SACOGLOSSA OR ACOCHLIDIA? 3D RECONSTRUCTION, MOLECULAR PHYLOGENY AND EVOLUTION OF AITENGIDAE (GASTROPODA: HETEROBRANCHIA)

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ABSTRACT

The amphibious 'bug-eating slug' *Aiteng ater* Swennen & Buatip, 2009 shows a worm-like, compact body shape lacking any cephalic tentacles or body processes. Anatomically it has been described as showing an unusual mix of sacoglossan and acochlidian characters, thus the systematic affinities are uncertain. The species is redescribed here with an integrative microanatomical and molecular approach. All major organ systems were three-dimensionally reconstructed from serial histological sections using AMIRA software. *Aiteng ater* has a prepharyngeal nerve ring with separate cerebral and pleural ganglia rather than cerebro-pleural ganglia, and no sacoglossan-like ascus is detectable histologically. The radula is triseriate rather than uniseriate, showing one lateral tooth on each side of the rhachidian tooth. A well-developed two-chambered heart is present. The vas deferens in *A. ater* splits off distal to the female glands. The intestine is short and opens into a small mantle cavity. Long cavities in the connective tissue are remains of dissolved calcareous spicules. Only a few characters thus remain to support a closer relationship of *A. ater* to Sacoglossa, i.e. the *Gascoignella*-like body shape lacking cephalic tentacles, the presence of an elysiid-like system of dorsal vessels, and an albumen gland consisting of follicles. Additionally we describe in microanatomical detail an equally small and vermiform new aitengid species from Japan. *Aiteng mysticus* n. sp. differs from *A. ater* in habitat, body size and colour, central nervous system and presence of a kidney. Both aitengid species resemble acochlidians in the retractility of the head, by possessing calcareous spicules, a prepharyngeal nerve ring with separated cerebral and pleural ganglia, a triseriate radula with an ascending and descending limb, but without sacoglossan-like ascus, and a special diaulic reproductive system. The prominent rhachidian tooth of Aitengidae, which is used to pierce insects and pupae in *A. ater*, and the large, laterally situated eyes closely resemble the anatomy of members of the limnic Acochlidiidae. The acochlidian nature of *Aiteng* is strongly indicated by our molecular analysis, in which it forms a basal hedylopsacean offshoot or the sister clade to limnic Acochlidiidae and brackish or marine Pseudunelidae within Hedylopsacea. Such a topology would, however, imply that Aitengidae have lost the most characteristic acochlidian apomorphy, the subdivision of the body into a headfoot complex and a free, elongated visceral hump. Also, the absence of cephalic tentacles gives the Aitengidae an appearance that is very different to other, strictly aquatic Acochlidia. Differences of the external morphology and the internal anatomy are discussed in the light of a habitat shift of Aitengidae within the Acochlidia.

INTRODUCTION

The Acochlidia and Sacoglossa were traditionally regarded as taxa of the 'Opisthobranchia' in morphological (e.g. Jensen, 1996; Dayrat & Tillier, 2002; Wägele & Klussmann-Kolb, 2005; Schrödl & Neusser, 2010) as well as molecular (e.g. Grande *et al*., 2004; Vonnemann *et al*., 2005; Händeler *et al*., 2009) studies. Recent molecular studies (e.g. Klussmann-Kolb

et al., 2008; Dinapoli & Klussmann-Kolb, 2010; Jörger et al., 2010) have changed our understanding of the phylogeny of Heterobranchia considerably. With a comprehensive euthyneuran taxon set, an analysis of mitochondrial cytochrome c oxidase subunit I (COI) and 16S rRNA genes and nuclear 18S and 28S rRNA genes has revealed the traditional 'Opisthobranchia' as polyphyletic (see Schrödl et al., 2011). Both Sacoglossa and Acochlidia have been shown to be part of an early (pan)pulmonate radiation (Jörger et al., 2010). The internal acochlidian topology revealed by molecular markers is congruent with that obtained by our morphology-based cladistic analysis (Schrödl & Neusser, 2010). However, a still undescribed putative member of the recently established Aitengidae Swennen & Buatip, 2009, named 'himitsu namekuji' (English: secret slug) when the specimens were found in Japan, clustered among hedylopsacean acochlids in the molecular analyses (Jörger et al., 2010).

