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SYSTEMATICS AND REDESCRIPTION OF THE EUROPEAN MEIOFAUNAL SLUG *MICROHEDYLE GLANDULIFERA* (KOWALEVSKY, 1901) (HETEROBRANCHIA: ACOCHLIDIA): EVIDENCE FROM MOLECULES AND MORPHOLOGY

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ABSTRACT

Despite the long history of meiofaunal research in Europe our knowledge of its Acochlidia—the most diverse, abundant and widespread group of interstitial slugs—is still fragmentary. Distribution ranges and modes of dispersal are unknown and taxonomic hypotheses based on traditional light-microscopical examination have never been tested against a modern integrative approach combining microanatomical techniques with molecular analyses. This study redescribes *Microhedyle glandulifera* (Kowalevsky, 1901), a key species for microhedylid taxonomy and focus of taxonomic disorder. Three-dimensional reconstructions from histological semithin serial sections reveal several previously unknown characters, in particular concerning the nervous system (e.g. presence of gastro-oesophageal ganglia). There are no jaws, but a 'cuticular element' is attached anteriorly to the radula cushion. Scanning electron microscopic examination shows a radula with the formula 34–38 × 1.1.1. *Microhedyle glandulifera* can be distinguished from other Microhedylidae by a combination of external and radular features, and the unique presence of triaxonic spicules. Population genetic analyses based on mitochondrial markers support *M. glandulifera* as a widespread European species known to range from the North Sea to the Sea of Marmara (eastern Mediterranean). Accordingly, northern Atlantic '*M. lactea*' and Mediterranean '*M. glomerans*' are confirmed as junior synonyms of *M. glandulifera*. Molecular data indicate a recent radiation of *M. glandulifera* in European waters and potential means of dispersal in meiofaunal slugs with low reproductive output and no pelagic larval stages are discussed. Based on our molecular phylogeny and revision of distinguishing morphological characters, four valid Microhedylidae species occur in European waters: *Pontohedyle milaschewitchii*, *Parhedyle tyrtowii*, *Parhedyle cryptophthalma* and *Microhedyle glandulifera*. Morphological and molecular evidence indicate that *Microhedyle odhneri* is a member of the genus *Parhedyle*, and possibly a junior synonym of *Parhedyle tyrtowii*.

INTRODUCTION

European waters have a long tradition of meiofaunal research and several of the first descriptions of meiofaunal taxa were made at marine research centres at Kristineberg (Sweden), Heligoland (Germany), Roscoff, Banyuls-sur-Mer (both France) or Sebastopol (Ukraine) (see Coull & Giere, 1988 on history of meiofauna research). Despite having the best-studied meiofauna in the world, our knowledge of certain European groups is still fragmentary concerning number of species, their phylogenetic relationships, distributional ranges and modes of dispersal.

In the meiofauna Acochlidia are the most successful group of heterobranch gastropods in regard to diversity and local densities (Swedmark, 1968; Poizat, 1986, 1991). Due to their vulnerability to degradation of their habitat (e.g. due to increasing pollution), they have been shown to be valuable indicator organisms for clean and well-oxygenated sediments (Poizat, 1984, 1985). A solid taxonomic framework is needed for marine biodiversity estimations, conservational efforts and ecological approaches. However, for Acochlidia in general—and European Microhedylidae in particular—systematics are complicated by (1) original descriptions that lack detail; (2) poor understanding of some distinguishing characters and their

intraspecific variation; (3) frequent loss of type material; and (4) imprecise type localities. *Pontohedyle milaschewitchii* (Kowalevsky, 1901) has recently been redescribed in detail (Jörger *et al*., 2008, 2009) and is well characterized by the presence of only one pair of bow-shaped head appendages. *Pontohedyle milaschewitchii* is thus considered a taxonomically unambiguous member of the European meiofauna, with reported collecting localities throughout the Mediterranean and Black Sea (see e.g. Kowalevsky, 1901; Arnaud, Poizat & Salvini-Plawen, 1986; Wawra, 1986; Poizat, 1991). In contrast, the taxonomic validity and distribution range of slender microhedylids with two pairs of head appendages is still uncertain. In one of his pioneering studies on meiofaunal gastropods, Kowalevsky (1901) described *Parhedyle tyrtowii* (Kowalevsky, 1901) and *Microhedyle glandulifera* (Kowalevsky, 1901) (both as *Hedyle*) from the Black Sea, Sea of Marmara and eastern Mediterranean, but unfortunately no type material remains from his studies (Wawra, 1974, 1978). While the original description of *P. tyrtowii* is detailed given the resources available at that time, *M. glandulifera* was only briefly described in comparison to *P. tyrtowii*. Later, *Microhedyle lactea* Hertling, 1930 was described from Heligoland (North Sea, Germany) as a geographic subspecies of Mediterranean and Black Sea *M. glandulifera* (see Hertling, 1930). Odhner (1937) elevated it (without further comment) to the rank of species and further sampling localities were reported from Banyuls-sur-Mer (France, Mediterranean) (Odhner, 1952) and Arcachon (France, Atlantic) (Marcus & Marcus, 1955). Additionally, Salvini-Plawen (1973) described *M. glomerans* Salvini-Plawen, 1973 from Secche della Meloria (Livorno, Italy). Without revising any material or providing any additional data, Rankin (1979) created the new species *M. napolitana* (Rankin, 1979) (as *Stellaspina*), referring only to a brief description of *M. glandulifera* found in Naples (Italy) by Marcus (1954). To resolve taxonomic issues, Wawra (1974) recollected *M. glandulifera* from its type locality in Greece. In a detailed taxonomic revision Wawra (1978) corrected the radula formula of *M. glandulifera* to 1.1.1. Comparing morphology of *Microhedyle* populations from Lesbos (Greece, type locality of *M. glandulifera*), Rovinj (Croatia), Livorno (Italy), Banyuls (France) and Heligoland (type locality of *M. lactea*), Wawra (1978) demonstrated the coloration of the digestive gland and numbers of rows of radula teeth to vary within and among populations rather than distinguishing the two species; thus, he considered *M. lactea* as junior synonym of a widespread Mediterranean and Atlantic *M. glandulifera*. In his later classification of Acochlidia, Wawra (1987) also synonymized *M. glomerans* and *M. napolitana* with *M. glandulifera*, although without detailed discussion. Here we give a morphological redescription of the key species *Microhedyle glandulifera* using modern technologies [i.e. scanning electron microscopy (SEM) of the radula and three-dimensional (3D) reconstruction from histological semithin sections] as a basis for a taxonomic revision of European Acochlidia.

Recent integrative taxonomic approaches testing traditional taxonomy against molecular data have revealed flocks of cryptic species across different meiofaunal taxa, e.g. polychaete annelids (Schmidt & Westheide, 1999; Schmidt & Westheide, 2000), proseriate flatworms (Casu & Curini-Galletti, 2004; Casu *et al*., 2009), gastrotrichs (Todaro *et al*., 1996; Leasi & Todaro, 2009) and acochlidian gastropods (Neusser, Jörger & Schrödl, 2011b). Minute body size, low reproductive output and the frequent absence of pelagic larvae (Swedmark, 1959, 1964) make meiofaunal taxa prone to reproductive isolation and potential cryptic speciation. Wawra's (1987) taxonomic hypothesis based on morphological characters (synonymizing Northern Sea and Atlantic *M. lactea*, Mediterranean *M. glomerans* and *M. napolitana* with *M. glandulifera*) is here tested with molecular markers.

MATERIAL AND METHODS

Sampling

Microhedylid Acochlidia were collected from nine different localities along the European coast, including the North Sea, Atlantic Ocean, Mediterranean Sea and Black Sea. Wherever possible, we collected at type localities (or additional localities reported in the original literature) and also covered some sites in between (Fig. 1, Table 1). For morphological redescription, *M. glandulifera* was collected near Rovinj, Croatia (Mediterranean), a locality where populations had been previously collected and exhaustively compared morphologically to *M. glandulifera* from the type locality on Lesbos, Greece (Wawra, 1978). Specimens were extracted from sand samples following the method described by Schrödl (2006). Living specimens were investigated under the light microscope, mainly for the presence and types of spicules. For molecular purposes and for radula preparation specimens were fixed in 96% ethanol. For histological work specimens were slowly anesthetized using $MgCl_2$ to prevent them from retracting into their visceral hump and subsequently fixed in 4% glutaraldehyde (buffered in cacodylate).

