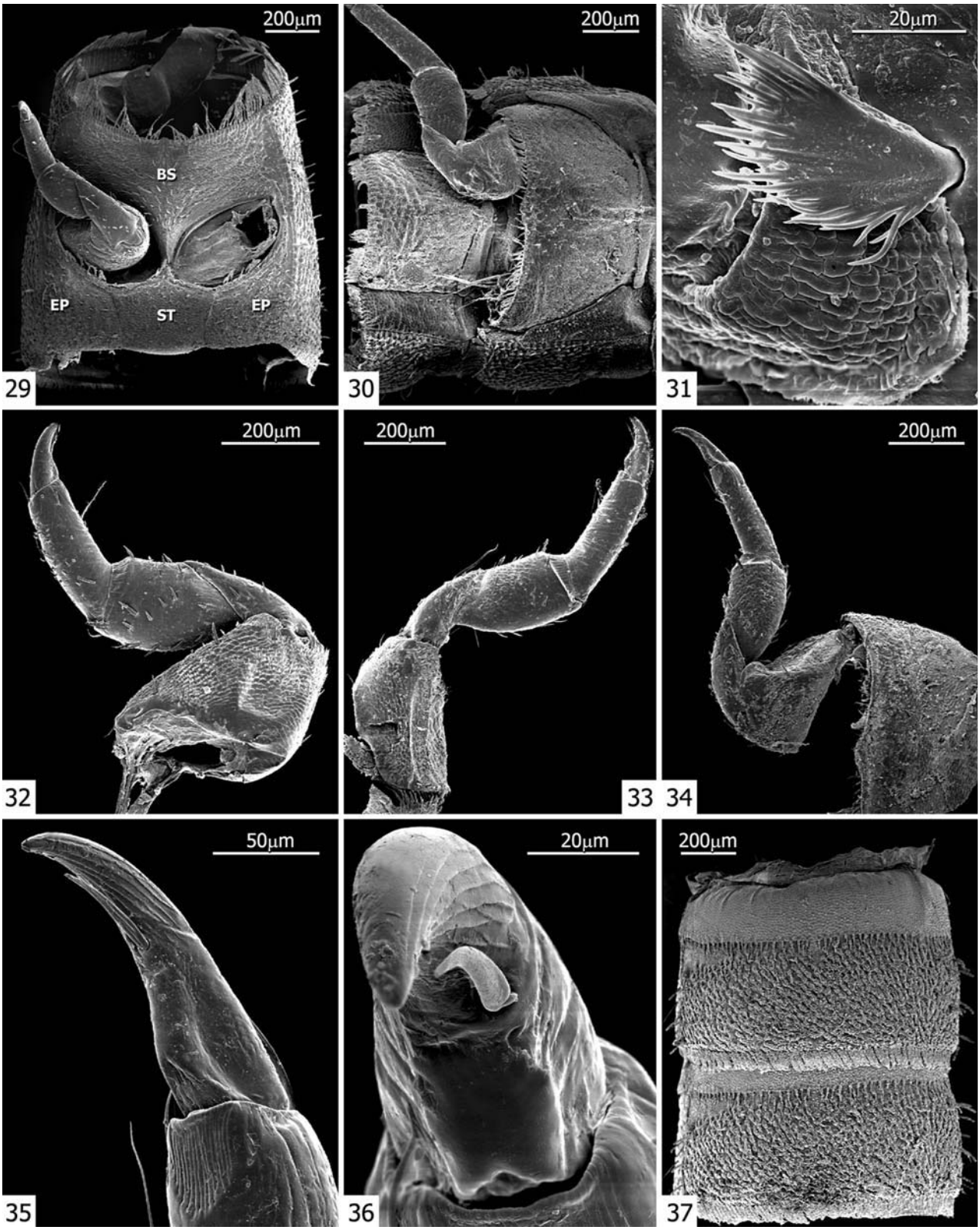
lindrical without flattened thoracic and abdominal segments; (2) dorsal side of segments without sagital carina; (3) abdominal pleura present on segments 1–7; (4) abdominal segment 9 rounded apically in dorsal view, flattened and strongly slanting posteriad.



Figs 24–28. *Hedyselmis opis* larva: 24 – maxillary palpus, ventral view; 25 – apical sensory field of maxillary palpus, apical view; 26 – labial palpus, ventral view; 27 – same, detail of apical sensory field; 28 – labium, ventral side. AS – admedian sensillum, LS – lateral seta. Scale: 0.1 mm.