The family Aitengidae was established as a monotypic sacoglossan family with a possible affinity to Acochlidia (Swennen & Buatip, 2009). Its sole species, the mysterious 'bug-eating slug' Aiteng ater Swennen & Buatip, 2009 was included into the 'top ten list of bizarre new species 2010' by the International Institute for Species Exploration at Arizona State University (http://species.asu.edu/Top10). Aiteng ater lives amphibiously in a mangrove forest in Thailand. The body length is 8–12 mm and the body shape is worm-like, lacking any cephalic tentacles or body processes. Anatomically it was described as showing an unusual mix of acochlidian and sacoglossan features, such as the prepharyngeal nerve ring characteristic for the Acochlidia, but the uniseriate radula, an ascus, a ramified digestive gland, a system of dorsal vessels and the albumen gland consisting of follicles—features which are all characteristic for Sacoglossa. The head and back of the slug bear strange 'white cigar-shaped bodies', which were interpreted as parasites by Swennen & Buatip (2009). Aiteng ater was preliminarily placed within Sacoglossa, but the authors expressed their doubts and the systematic affinities remained uncertain. The present study aims to re-examine A. ater with a microanatomical approach using computer-based three-dimensional (3D) reconstructions, as used e.g. for Acochlidia (Neusser et al., 2006; Neusser & Schrödl, 2007, 2009; Jörger et al., 2008, 2009; Neusser, Heß & Schrödl, 2009a; Neusser, Martynov & Schrödl, 2009b; Brenzinger et al., 2010; Neusser, Jörger & Schrödl, 2011) and to compare it to the 'secret slug' from Japan, which is also reconstructed in the present study in the same way. Combining evidence from detailed micromorphological descriptions and molecular analyses of both aitengid species we aim to clarify the systematic relationships and evolutionary history of the Aitengidae.

MATERIAL AND METHODS

Material

One paratype of Aiteng ater was obtained from the Zoological Museum, University of Amsterdam (ZMA) for semithin sectioning. One specimen of A. ater was collected at the type locality by Dr Swennen (Prince of Songkla University, Thailand) in October 2009 and was provided for the examination of the radula. Several specimens of Aiteng mysticus n. sp. were collected by H.F. and Y.K. on different islands of Okinawa Prefecture, Ryukyu Islands, Japan, in April 1992, March 1993, May 2008 and June 2009. The latter specimens were relaxed in 7.5% $MgCl_2$, fixed in 10% formalin and preserved in 75% ethanol for semithin sectioning and scanning electron microscopy (SEM) or fixed in 99% ethanol for molecular studies. Details of collecting sites are given in Table 1 and a summary of all material used in the morphological study in Table 2.

Table 1. Collecting date and localities of *Aiteng mysticus* n. sp. in Okinawa Prefecture, Ryukyu Islands, Japan.

Locality no.	Locality	GPS data	Date/collected by
1	Shimozaki, Nikadori, Hirara, Miyako Island	24°49′49″N, 125°16′42″E	04.1992 and 05.2008/HF, YT
2	Matsubara, Hirara, Miyako Island	24°47′01″N, 125°16′05″E	05.2008/HF, YT
3	Nakamoto, Kuroshima Island	24°13′42″N, 123°59′58″E	03.1996/YK
4	NW of Yonaguni Airport, easternmost corner of Higashi-bokujô, Yonaguni Island	24°28′04″N, 122°58′15″E	06.2009/HF, YT

HF, Hiroshi Fukuda; YK, Yasunori Kano; YT, Yuki Tatara.

Table 2. Material examined for morphological study.

Species	Locality (no., see Table 1)	Type of investigation and storage	Museum no.
Aiteng mysticus n. sp.	1	Specimen in 75% ethanol (H)	ZSM Mol 20110185
		Section series (P)	ZSM Mol 20110186
		Radula on SEM stub (P)	ZSM Mol 20110187
		Specimen in 99% ethanol (P)	NSMT Mo 77319
Aiteng mysticus n. sp.	2	Section series (P)	ZSM Mol 20110188
		Specimen in 99% ethanol (P)	OKCAB M21473
Aiteng mysticus n. sp.	4	Specimen in 5% formalin and radula on SEM stub (P)	OKCAB M21474
Aiteng ater	Pak Phanang Bay, Gulf of Thailand	Section series (P)	ZMA 409068
		Radula on SEM stub	ZSM Mol 20110189

Abbreviations: H, holotype; NSMT, National Museum of Nature and Science, Tokyo, Japan; OKCAB, Laboratory of Conservation of Aquatic Biodiversity, Faculty of Agriculture, Okayama University, Japan; P, paratype; ZMA, Zoological Museum, University of Amsterdam, The Netherlands; ZSM, Bavarian State Collection of Zoology, Germany.