Morphological analysis of Microhedyle glandulifera

For histological work, specimens were embedded in Spurr's low-viscosity epoxy resin (Spurr, 1969), following the protocol previously used for micromolluscs (e.g. Neusser *et al*., 2006). Semithin serial sections (1.5 μm) of eight individuals (all subadult juveniles) were prepared using a Histo Jumbo diamond knife (Diatome, Biel, Switzerland) with a rotation microtome (HM 360, Zeiss, Germany) and glue on the lower cutting edge, after the method described by Ruthensteiner (2008). Sections were stained with a 1:1 dilution of Richardson's Blue for 20–25 s (Richardson, Jarett & Finke, 1960). Every section was photographed through a Leica DMB-RBE microscope (Leica Microsystems, Wetzlar, Germany) with mounted Spot CCD camera (Spot Insight, Diagnostic Instruments, Sterling Heights, MI, USA). Photographs were then edited (i.e. downsized, converted to greyscale, un-sharp masked and contrast enhanced) with standard picture-editing software. A computer-

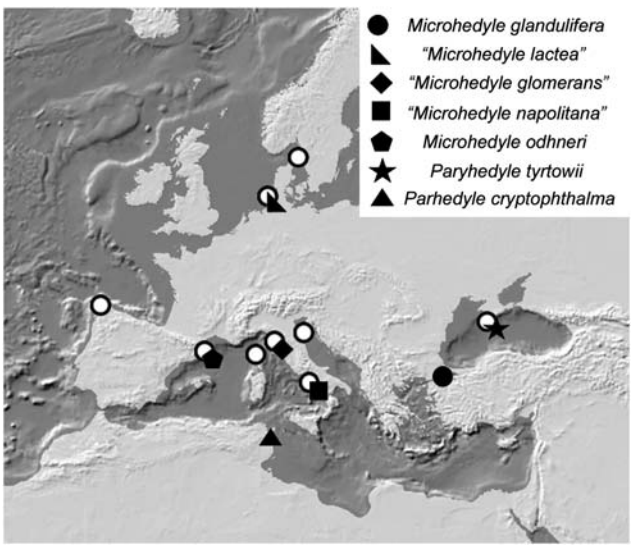

Figure 1. Type localities of European Microhedylidae species (solid symbols) and sampling localities for the present study (open circles, see also Table 1).

Table 1. Collecting sites and remarks on the habitat of European Microhedylidae sampled.

Collecting site	Location	GPS (retrieved from Google Earth)	Habitat description
Kristineberg	Bonden Island, Bohuslän, Sweden, North Sea	—	Subtidal, 20 m, coarse sand and shell gravel
Ferrol	La Coruna, Galicia, Spain, Atlantic Ocean	43°16′12″N, 08°12′11″E	Subtidal, 41 m, medium-grained sand
Canet-Plage	Languedoc-Roussillon, France, Mediterranean Sea	42°39′55″N, 03°02′06″E	Subtidal, 1 m, fine sand
Calvi	Bay of Revellata, Corsica, France, Mediterranean Sea	42°33′57″N, 8°44′15″E	Subtidal, 22 m, coarse sand and shell gravel
Livorno	Secche della Meloria, Tuscany, Italy, Mediterranean Sea	43°33′01″N, 10°13′08″E	Subtidal, 3–4 m, coarse sand
Cape Kamenjak	Premantura, Istria, Croatia, Mediterranean Sea	44°46′03″N 13°54′58″E	Subtidal, 6–9 m, coarse sand
Rovinj	Istria, Croatia, Mediterranean Sea	45°04′05″N, 13°02′14″E	Subtidal, 2–3 m, coarse sand
Sebastopol	Cape Fiolent, Crimea, Ukraine, Black Sea	—	Subtidal, 15 m
Heligoland	Germany, North Sea	—	Subtidal, coarse sand

Table 2. Acochlidian specimens used for phylogenetic analysis of European Microhedylidae, with sampling localities, museums voucher numbers (ZSM, Bavarian State Collection of Zoology), DNA voucher accession numbers and GenBank accession numbers.

Species	Collecting sites	ZSM number	DNA Bank accession number	GenBank accession number		
				28S rRNA	16S rRNA	COI
Hedylopsis spiculifera	Rovinj	20080951	AB35081816	HQ168443	HQ168417	HQ168455
Pontohedyle milaschewitchii	Cape Kamenjak	20080054	AB34404241	JF828043	HQ168422	HQ168459
Parhedyle cryptophthalma	Naples	20100584	AB34599403	JF828041	JF828042	JF828033
Parhedyle tyrtowii	Sebastopol	20091369	AB35081774	JF819813*	—	JF819818*
Microhedyle odhneri	Canet-Plage	20090571	AB35081818	JF819814*	—	JF819819*
Microhedyle glandulifera	Cape Kamenjak	20081019	AB35081799	HQ168449	HQ168424	HQ168461
Microhedyle glandulifera	Rovinj	20080056	AB34404242	—	JF819815*	JF819777*
"*Microhedyle glomerans*"	Livorno	20080413	AB35081799	—	JF819816*	JF819780*
"*Microhedyle lactea*"	Kristineberg	20080136	AB34404283	—	JF819817*	JF819778*

Sequences generated for the present study are marked with *.

based 3D reconstruction of the nervous and digestive systems of *M. glandulifera* was created (based on ZSM Mol 20090600) using Amira v.4.1 software (Visage Imaging GmbH, Germany), following the method described by Ruthensteiner (2008). All section series are deposited in the Mollusca Department of the Bavarian State Collection for Zoology (museums numbers: ZSM Mol 20090600, 20100610, 20100612–615).

For examination of the radulae by SEM, five specimens of *M. glandulifera* were dissolved in a proteinase K solution (90 µl ATL buffer + 10 µl proteinase derived from the Qiagen DNeasy Blood and Tissue Kit). Subsequently, radulae were rinsed several times in ultrapure water and placed onto SEM stubs with self-adhesive carbon stickers. The stubs were coated with gold for 120 s in a Polaron Sputter Coater and viewed with a LEO 1430 VP SEM (15 kV).

DNA extraction, PCR and sequencing

DNA was extracted from entire specimens using the Qiagen DNeasy Blood and Tissue Kit according to the manufacturer's protocol. For phylogenetic analyses portions of three markers were amplified with PCR using the protocols and primers described by Jörger *et al.* (2010a): nuclear 28S rRNA and mitochondrial 16S rRNA and cytochrome oxidase *c* subunit I (COI). PCR products were cleaned with ExoSAP-IT (Affymetrix) and sequenced using the PCR primers in both directions by the Genomic Service Unit (GSU) of the Department of Biology of the Ludwig-Maximilians-University Munich (Big Dye v.3.1; ABI 3730 capillary sequencer). Sequence data of microhedylacean *Parhedyle cryptophthalma*, *Pontohedyle milaschewitchii* and the hedylopsacean *Hedylopsis spiculifera* were retrieved from GenBank (Table 2).

For population genetic studies on Mediterranean *Microhedyle glandulifera* (including '*M. lactea*' and '*M. glomerans*') the partial COI (654 bp) was sequenced as described above from 36 individuals belonging to seven populations.

All sequences generated were deposited in GenBank (see Tables 2, 3). DNA vouchers are available from the DNA Bank of the Bavarian State Collection of Zoology (ZSM) and (if available) voucher specimens were deposited in ZSM.

Phylogenetic analyses

Sequences were edited with Geneious Pro v.5.2 (Biomatters) and checked with BLAST searches (Altschul *et al.*, 1990) against potential contaminations via the NCBI webpage (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Alignments for each marker were obtained with MUSCLE v.3.8 (Edgar, 2004) and the COI alignment was afterwards checked manually according to amino acid translation. We concatenated the resulting alignments using BioEdit (Hall, 1999). Phylogenetic analyses of the combined dataset (28S + 16S + COI) were conducted using RAxML v.7.0.4 (Stamatakis, 2006). Data were analysed in three partitions (according to each marker) under the GTR + Γ + I model, selected as best-fitting model of nucleotide substitution with jModeltest (Posada, 2008). Analyses were conducted following the RAxML manual ("hard and slow way"), with hedylopsacean *Hedylopsis spiculifera* defined as outgroup. Statistical support for each node was estimated via multiple nonparametric bootstrapping (1,000 replicates).

Table 3. Mitochondrial COI sequences generated within this study, for population genetics on *Microhedyle glandulifera* (including '*M. glomerans*' and '*M. lactea*').