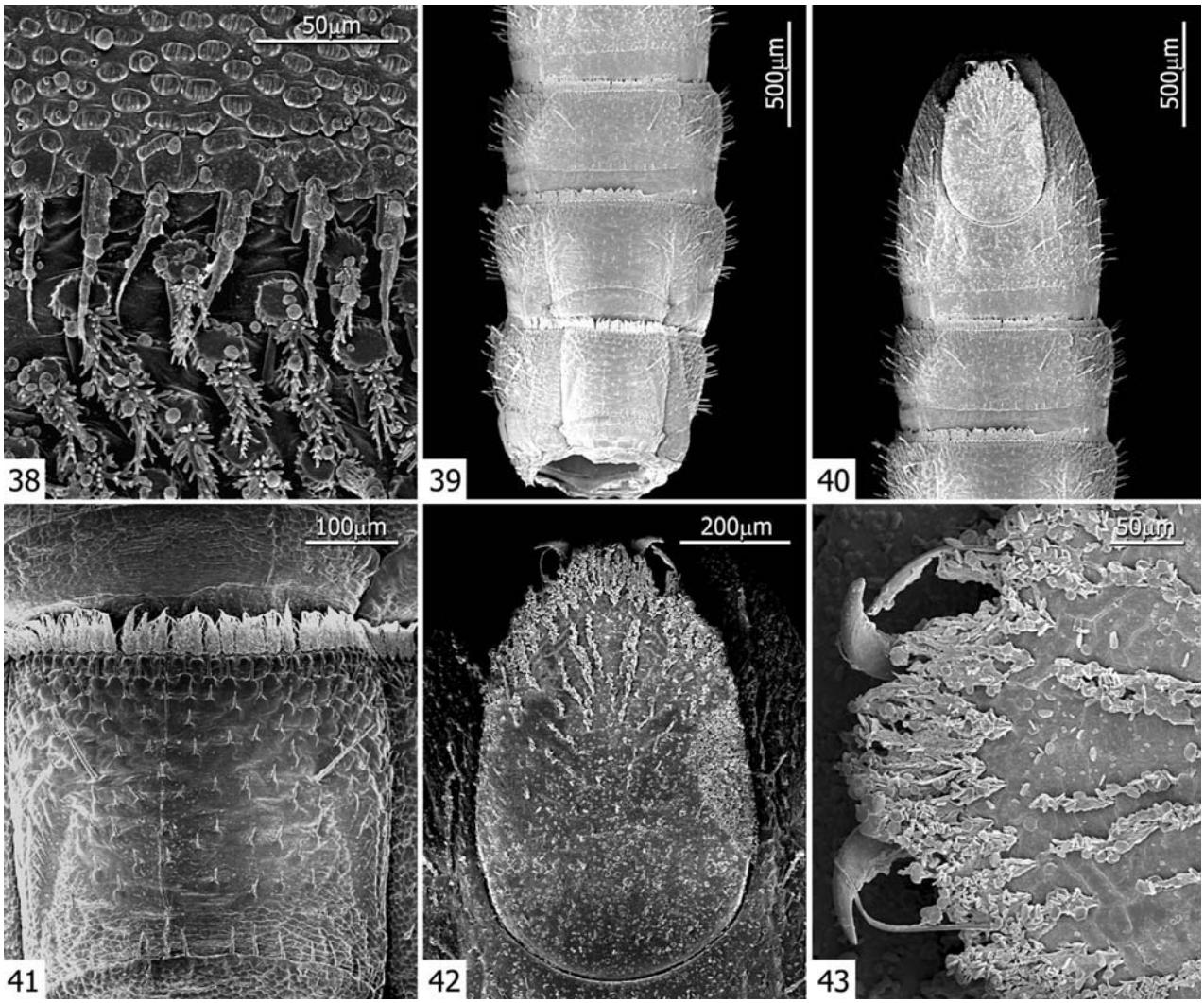
Habitus (Fig. 1). Length 8.3–8.8 mm, greatest width 1.2–1.3 mm. Colour dark brown, ventral side and legs slightly lighter. Body elongate, subcylindrical, parallel-sided, convex dorsally, almost flat ventrally. Biforous spiracles present laterally on mesothorax and abdominal segments 1–8 (Fig. 2). Integument with dense, light, tufted scales (Figs 3, 4) and two types of sparse yellow setae: (1) long acuminate setae (Fig. 5); (2) shorter spiky setae (Fig. 6). Dorsal side of cranium, dorsal and lateral sides of thorax and abdomen microgranulate; ventral side rugose; genae microreticulate.