Embedding and sectioning

Specimens were decalcified in Bouin's solution overnight and dehydrated in an acetone series (70, 90, 100%). For semithin sectioning two specimens of *A. mysticus* were embedded in Spurr's low-viscosity resin (Spurr, 1969) and the paratype of *A. ater* was embedded in Epon (Luft, 1961). Three series of ribboned serial semithin sections of 2 μm thickness were prepared using a diamond knife (Histo Jumbo, Diatome, Biel, Switzerland) with contact cement on the lower cutting edge to form ribbons (Ruthensteiner, 2008). Sections were stained with methylene-azure II (Richardson, Jarett & Finke, 1960). The sections of *A. mysticus* were deposited at the Bavarian State Collection of Zoology, Germany (ZSM), Mollusca Section (ZSM Mol 20110186 and 20110188); the sections of *A. ater* were deposited at ZMA (ZMA 409068).

3D reconstruction

Digital photographs of every second section were taken with a CCD microscope camera (Spot Insight, Diagnostic Instruments, Sterling Heights, MI, USA) mounted on a DMB-RBE microscope (Leica Microsystems, Wetzlar, Germany). Images were converted to 8-bit greyscale format, contrast enhanced and unsharp masked with standard image-editing software. A computer-based 3D reconstruction of all major organ systems was conducted with the software AMIRA 5.2 (Amira Visaging GmbH, Germany) following the procedure of Ruthensteiner (2008). The 3D reconstruction of *A. ater* was based on the paratype series and that of *A. mysticus* on the series ZSM Mol 20110188.

Scanning electron microscopy

One specimen of *A. mysticus* from Miyako Island, Japan, preserved in 75% EtOH, one specimen of the same species from Yonaguni Island, Japan, preserved in 5% formalin and one specimen of *A. ater* from Thailand were used for SEM examination of radulae. Specimens were macerated in 10% KOH overnight. Remaining tissue was removed with fine dissection pins. Radulae were mounted on specimen stubs and sputter-coated with gold for 135 s (SEM-Coating-System, Polaron) and examined with a LEO 1430 VP (Leo Elektronenmikroskopie GmbH, Oberkochen, Germany) at 15 kV.

Molecular studies

One alcohol-preserved specimen of *A. ater* from the type locality was available for molecular study. DNA was extracted by K. Händeler (University of Bonn, Germany) using the Qiagen Blood and Tissue Kit according to manufacturer's recommendations. Four genetic markers were sequenced following the protocols and using the same primers as described by Händeler *et al.* (2009) for partial mitochondrial COI and 16S rRNA genes, and following Jörger *et al.* (2010) for nuclear 18S rRNA and partial 28S rRNA genes. Sequences were edited using Geneious Pro™ 5.1 (Biomatters Ltd). To supplement sequence data available from public databases we additionally sequenced the sacoglossan *Platyhedyle denudata* and the acochlidian *Parhedyle cryptophthalma*, *Ganitus evelinae* and *Palliohedyle* sp. as described above (see Table 3 for collection details and Table 4 for GenBank accession numbers).

The sampled Aitengidae were analysed in a dataset containing 35 heterobranch taxa with a focus on Acochlidia and Sacoglossa (Table 4). We aimed to cover known acochlidian and sacoglossan diversity by including at least one representative of each genus for Acochlidia (only lacking monotypic *Tantulum elegans*) and one sacoglossan representative per family following the classification of Jensen (1996). Other outgroups were chosen to cover a variety of euopisthobranch and panpulmonate taxa (see Jörger *et al.*, 2010). The alignments for each marker were generated using Muscle (Edgar, 2004). To remove ambiguous regions the alignments of 18S, 28S and 16S rRNA were masked with Gblocks (Castresana, 2000; Talavera & Castresana, 2007) using the options for a less stringent selection; the COI alignment was checked manually according to translation into amino acids. We performed maximum-likelihood analyses using RAxML v.7.0.3 (Stamatakis, 2006) according to the programmer's instructions ('hard and slow way') of the concatenated datasets combining 18S + 28S, 18 + 28S + COI, 18S + 28S + COI + 16S and 28S + COI + 16S with the GTR + Γ + I model, chosen via the Akaike Information Criterion implemented in jModeltest (Posada, 2008) and with one partition for each marker. The acteonoid *Rictaxis punctocaelatus* was defined as outgroup.