Species	Collecting sites	ZSM number	DNA Bank voucher	GenBank accession number (COI)
M. glandulifera	Cape	20080056	AB34404242	JF819777
	Kamenjak	20081019	AB35081799	HQ168461§
		20091332	AB35081756	JF819791
		20091168	AB34858195	JF819786*
		20091169	AB34404297	JF819787
		20100411	AB34402384	JF819792
		20100412	AB35081778	JF819793
		20100413	AB34858243	JF819794
		20100414	AB34858183	JF819795
		20100415	AB34858186	JF819796
		20100416	—	JF819797
		20100417	AB35081836	JF819798
		20110027	AB35081779	JF819811
		20110028	AB34499233	JF819812
	Rovinj	20100419	AB35081749	JF819799
		20100420	AB34858209	JF819800
		20100421	AB34858194	JF819801
		20100422	AB34404240	JF819802
		20100423	AB35081781	JF819803
		20100424	AB34599359	JF819804
		20100425	AB34404230	JF819805
	Calvi	20080959	AB35081807	JF819781
		20080960	AB35081815	JF819782
		20091178	AB34858185	JF819790
'*M. glomerans*'	Livorno	20080413	AB34858172	JF819780
'*M. lactea*'	Ferrol	20080392	AB35081748	JF819779
	Kristineberg	20080136	AB34404283	JF819778
		20091170	AB35081831	JF819788*
		20091171	AB35081820	JF819789
		20081017	AB35081825	JF819783
		20081018	AB35081761	JF819784
	Heligoland	20100426	AB34599395	JF819806
		20100427	AB34858168	JF819807
		20100428	AB34858180	JF819808
		20100429	AB34858189	JF819809
		20100430	AB35081763	JF819810

§marks sequence retrieved from GenBank. * marks two sequences that were not included in population genetic analyses due to missing data.

Population genetic analyses

Haplotype networks of *M. glandulifera* based on mitochondrial COI sequences were inferred using statistical parsimony as implemented in TCS v.1.21 (Clement, Posada & Crandall, 2000) under the default settings (95% confidence criterion). Population genetic analyses were conducted in Arlequin v.3.5 (Excoffier & Lischer, 2010): to describe the genetic diversity of each sample, the number of haplotypes, haplotype and nucleotide diversity, and mean number of pairwise differences between populations were estimated. Additionally, pairwise F_{st} values between populations were calculated (with 1,000 permutations) and a hierarchical analysis of molecular variance (AMOVA) was conducted comparing Atlantic (Ferrol, Kristineberg and Heligoland) and Mediterranean populations (Rovinj, Cap Kamenjak, Livorno and Calvi).

SYSTEMATIC DESCRIPTION

MICROHEDYLIDAE Odhner, 1937

Microhedyle Hertling, 1930

Microhedyle glandulifera (Kowalevsky, 1901)

(Figs 2–6)

Hedyle glandulifera Kowalevsky, 1901: 1–32, pl. IV, figs 52–55.
Microhedyle glandulifera lactea Hertling, 1930: 1–11.
Microhedyle lactea Odhner, 1937: 51–64.
Microhedyle glomerans Salvini-Plawen, 1973: 123–125.
Stellaspina napolitana Rankin, 1979: 96–97.

Description: a slender, minute interstitial microhedylid (Fig. 2A), 1.5–2.5 mm in length. Head bears two pairs of thin appendages (oral tentacles and rhinophores) which are roundish in section and slightly tapering. Four main types of calcareous spicules: (1) monaxonic spicules (30–70 μm) in headfoot and visceral sac (Fig. 2D); (2) large triaxonic spicules (30–60 μm) in visceral sac (Fig. 2B, D); (3) short bean-shaped or oval spicules (15–20 μm) near posterior end of radula (Fig. 2C); (4) tiny structures like strings of beads (S-, C- or ring-shaped, 5–15 μm) distributed over entire body (Fig. 2D). Transitional forms between monaxonic and triaxonic, and occasional tetraxonic or pentaxonic, spicules were observed in some individuals. Absence of monaxonic/triaxonic spicules was recorded in specimens held in captivity for several months (see Discussion for interpretation).

Digestive system consists of oral tube, pharynx (containing radula), oesophagus, paired salivary glands, digestive gland and intestine, and follows general body plan described for other Microhedylacea (Neusser *et al*., 2006; Jörger *et al*., 2008; Neusser, Martynov & Schrödl, 2009b). Bulbous muscular pharynx 140 μm with two joined cavities, containing both rami of hook-shaped radula (Fig. 4A). Radula 100 μm (formula 34–38 × 1.1.1); rhachidian tooth triangular with central cusp and two small denticles on either side; lateral teeth rectangular with one rounded denticle at anterior margin and a corresponding notch in posterior margin (Fig. 3A–C). V-shaped 'cuticular element' (30 μm long, 6–18 μm wide) with two symmetrical sides and central groove is located in anterior part of pharyngeal cavity, attached by posterior end to lower muscle of radula cushion where oldest teeth of ventral radula ramus terminate (Fig. 4A, B). 'Cuticular element' consists of noncellular material with same properties and appearance as chitinous cuticle under light microscope; visible in histological sections but difficult to detect on whole mounts.

Central nervous system (CNS) euthyneurous, slightly epiathroid, following typical acochlidian plan: paired cerebral, rhinophoral, pedal, pleural, buccal and gastro-oesophageal ganglia; three separated, single ganglia on visceral nerve cord; single osphradial ganglion (Figs 5, 6A, C). Cerebral, pedal and pleural ganglia form prepharyngeal nerve ring; ganglia of visceral cord in posterior part of pharynx; only buccal and gastro-oesophageal ganglia are postpharyngeal.

In anterior head region (i.e. cephalic tentacles up to posterior end of pedal ganglia, total length of 175 μm) a mass of accessory ganglia (defined as ganglia-like aggregations of neuronal tissue without subdivision into cortex and medulla, according to Neusser *et al*., 2006) (Figs 5, 6B) forming two complexes on right and left sides of body, connected to cerebral ganglia by cerebral nerves. Form and size of accessory ganglia differ slightly between both sides of body, but are separated into same main portions: large anterior part connected to cerebral ganglia by rhinophoral and labiotentacular nerves; other