Head (Fig. 7) rounded, prognathous, partially retracted into prothorax, about as long as wide, without tooth on anterior margin between base of antenna and clypeus; epicranial sutures hardly visible. Stemmata clustered, forming a single "eye", distally surrounded by several distinctly long thin setae (Fig. 8). Antenna three-segmented (Fig. 9); scape widest, with subapical row of scale-like setae; pedicel elongate, about twice as long as scape; flagellum setiform with apical sensillum with blunt tip; sensorium as long as flagellum (Fig. 10). Labrum short, about twice as wide as long, with pair of distinctly long admedian setae; anterior third covered by dense yellowish branched scales (Fig. 11); tormae curved (Figs 12, 13); epipharynx with dense, mesally directed short setae on both sides (Fig. 13). Clypeus wider than long, microgranulate; frontoclypeal suture well indicated. Mandible (Figs 14–17) well developed, palmate, with three oblique apical teeth; incisor area with long curved articulated setose process (Figs 14–16); prostheca and mola absent; outer margin subapically with distinct tuft of long branched setae; ventral condyle rounded; ventral side with small tuft of short setae near insertion of articulated setose process (Fig. 14 arrow); dorsal side with oblique grooves beneath subapical setal tuft (Fig. 17). Maxilla (Figs 18–25) slender; cardo small, transverse, triangular with one mesal spiky seta (Fig. 20); stipes elongate, ventrally with transverse prebasal tuft of branched setae (Fig. 23) and subapical long acuminate sensillum (Fig. 22 arrow); outer margin with adherent branched setae; galea and lacinia subequally long, with truncate apex and with brush of stout, peg-like setae (Fig. 21); maxillary palpus



Figs 29–37. *Hedyselmis opis* larva: 29 – prosternum (BS – basisternum, EP – epimeron, ST – sternellum), ventral view; 30 – metasternum, ventral view; 31 – spiky sensillum on trochanter, ventral view; 32 – left fore-leg, dorsal view; 33 – left mid-leg, ventral view; 34 – right hind-leg, ventral view; 35 – claw, ventral view; 36 – same, detail of ventral seta; 37 – abdominal tergites 1–2, dorsal view.

(Fig. 24) four-segmented, slightly longer than galea; apical sensory field of distal segment with some four types of sensilla (Fig. 25). Labium (Figs 26–28). Prementum short, transverse, membranous; ligula setose with two admedian sensilla; labial palpi two-segmented; segment 1 subglobular with dense row of long setae; segment



Figs 38–43. *Hedyselmis opis* larva: 38 – detail of abdominal dorsal surface; 39 – venter of abdominal segments 3–6; 40 – venter of abdominal segments 7–9; 41 – detail of abdominal sternum; 42 – abdominal operculum; 43 – apex of operculum with apical claws.

2 peg-like, with apical sensory field (Fig. 27); palpiger slightly wider than segment 2. Postmentum undivided, elongate, widened apicad, with paired, distinctly long lateral setae; surface in apical fourth and along midline covered by long branched setae (Fig. 28). Hypopharynx simple**.**

Thorax. Protergum slightly longer than wide, widest in posterior half; anterior margin feebly arcuate; lateral sides slightly converging anteriad; disc without depressions or tubercles, with several bald microreticulate spots on posterior half (Fig. 1). Meso- and metatergum wider than long, about half as long as protergum, both with small admedian microreticulate spot on each side. Venter of prothorax (Fig. 29) with: (1) transverse basisternum (acuminately projected between coxae) fused with episterna; (2) paired epimera widened behind procoxae; (3) subpentagonal sternellum with short anterior acuminate projection between procoxae. Cervicosternum longer than wide, lightly sclerotized, folded between head capsule and probasisternum. Venter of mesothorax with small rudiments of episternum and epimeron separated by coxal cavities, basisternum large, transverse, fused with transversally triangular intersternite; portion behind coxae membranous; laterotergite bearing spiracle fused with tergum. Metathorax (Fig. 30) with episternum large; epimeron small, reduced; basisternum large, narrowed anteriad; portion behind coxae same as in mesothorax. Legs short (Figs 32–34), all subequal in length; each coxa subtriangular in ventral view, transverse in resting position, excavated ventrally; trochanter almost as long as femur, each ventrally with flattened spiky sensillum (Fig. 31); tibia slightly longer than femur, with few hair-like setae; claw (Fig. 35) slightly shorter than half length of tibia, moderately curved, grooved in distal half, with single stout seta on the middle of the ventral surface (Fig. 36).