SYSTEMATIC DESCRIPTIONS
AITENGIDAE Swennen & Buatip, 2009
Aiteng Swennen & Buatip, 2009

Type species: *Aiteng ater* Swennen & Buatip, 2009, by original designation.

Aiteng ater Swennen & Buatip, 2009
(Figs 1–4, 5A, 6)

Aiteng ater Swennen & Buatip, 2009: 495–500, figs 1B–M, 2A–H.

Table 3. Collection data of the species for which molecular data were generated.

Species	ZSM no.	Locality	GPS data	Date/collected by
Aiteng ater	—	Pak Phanang Bay, Thailand, Gulf of Thailand	8°29′18″N, 100°10′55″E	09.2007/CS
Aiteng mysticus n. sp.*	—	Matsubara, Miyako, Okinawa, Japan	24°47′01″N, 125°16′05″E	05.2008/HF,YT
Aiteng mysticus n. sp.§	—	Shimozaki, Nikadori, Miyako, Okinawa, Japan	24°49′49″N, 125°16′42″E	05.2008/HF,YT
Palliohedyle sp.	Mol 20100356	Tambala River near Manado, Sulawesi, Indonesia	1°24′11″N, 124°41′08″E	11.2009/KJ
Pontohedyle milaschewitchii	Mol 20080054	Cap Kamenjak, Istria, Croatia, Mediterranean Sea	44°46′03″N, 13°54′58″E	09.2005/KJ
Parhedyle cryptophthalma	Mol 20100584	Bacoli, Naples, Italy, Mediterranean Sea	40°47′19″N, 14°03′54″E	09.2009/MS
Ganitus evelinae	Mol 20100328	Sina da Pedra, Ilhabela, Brazil, Atlantic Ocean	23°46′43″S, 45°21′33″W	03.2010/MS
Platyhedyle denudata	Mol 20091351	Secche della Meloria, Livorno, Italy, Mediterranean Sea	43°33′01″N, 10°13′08″E	09.2009/MS

CS, Cornelis Swennen; HF, Hiroshi Fukuda; KJ, Katharina Jörger; MS, Michael Schröd; YT, Yuki Tatara; ZSM, Bavarian State Collection of Zoology, Germany.
*as Aitengidae sp. in Jörger *et al.* (2010). §COI sequence only.