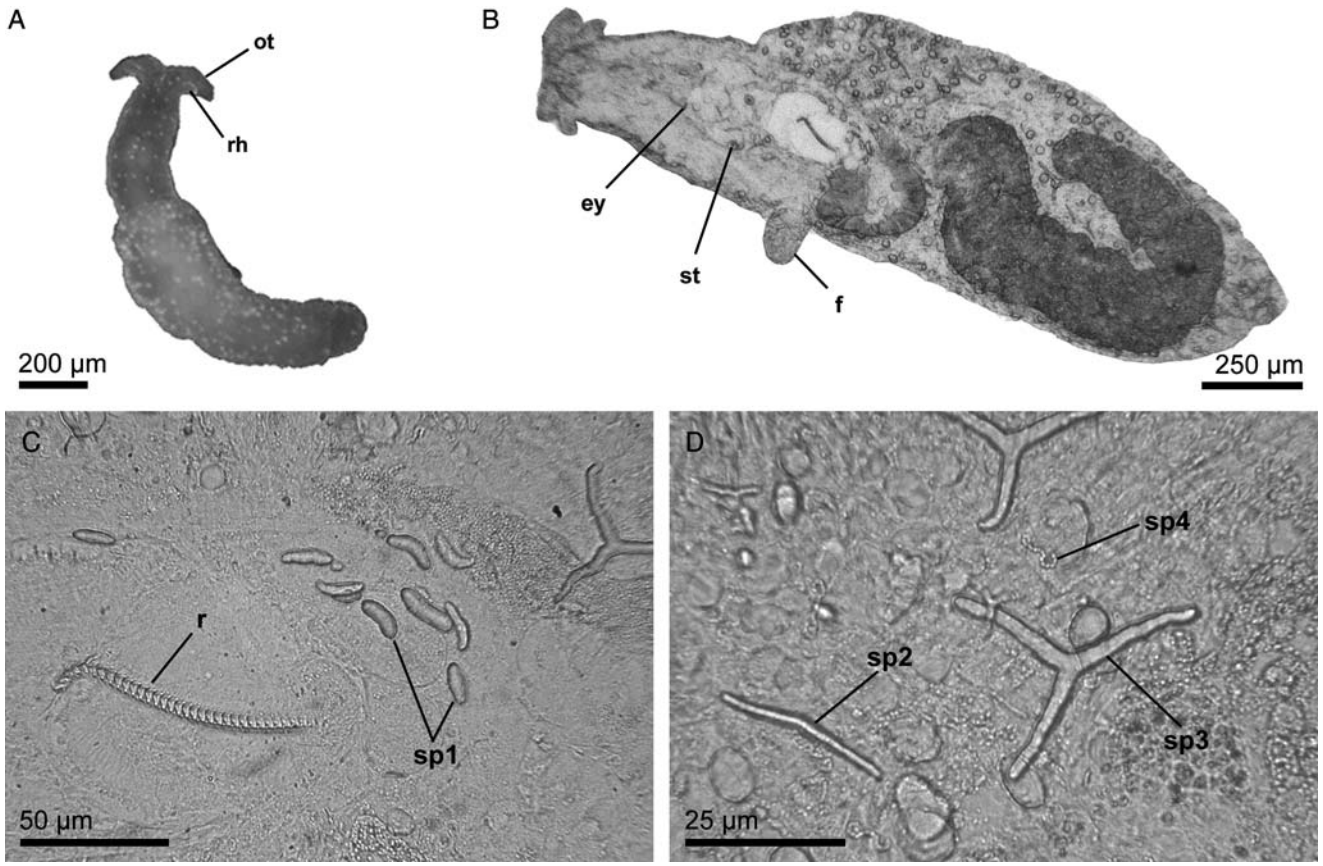

Figure 2. External morphology and spicules in *Microhedyle glandulifera*. **A.** Living specimen under dissecting microscope. **B.** Overview of an entire juvenile specimen. **C.** Accumulation of oval to bean-shaped spicules posterior to the radula. **D.** Monaxonic, triaxonic and bead string-like spicules. Abbreviations: ey, eye; f , foot; ot, oral tentacle; r, radula; rh, rhinophore; sp1, oval to bean-shaped spicules; sp2, monaxonic spicule; sp3, triaxonic spicule; sp4, bead string-like spicule; st, statocyst.

portion extends posteriorly on outer side of cerebral and pleural ganglia, and is innervated by a cerebral nerve (interpreted as Hancock's nerve). Additional pair of small accessory ganglia in foot near anterior end of pedal ganglia; no connection between these and remaining accessory ganglia. Spherical cerebral ganglia (65 µm diameter) connected via thick commissure. Slightly ventrally the strong labiotentacular nerve emerges from cerebral ganglion; rhinophoral nerve emerges dorsally. At base of rhinophoral nerve Hancock's nerve leads posterolaterally to flanking accessory ganglia. Anterolateral to cerebral ganglia Hancock's nerve leaves outer side of accessory ganglia and extends to barely visible groove in epidermis regarded as Hancock's organ (Fig. 6E). From posterior part of cerebral ganglia the thin static nerve emerges, innervating statocysts. Pigmented eyes (15 µm diameter) anteroventral to cerebral ganglia (Fig. 6D). Posterior to eyes, small rhinophoral ganglia (25 µm) close to cerebral ganglia, connected to latter via thin connective. Pedal ganglia (50 µm) bear short strong commissure; three nerves emerge ventrally from each ganglion, innervating anterior and posterior part of foot. Paired statocysts (20 µm) with one statolith each, attached posterodorsally to pedal ganglia; innervated by thin cerebral static nerve. Pleural ganglia (30 µm) posterior to cerebral and dorsal to pedal ganglia, with very short connective to cerebral ganglia and longer one to pedal ganglia (i.e. epiathroid condition of CNS). Three ganglia on visceral nerve cord: left parietal ganglion (25 µm), fused subintestinal/visceral ganglion (40 µm) and fused supraintestinal/parietal ganglion (40 µm), with smaller osphradial ganglion (25 µm) attached posteriorly via short connective. Thick visceral nerve emerges from large subintestinal/visceral ganglion and leads posteriorly to visceral sac. Buccal ganglia (35 µm) connected by commissure; slightly smaller elongated gastro-oesophageal ganglia (30 µm) nestle dorsally on buccal ganglia, connected to latter by thin connectives.

Phylogenetic analysis

In our maximum-likelihood analyses of European Microhedylidae *Pontohedyle milaschewitchii* forms the sister group to remaining Microhedylidae (Fig. 7), uniting the genera *Parhedyle* and *Microhedyle* (bootstrap probability BS = 72%). The species collected at the type locality of *M. odhneri* clusters among species of *Parhedyle* (BS = 94%), sister to *Parhedyle tyrtowii* (BS = 97%). Direct comparison of mitochondrial COI sequences show 98.25% identity between Black Sea *Parhedyle tyrtowii* and the microhedylid collected as '*M. odhneri*'. Atlantic '*M. lactea*' and Mediterranean '*M. glomerans*' form a clade with Mediterranean *M. glandulifera* (BS = 90%).

Population genetic and demographic analyses

Network analysis shows one connected haplotype network for populations of *M. glandulifera* throughout the Mediterranean and along the Atlantic Coast to the North Sea (as '*M. lactea*') (Fig. 8). The specimen collected at the type locality of *M. glomerans* (Livorno, Italy) nests within the Mediterranean *M. glandulifera*, sharing a common haplotype with two

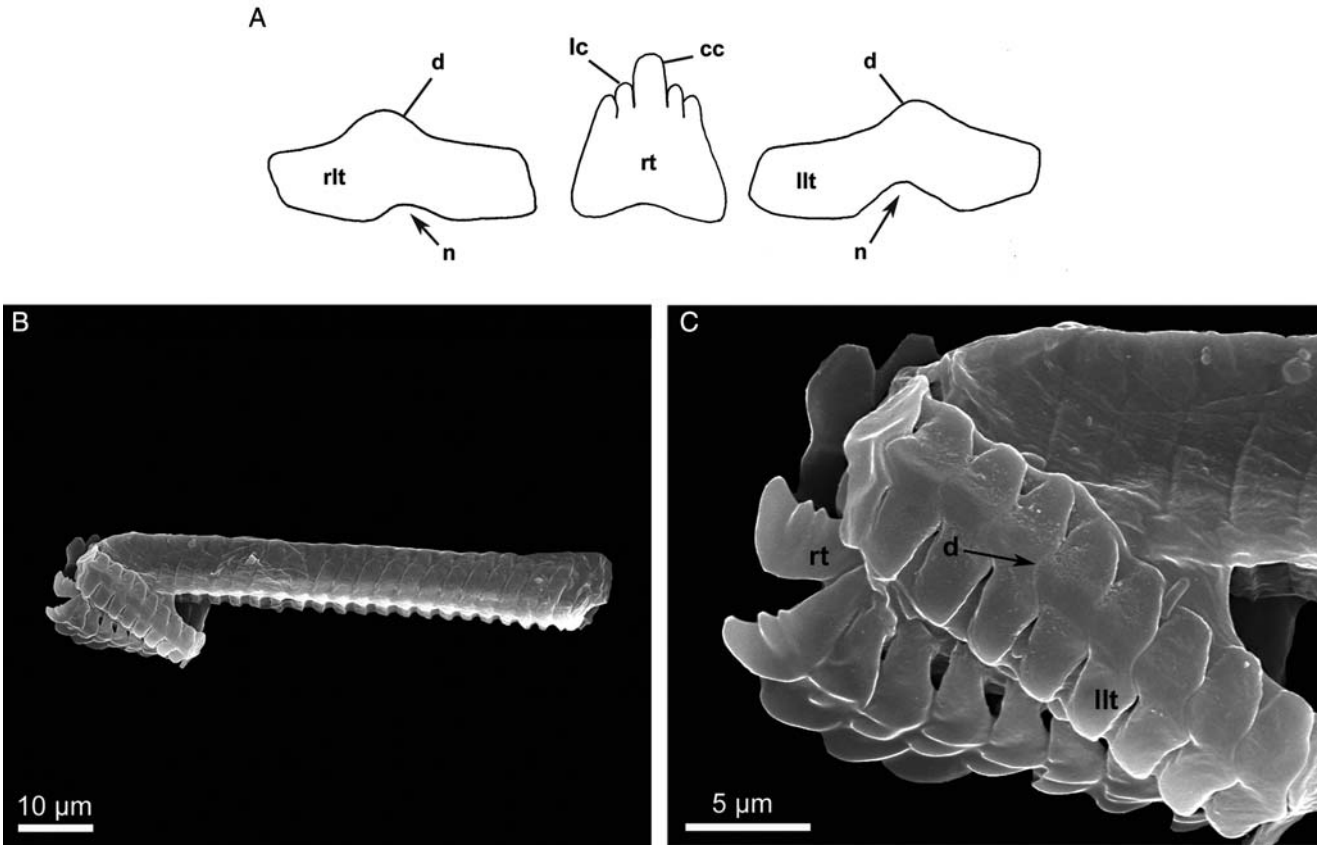

Figure 3. Radula of *Microhedyle glandulifera*. **A.** Schematic drawing of rhachidian and lateral teeth. **B, C.** Scanning electron micrographs. Abbreviations: cc, central cusp; d, denticle; lc, lateral cusp; llt, left lateral tooth; n, notch; rlt, right lateral tooth; rt, rhachidian tooth.