Abdomen (Figs 37–43). Abdominal segments 1–8 simple, subequal in length, wider than long, distal margin of segments with row of spiky scales (Figs 37, 41); segments 1–7 with distinct tergum, paired elongate pleura and subquadrate sternum (Figs 39–41), segment 8 with all sclerites fused; segment 9 with apex rounded, posterior

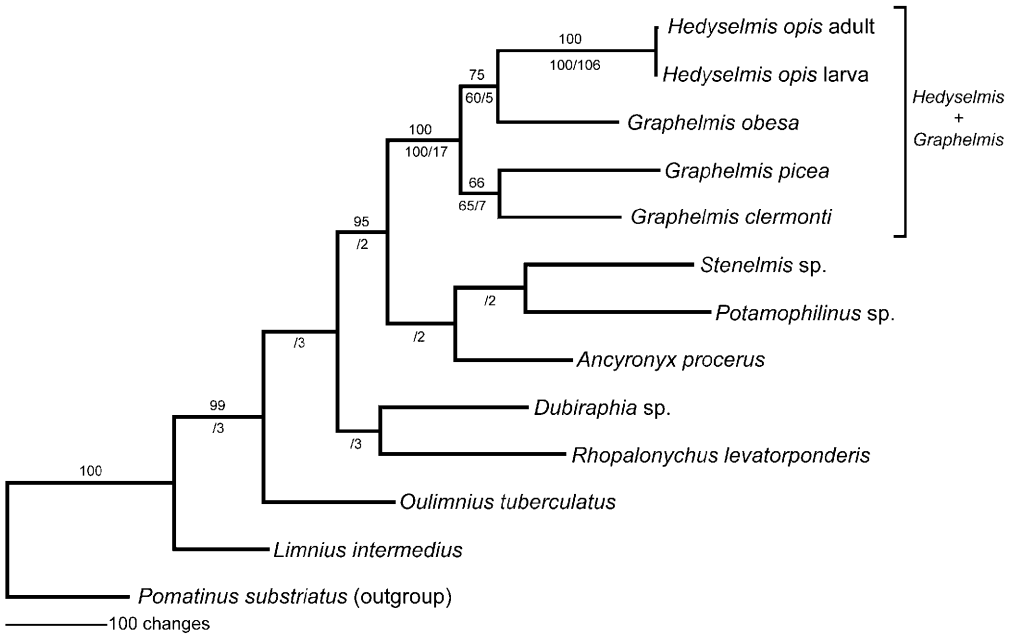


Fig. 44. Single most parsimonious tree obtained using the combined dataset. Above branches, posterior probability values (×100) of the Bayesian analyses (only if > 0.5); below branches, bootstrap (only if more than 50%) / Bremer support values of the parsimony analyses.

0.6 flattened dorsally and strongly slanting posteriad; operculum (Fig. 42) oval, reaching apex of segment 9 with dense setae along distal margin; opercular claws well developed (Fig. 43). Anterior portion of segments (normally concealed by preceding segment) with grooved microgranules; exposed portion covered by dense, shortly branched setae inserted on small tubercles; line between these portions with hair-like setae (Fig. 38).

DISCUSSION

In Coleoptera almost identical sequences for the genes used in the present work are found only within the same species, usually from the same or closely linked populations (see e.g. Ribera et al., 2003). Although there are almost no available molecular data on species of Elmidae, it is very likely that the same is true for this family.

Some species in very closely related species complexes of recent origin may have nearly identical or identical mitochondrial haplotypes, even though they can be separated using morphological characters (e.g. Ribera & Vogler, 2004). In our case, the genus has only two known, well-characterised species with presumably allopatric distributions (Jäch & Boukal, 1997) and it is highly unlikely that there were larvae of a very closely related undescribed species of the same genus at the same locality (with no adults). Thus, it is highly likely the described larva belongs to *Hedyselmis opis*.

All our phylogenetic analyses support a close relationship between *Hedyselmis* and *Graphelmis*, as suggested by Jäch & Boukal (1997). *Hedyselmis* and *Graphelmis* adults share several common characters (shape of prosternal process, fused third and fourth elytral stria, basal teeth on tarsal claws, shape of male pregenital segment), supporting this very close relationship (Jäch & Boukal, 1997 and F. Čiampor Jr, unpubl. observ.). The larva of *Graphelmis* has not been formally described, but it is mentioned in Glaister (1999). Based on this work, as well as personal communication with the author, some larvae collected in Malaysia were preliminary assigned to the genus *Graphelmis*, although further study is needed to support this identification. These larvae are generally similar to those of *Hedyselmis*, differing only in having less flattened integument scales, paired spines on dorsal side of the abdominal segment 9, slightly longer legs and lighter colouration with a yellowish pattern.

Jäch & Boukal (1997) suggest that *Cephalolimnius* Delève, 1973 is also a close relative of *Hedyselmis.* This assumption is supported by the wing venation and morphology of male unpaired sclerite of spiculum gastrale. Unfortunately specimens of this genus were not available for study, so the relationship *Hedyselmis – Cephalolimnius* could not be tested.

Although our study suggests that *Graphelmis* may be paraphyletic with respect to *Hedyselmis*, we refrain here from introducing any formal nomenclatorial change. Our sampling of *Graphelmis*, as well as the knowledge of their larval stages, is insufficient to allow a definitive inclusion of *Hedyselmis* within *Graphelmis*.

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