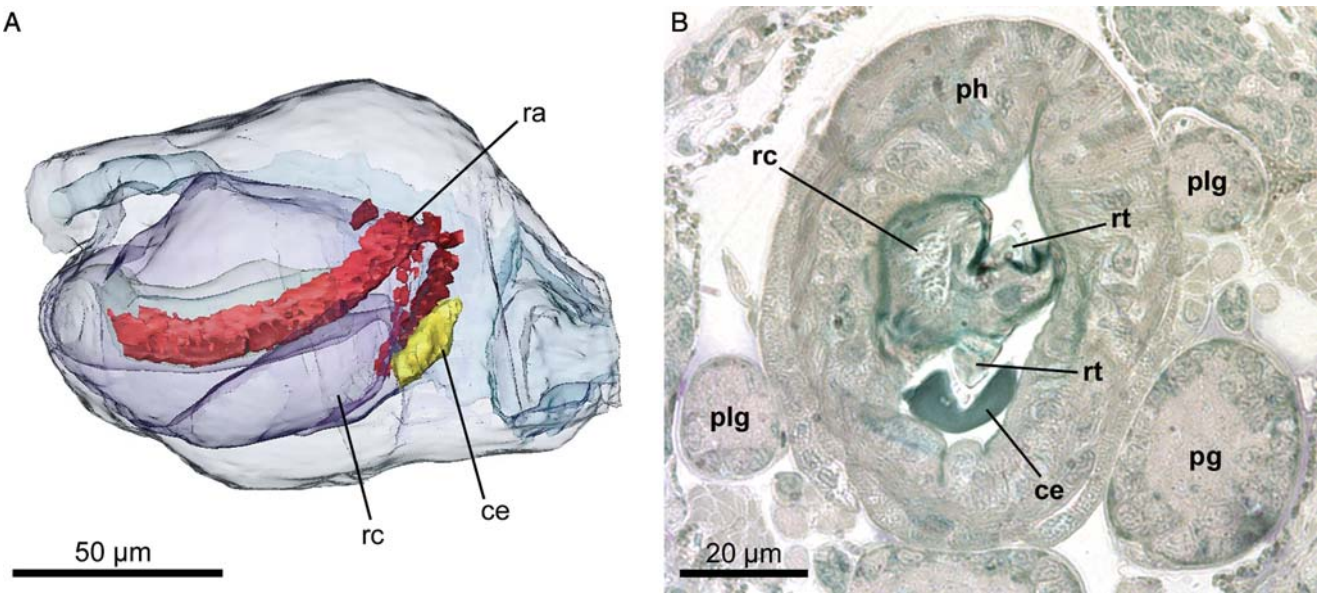

Figure 4. Three-dimensional reconstruction (**A**) and semithin cross-section (**B**) of the pharynx of *Microhedyle glandulifera* showing the position of the radula, radular cushion and 'cuticular element'. Abbreviations: ce, cuticular element; pg, pedal ganglion; ph, pharynx; plg, pleural ganglion; ra, radula; rc, radular cushion; rt, rhachidian tooth.

specimens of *M. glandulifera* collected at Rovinj and Cape Kamenjak (Croatia).

Sequences of 34 specimens (two specimens were excluded from the analyses due to missing data; Table 3) yielded 17 different mitochondrial haplotypes, 10 of which are represented by single individuals only. Populations from Heligoland ($n = 5$), Corsica ($n = 3$), Rovinj ($n = 7$) and Cape Kamenjak ($n = 13$) show relatively high haplotype diversities, ranging from

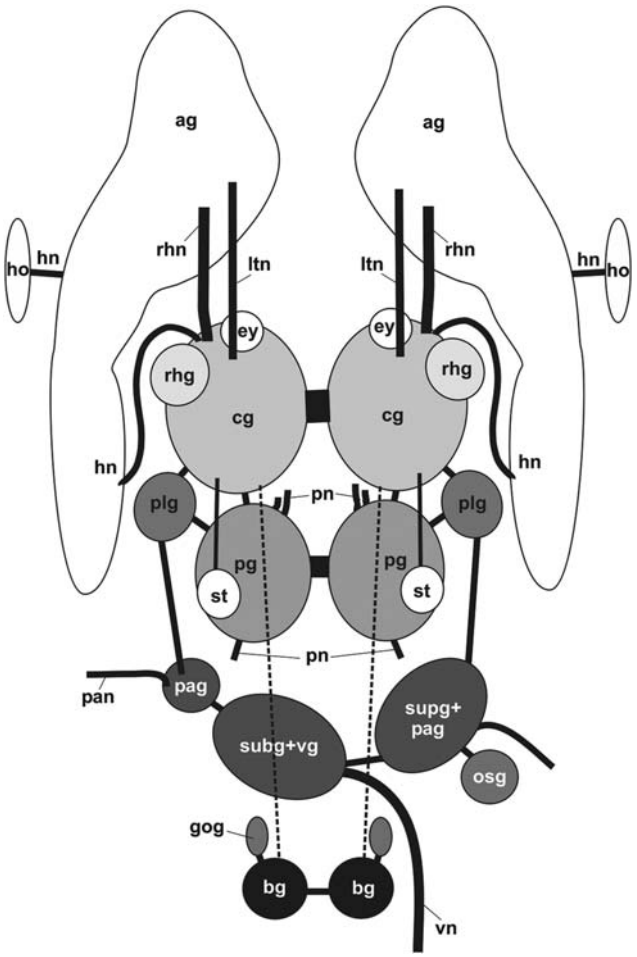

Figure 5. Schematic overview over the CNS of *Microhedyle glandulifera* (not to scale). Abbreviations: ag, accessory ganglion; bg, buccal ganglion; cg, cerebral ganglion; ey, eye; gog, gastro-oesophageal ganglion; hn, Hancock's nerve; ho, Hancock's organ; ltn, labiotentacular nerve; osg, osphradial ganglion; pag, parietal ganglion; pan, parietal nerve; pg, pedal ganglion; plg, pleural ganglion; pn, pedal nerve; rhg, rhinophoral ganglion; rhn, rhinophoral nerve; st, statocyst; subg + vg, subintestinal/visceral ganglion; supg + pag, supraintestinal/parietal ganglion; vn, visceral nerve.

0.89 to 1.00. Among the same populations, nucleotide diversity is very low (0.0036–0.0062). Average pairwise nucleotide differences were 2.41–4.09. Haplotype diversity in the Kristineberg population ($n = 4$) is comparably low (0.50) and also has low nucleotide diversity (0.0022, corresponding to 1.50 pairwise nucleotide differences). 'Populations' from Ferrol and Livorno were not considered, because they were each represented by only one individual. AMOVA analyses based on grouped datasets comparing Atlantic with Mediterranean populations showed considerably higher variation within than between these two groups (Table 4); only the F_{st} value comparing the Kristineberg to Cape Kamenjak populations is significant, but very low (0.009).

DISCUSSION

Morphology of Microhedyle glandulifera

Since the original description of *Microhedyle glandulifera* by Kowalevsky (1901), four more microhedylid species have been described in comparison to *M. glandulifera*, which has therefore become a key species for taxonomic descriptions in Microhedylidae (Hertling, 1930; Odhner, 1952; Marcus, 1953; Marcus & Marcus, 1955; Westheide & Wawra, 1974). The original description of *M. glandulifera* was, however, brief and fragmentary (e.g. lacking details on radula morphology), until it was revised by Wawra (1978) who added valuable details on spicules, radula morphology and sperm structure. Using SEM we confirm Wawra's (1978) description of the radula with a formula of 1.1.1, correcting the earlier description by Marcus (1954), who probably interpreted the denticle on the lateral teeth and the corresponding notch as cleavage in the teeth, resulting in a radula formula 2.1.2 (see Salvini-Plawen, 1973; Wawra, 1978). The radula (Fig. 3) is highly similar to the one described for Western Atlantic *M. remanei* (Marcus, 1953) apart from the absence of the second potential denticle on the lateral tooth and less prominent lateral cusps on the rhachidian tooth (Neusser *et al.*, 2006: fig. 4D). The radula of the microhedylid *Pontohedyle milaschewitchii* has the same formula ($n \times 1.1.1$), but can be clearly differentiated by the presence of three lateral cusps on the rhachidian tooth and a pointed (*vs* round) denticle on the lateral tooth (Jörger *et al.*, 2008: fig. 7C). The present study redescribes *M. glandulifera* in microanatomical detail, confirming some unusual features such as the 'cuticular element' in the pharynx and presenting novel data on the nervous system (e.g. paired gastro-oesophageal ganglia attached to the buccal ganglia). The presence of Hancock's organs as described by Edlinger (1980) was confirmed, such as the presence of an osphradial ganglion as illustrated (but not described) in a comparative study of heterobranch nervous systems (Huber, 1993: fig. 13; as *Unela*).

The 'cuticular element' in *M. glandulifera* was first described by Wawra (1978) as a bilateral separated structure fused only at its base. In our populations of *M. glandulifera* this is a V-shaped structure with a central groove surrounding the oldest portion of the radula. Its function is still a matter of speculation, but since it is only attached at its base to the radula cushion, and otherwise hanging loosely within the pharynx cavity, we consider it unlikely that it forms a jaw-like counterpart of the radula as suggested by Wawra (1978). It might instead be interpreted as protective sheet for the surrounding tissue or a sort of chute for the radula. Due to the attachment site on the radula cushion we do not consider the (paired or fused) 'cuticular element' to be homologous with paired jaws reported for microhedylacean Ganitidae or other Euthyneura.

Microhedyle glandulifera can be distinguished from other Microhedylidae by the unique presence of large triaxonic spicules (Fig. 2C), while the other types of spicules (i.e. monaxonic, bead-string-like, and oval or bean shaped) can also be found in other microhedylids. Triaxonic spicules have so far only been reported from an undescribed *Asperspina* (Asperspinidae) from North Carolina, USA (Rieger & Sterrer, 1975; as *Hedylopsis*). On one occasion we noticed the absence of triaxonic and monaxonic spicules in specimens of *M. glandulifera* (from Cape Kamenjak, with identical COI sequences to the remaining material) after being held in captivity for several months. Thus, while the presence of triaxonic spicules is characteristic for *M. glandulifera*, their absence is insufficient for species delineation. Rieger & Sterrer (1975) suggested that spicules—characteristic for many meiofaunal organisms—might be a by-product of metabolic processes. Long-time captivity and absence of natural food resources might hinder these metabolic processes, or slightly acidic conditions could potentially lead to dissolution of calcareous spicules. Spicules were also suggested to have a stabilizing effect for the surrounding tissue when moving through the interstitial habitat (Rieger & Sterrer, 1975). A conspicuous accumulation of monaxonic spicules in *Pontohedyle milaschewitchii* for example

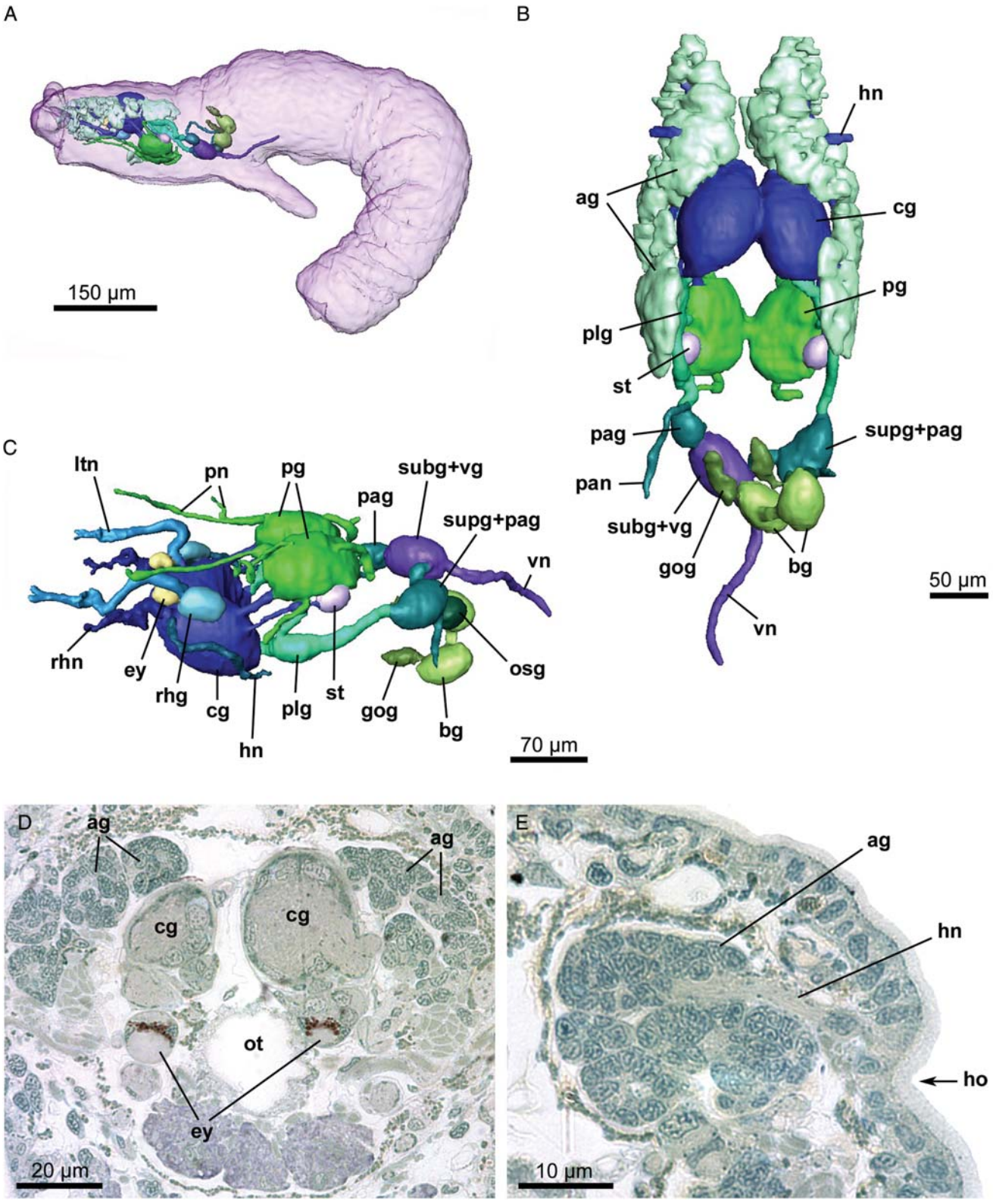

Figure 6. CNS of *Microhedyle glandulifera*. **A–C.** 3D reconstructions of CNS. **A.** Position of CNS in body. **B.** Dorsal view with accessory ganglia. **C.** Lateroventral view (accessory ganglia omitted). **D–E.** Semithin cross-sections. **D.** Cerebral ganglia with eyes. **E.** Accessory ganglia, Hancock's nerve and Hancock's organ. Abbreviations: ag, accessory ganglion; bg, buccal ganglion; cg, cerebral ganglion; ey, eye; gog, gastro-oesophageal ganglion; hn, Hancock's nerve; ho, Hancock's organ; ltn, labiotentacular nerve; osg, osphradial ganglion; ot, oral tube; pag, parietal ganglion; pan, parietal nerve; pg, pedal ganglion; plg, pleural ganglion; pn, pedal nerve; rhg, rhinophoral ganglion; rhn, rhinophoral nerve; st, statocyst; subg + vg, subintestinal/visceral ganglion; supg + pag, supraintestinal/parietal ganglion; vn, visceral nerve.

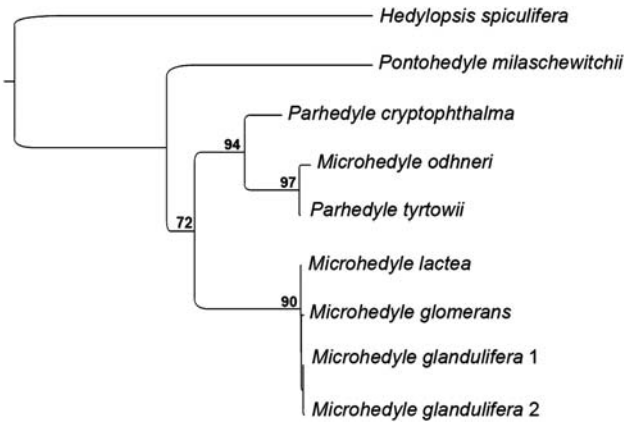

Figure 7. Phylogenetic analysis of European Microhedylidae. Maximum-likelihood tree generated with RAxML based on the concatenated dataset of 28S rRNA, 16S rRNA and COI. Bootstrap values >50% given above nodes.

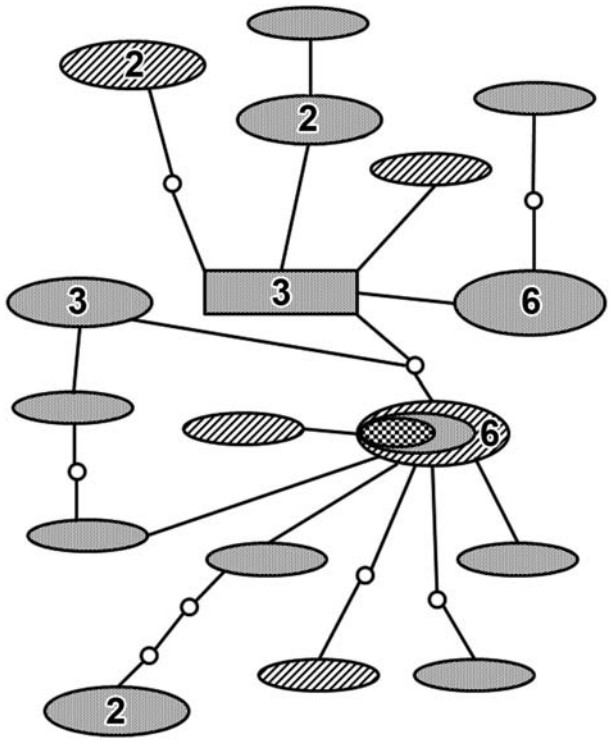

Figure 8. Statistical parsimony haplotype network of *Microhedyle glandulifera* (including '*M. lactea*' and '*M. glomerans*') based on mitochondrial COI (654 bp), generated with TCS 1.21 (Clement *et al.*, 2000). Square indicates haplotypes likely to be ancestral in this network; small, open circles represent unsampled haplotypes; numbers indicate frequency of haplotypes occurrence (if higher than one). Shading: dotted, Mediterranean; hatched, North Sea; chequered, European Atlantic.

was interpreted as a stabilizer of the head region (Jörger *et al.*, 2008). Due to their triaxonic shape, spicules of *M. glandulifera* might have an even better 3D stabilizing effect, e.g. as squeeze protection of certain organs.

Synonymy of Microhedyle glandulifera

Microhedyle lactea from Heligoland was distinguished from *M. glandulifera* by the lack of coloration in salivary and digestive glands and more rows of radula teeth (38–44 *vs* 34–35) (Hertling, 1930). Wawra (1978) demonstrated that the number of rows of teeth depends on the developmental stage and varies considerably within and among populations; this is confirmed by our observations. In several Acochlidia the coloration of the digestive glands is variable, probably depending on type and availability of food resources (Jörger *et al.*, 2008; Neusser *et al.*, 2009b; personal observations) and must thus as be treated with caution as a taxonomic character.

Table 4. AMOVA of *Microhedyle glandulifera*, Atlantic populations (Heligoland, Kristineberg and Ferrol) *vs* Mediterranean populations (Cape Kamenjak, Rovinj, Livorno and Calvi).

Source of variation	df	Sum of squares	Variance components	Percentage of variation
Among groups	1	4.421	0.26457 Va	16.60
Among populations within groups	5	4.578	−0.13511 Vb	−8.48
Within populations	27	39.531	1.46410 Vc	91.88

There are several indications that the description of *M. glomerans* from Secche della Meloria (Livorno, Italy) by Salvini-Plawen (1973) was only based on one fixed individual, e.g. due to the lack of spicules, which is not commented on as a characteristic feature, and the descriptions of external morphology "in fixed material". Salvini-Plawen (1973) mentioned that *M. glandulifera* occasionally curls up, instead of contracting, and we support Wawra's (1987) interpretation of the eponymous curling up of *M. glomerans* as a fixation artefact. The unusual "subpharyngeal position of the visceral cord" can also be interpreted as an artefact, since the relative position of ganglia varies with the stage of contraction (personal observations) and the supposedly distinguishing "folded digestive gland" depends on the (non)stretched stage of the visceral hump and has been frequently observed in Microhedylidae (personal observation). Salvini-Plawen (1973) described *M. glomerans* without eyes, but had only a whole mount of the head region available, on which barely pigmented eyes can easily be overlooked. Reinvestigation of the type material by Wawra (1987) led to the synonymization of *M. glomerans* with *M. glandulifera*, and is supported by our observations.

Microhedyle napolitana was named by Rankin (1979) based on a literature record containing a brief description by Marcus (1954) of '*M. glandulifera*' found in Naples (Italy). Wawra (1987) considered it against good taxonomic practice to establish new species based on other authors' short notes only. In the absence of any reliable distinguishing characters we follow Wawra (1987) in synonymizing *M. napolitana* with *M. glandulifera*. Morphological evidence thus supports Wawra (1978) in considering *M. lactea*, *M. glomerans* and *M. napolitana* as junior synonyms of the widespread Mediterranean and Atlantic *M. glandulifera*.

Though poorly known and only reported from its type locality at Canet-Plage (France, Mediterranean), '*Microhedyle odhneri*' has not yet been proposed as synonym of *M. glandulifera*. The original description of *M. odhneri* was based on fixed material; it briefly mentions a microhedylid body shape with two pairs of head tentacles and radula with a formula 39–48 × 1.1.1 (Marcus & Marcus, 1955). Information on spicules is lacking, but empty spaces in the epidermis are mentioned that resemble cavities of dissolved spicules. Unfortunately, the type material of *M. odhneri* could not be found in the Marcuses' collection in São Paulo and might be lost (C. Magenta Cuñha, personal communication). We collected new material at the type locality at Canet-Plage and light-microscopic investigation revealed the presence of plate-

like spicules with holes and fine bead string-like spicules. While the latter have been reported for the genera *Parhedyle* and *Microhedyle*, plate-like spicules are characteristic of *Parhedyle* (Wawra, 1987; Jörger *et al*., 2010b). Light-microscopic and SEM examination of the radula reveals the unusual asymmetric 1.1.2 formula (own unpublished data) known for *P. cryptophthalma* and suspected for *P. tyrtowii* (Westheide & Wawra, 1974; Jörger *et al*., 2010b), rather than a radula with the formula 1.1.1, as reported by Marcus & Marcus (1955); the small inner lateral tooth was obviously overlooked by the original authors due to inadequate methodology. We conclude that *M. odhneri* generically differs from *M. glandulifera*, since spicule types and radula shape indicate inclusion in *Parhedyle*.

Worldwide *Microhedyle* species have been compared morphologically by Neusser *et al*. (2006). *Microhedyle glandulifera* can be clearly distinguished from its Western Atlantic congener *M. remanei* by details of radula morphology (see Discussion above), the presence of large triaxonic spicules and commonly pigmented eyes (*vs* lack of eyes) (Kirsteuer, 1973; Neusser *et al*., 2006). However, the latter two features need to be treated with caution. The eyes (traditionally considered a reliable character for species delineation in Acochlidia; e.g. Odhner, 1938, 1952; Marcus, 1954; Salvini-Plawen, 1973) are a rather unreliable character since intensity of pigmentation can be variable between and within populations and barely pigmented eyes can easily be overlooked (Jörger *et al*., 2010b; Neusser *et al*., 2011b). Differences from Western Atlantic *M. nahantensis* (Doe, 1974) also refer to the difference in spicule types (Doe, 1974). Having roundish plate-like spicules (Doe, 1974) that are elsewhere only present in the genus *Parhedyle* raises doubts on its placement within *Microhedyle*; re-examination of the radula morphology by SEM is needed to clarify the generic affiliation of *M. nahantensis*.

With present knowledge, *Microhedyle glandulifera* seems morphologically characterized by a unique combination of characters: slender microhedylid with two head appendages; radula formula 1.1.1 with the rhachidian tooth bearing one central and two lateral cusps and lateral tooth with one central denticle; presence of monaxonic, triaxonic, bean-shaped and bead-string-like spicules.

More comparative data are needed to evaluate the value of additional microanatomical characters for species delineation in Acochlidia. In contrast to Hedylopsacean taxa, Microhedylacea have a reduced, aphallic reproductive system providing little comparable characters, and excretory and digestive systems (with the exception of the radula) also offer little distinguishing details (see e.g. Jörger *et al*., 2008; Neusser *et al*., 2009b; Schrödl & Neusser, 2010). The acochlidian nervous system and sensory organs have been discussed as valuable characters for phylogenetic analyses (Neusser, Jörger & Schrödl, 2007) and microanatomical redescriptions have indeed revealed a variety of previously unknown features, e.g. an osphradium in limnic *Strubellia* (Brenzinger *et al*., 2011b) and the detection of an unpaired osphradial ganglion in *Parhedyle crypthophthalma* (Jörger *et al*., 2010b) and *M. glandulifera* (Huber, 1993; present study). However, detailed studies on acochlidian nervous systems have also shown a high variety and partial inconsistency of some nervous features e.g. position of cerebral nerves and the difficulties of detection of all parts of the nervous system in single individuals (Sommerfeldt & Schrödl, 2005; Neusser *et al*., 2006, 2007, 2009b, 2011b; Neusser & Schrödl, 2007, 2009; Jörger *et al*., 2008, 2010b; Neusser, Heß & Schrödl, 2009a; Brenzinger *et al*., 2011a). Homologies of the cerebral nerves are still unclear and comparative studies at the population level and across different ontogenetic stages are needed to evaluate the degree of intraspecific variation in nervous features and thus their value for phylogenetic purposes.

In summary, the distinction and classification of Microhedylidae by morphological characters can be difficult due to their generally small sizes and regressive uniformity (Neusser *et al*., 2009a; Schrödl & Neusser, 2010); thus molecular data are needed for independent and ecologically unbiased assessment.

Molecular systematics

We used molecular markers to test morphology-based hypotheses on the identification of *M. glandulifera*. Phylogenetic analysis of molecular markers (nuclear 28S rRNA and mitochondrial 16S rRNA and COI) showed two main results. (1) All specimens of *M. glandulifera* form a clade that also includes specimens of '*M. lactea*' and a potential '*M. glomerans*', confirming morphology-based taxonomic assumptions by Wawra (1978, 1987) and herein. (2) In contrast, microhedylid specimens collected at the type locality of *M. odhneri* cluster with a specimen of *Parhedyle tyrtowii* from the type locality in the Black Sea. As also indicated by radular and spicule features, '*Microhedyle*' *odhneri* differs from *M. glandulifera* and should be transferred to the genus *Parhedyle*; the sequence similarity with *Parhedyle tyrtowii* is high enough (98.25% identity in COI) to suggest conspecificity. At the present stage of knowledge the genus *Parhedyle* (including the two valid species *P. cryptophthalma* and *P. tyrtowii*) seems to be well supported based on molecular (Fig. 7) and morphological data (radula formula 1-1-2, presence of plate-like spicules) (Kowalevsky, 1901; Westheide & Wawra, 1974; Wawra, 1987; Jörger *et al*., 2010b).

Microhedyle glandulifera has a derived position in our phylogenetic analyses although taxon sampling was limited. An improved sampling of Microhedylidae (including Western Atlantic *M. remanei* and Northwestern Atlantic *M. nahantensis*) is needed to clarify the relationships within Microhedylidae and between the genera *Microhedyle*, *Parhedyle* and *Pontohedyle*. Cladistic analyses based on morphological characters render Microhedylidae paraphyletic due to the inclusion of the tropical family Ganitidae (characterized by uniserate radulae with dagger-shaped teeth) and relationships within Microhedylidae remain unresolved due to a lack of reliable characters and conservative coding procedures (see Schrödl & Neusser, 2010). In accordance with the present study, previous molecular approaches showed *Pontohedyle* as a basal offshoot within Microhedylidae but, as in morphological analyses, rendered the family paraphyletic due to the inclusion of Ganitidae (Jörger *et al*., 2010a; Neusser *et al*., 2011a).

Cryptic species vs *a single widely distributed species*

Microhedyle glandulifera specimens (including those referring to synonymous species) form a clade in the phylogenetic analysis, indicating their monophyletic origin from within Microhedylidae *s. l.* The question is whether or not there is genetic structure within this clade indicating limited gene flow among populations and/or cryptic speciation. *Microhedyle glandulifera* extends from the Black Sea through the Mediterranean to the North Sea, covering an area of several thousand kilometres of coastline and different hydrographic conditions. Wide distributions of tiny meiofaunal taxa with supposedly low dispersal abilities (the "meiofaunal paradox", Giere, 2009) remain controversial (Schmidt & Westheide, 2000; Westheide *et al*., 2003; Boeckner, Sharma & Proctor, 2009). Although molecular data have supported the existence of some truly widespread and even amphi-atlantic species, e.g. among polychaete annelids (Schmidt & Westheide, 2000; Westheide *et al*., 2003), several studies across different meiofaunal taxa have revealed that species formerly considered to be cosmopolitan or

at least amphi-atlantic are flocks of cryptic species (Todaro *et al.*, 1996; Schmidt & Westheide, 1999; Schmidt & Westheide, 2000; Casu & Curini-Galletti, 2004; Casu *et al.*, 2009; Leasi & Todaro, 2009). Based on the reported collecting sites some species of Acochlidia have wide distributional ranges, e.g. *M. remanei* in the Western Atlantic and *Pseudunela cornuta* from Solomon Islands and Hong Kong (Marcus, 1953; Challis, 1970; Kirsteuer, 1973; Hughes, 1991; Neusser *et al.*, 2006). Reported populations have however never been compared in microanatomical detail or with molecular approaches; their ranges thus still need to be confirmed. In a first integrative taxonomic approach combining data from detailed 3D reconstructions on the anatomy and molecular mitochondrial markers, Neusser *et al.* (2011b) on the one hand revealed the presence of cryptic species inhabiting nearby beaches and on the other hand geographically distant populations of one species of *Pseudunela* (from Indonesia and Fiji).

For Mediterranean *M. glandulifera*, molecular data from mitochondrial COI and 16S rRNA sequences show no or only minor differences from Atlantic '*M. lactea*' and Mediterranean '*M. glomerans*'. In network analyses, using the barcoding marker COI, which is known to be rather fast-evolving and shows good resolution for the separation of species (Hebert *et al.*, 2003a; Hebert, Ratnasingham & deWaard, 2003b), all sampled populations of *M. glandulifera* are united in one haplotype network with mixed haplotypes, which are interconnected by only a few changes in nucleotides (Fig. 8). These similarities support the wide-ranging distribution of a single species *M. glandulifera* and rejects cryptic speciation. The present study provides a first glimpse of population genetic structure in a mesopsammic gastropod. However, the dataset is still limited in specimens per population, populations sampled and choice of genetic markers. Based on COI no genetic structure differentiating Atlantic and Mediterranean populations of *M. glandulifera* could be detected. Only populations from Kristineberg (Sweden) and Cape Kamenjak (Croatia) show a significant but low F_{st} value. These populations represent the most distant areas within our dataset and the presence of some genetic structure between the two might indicate some emerging genetic differentiation between Mediterranean and Atlantic populations. Faster evolving molecular markers such as AFLPs or genomic microsatellites applied to larger sample sets are needed for future population genetic studies of European microhedylids.

A molecular clock analysis calibrated with fossils from different heterobranch outgroups dated the origin of Acochlidia to the early Mesozoic and the main radiations events within Acochlidia were estimated to have taken place in the Jurassic, with diversification of Microhedylidae in the late Jurassic or Cretaceous (Jörger *et al.*, 2010a). The establishment of molecular clocks for more recent acochlid radiations (genus or species level) is hindered by the lack of a fossil record and likely incomplete knowledge of extant diversity of the group.

Our preliminary data could suggest gene flow between populations in *M. glandulifera*. Owing to the low number (maximum 35) of yolk-rich eggs (Wawra, 1978) which indicates lecithotrophic development (Swedmark, 1959, 1968), it is unlikely that larvae play a major role in long-distance dispersal. Studies on dispersal of meiofaunal taxa, however, suggest that adults are the main dispersal stage (Palmer, 1986, 1988; Boeckner *et al.*, 2009). While e.g. copepods and some polychaetes are considered to be capable of actively dispersing through the water column to colonize new areas (Boeckner *et al.*, 2009), based on their external morphology acochlidian slugs seem less prone to active dispersal in the water column. Boeckner *et al.* (2009) observed meiofaunal taxa high in the water column even at calm, low-energy sites, concluding that even slight turbulence might be sufficient to suspend meiofaunal organisms and allow their dispersal by currents. While occasional dispersal by accidentally suspended individuals should be considered, Acochlidia actively entering the water column to facilitate dispersal (e.g. from over-populated or nutrient-poor habitats) remains pure speculation. To our knowledge no acochlids have ever been found in plankton samples. Soft-bodied slugs floating in the water column without protection are probably at high risk of predation and more secure means of adult dispersal e.g. by rafting on suspended sand grains (as reported for other meiofaunal taxa; Hicks, 1988; Jokiel, 1990) or even by hitchhiking on larger benthic animals as speculated by Neusser *et al.* (2011b) and Brenzinger *et al* (2011b) might be more likely.

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