**Table 4.** Taxon sampling and GenBank accession numbers for the gene sequences used in the present study.

Taxon	Family	Species	18S	28S	16S	COI
PANPULMONATA						
Incerta sedis	Aitengidae	Aiteng ater	JF828036*	JF828037*	JF828038*	JF828031*
		Aiteng mysticus n. sp. §	HQ168428	HQ168441	HQ168415	HQ168453
Acochlidia	Hedylopsidae	Hedylopsis ballantinei	HQ168429	HQ168442	HQ168416	HQ168454
	Pseudunelidae	Pseudunela sp.†	HQ168431	HQ168444	HQ168418	HQ168456
	Acochlidiidae	Strubellia paradoxa	HQ168432	HQ168445	HQ168419	HQ168457
	Acochlidiidae	Acochlidium fijiense	HQ168433	HQ168446	HQ168420	HQ168458
	Acochlidiidae	Palliohedyle sp.	—	JF828039*	JF828040*	JF828032*
	Asperspinidae	Asperspina sp.	HQ168434	HQ168447	HQ168421	—
	Microhedylidae	Pontohedyle milaschewitchii	HQ168435	JF828043*	HQ168422	HQ168459
	Microhedylidae	Parhedyle cryptophthalma	—	JF828041*	JF828042*	JF828033*
	Microhedylidae	Microhedyle glandulifera	HQ168437	HQ168449	HQ168424	HQ168461
	Ganitidae	Paraganitus ellynnae	HQ168436	HQ168448	HQ168423	HQ168460
	Ganitidae	Ganitus evelinae	—	JF828044*	JF828045*	JF828034*
Sacoglossa	Volvatellidae	Volvatella viridis	HQ168426	HQ168439	HQ168413	HQ168451
	Cylindrobullidae	Cylindrobulla beauii	EF489347	EF489371	EF489321	—
	Juliidae	Julia exquisita	—	GQ996653	EU140895	GQ996661
	Oxynoidae	Oxynoe antillarum	FJ917441	FJ917466	FJ917425	FJ917483
	Platyhedylidae	Gascoignella nukuli	HQ168427	HQ168440	HQ168414	HQ168452
	Platyhedylidae	Platyhedyle denudata	—	JF828046*	—	JF828035*
	Caliphyllidae	Cyerce nigricans	AY427500	AY427463	EU140843	DQ237995
	Plakobranchidae	Plakobranchus ocellatus	AY427497	AY427459	DQ480204	DQ237996
	Elysiidae	Elysia viridis	AY427499	AY427462	AY223398	DQ237994
	Limapontiidae	Limapontia nigra	AJ224920	AY427465	—	—
	Boselliidae	Bosellia mimetica	AY427498	AY427460	EU140873	GQ996657
	Hermaeidae	Hermaea cruciata	—	GU191025	GU191042	GU191058
Siphonarioidea	Siphonaridae	Siphonaria concinna	EF489334	EF489353	EF489300	EF489378
Amphiboloidea	Amphibolidae	Phallomedusa solida	DQ093440	DQ279991	DQ093484	DQ093528
Hygrophila	Lymnaeidae	Lymnaea stagnalis	EF489345	EF489367	EF489314	EF489390
Stylommatophora	Arionidae	Arion silvaticus	AY145365	AY145392	AY947380	AY987918
Systellommatophora	Onchidiidae	Onchidella floridana	AY427521	AY427486	EF489317	EF489392
Glacidorboidea	Glacidorbidae	Glacidorbis rusticus	FJ917211.1	FJ917227.1	FJ917264.1	FJ917284.1
EUOPISTHOBRANCHIA						
Umbraculoidea	Tylodinidae	Tylodina perversa	AY427496	AY427458	—	AF249809
Anaspidea	Akeridae	Akera bullata	AY427502	AY427466	AF156127	AF156143
Cephalaspidea s.s.	Diaphanidae	Toledonia globosa	EF489350	EF489375	EF489327	EF489395
'LOWER HETEROBRANCHIA'						
Acteonoidea	Acteonidae	Rictaxis punctocaelatus	EF489346	EF489370	EF489318	EF489393

*Sequences generated in the present study. §Aitengidae sp. in Jörger et al. (2010), described as new in the present study. †P. marteli Neusser et al. (2011).

Central nervous system (CNS) (Fig. 1A, C, D): CNS euthyneurous with paired cerebral (cg), optic (og), pedal (pg), pleural (plg), buccal (bg) and gastro-oesophageal ganglia (gog) and four distinct ganglia on visceral nerve cord (Figs 1C, 2B, 3). All ganglia prepharyngeal, except buccal and gastro-oesophageal ganglia (Fig. 1D). Cerebral, pedal and pleural ganglia linked by short connectives forming prepharyngeal nerve ring (Figs 1D, 2B, 3). Cerebral ganglia (Figs 1C, 2B, 3) linked by short commissure. Labiotentacular nerve (ltn) (Figs 1C, D, 2A, 3) emerges anteriorly from cerebral ganglion. Optic

Figure 1. 3D reconstruction of *Aiteng ater*. **A.** General microanatomy, dorsal view. **B.** Mantle cavity, dorsal view. **C.** Central nervous system, dorsal view. **D.** CNS and anterior part of digestive system, left view. **E.** Digestive system (only main branch of digestive gland reconstructed), right view. **F.** Circulatory and excretory systems, dorsal view. **G.** Reproductive system, dorsal view. **H.** Anterior copulatory organs, ventral view. **I.** Female reproductive system including sperm storing receptacles, right view. Abbreviations: a, anus; alg, albumen gland; am, ampulla; ao, aorta; apg, anterior pedal gland; at, atrium; bc, bursa copulatrix; bg, buccal ganglion; cg, cerebral ganglion; cns, central nervous system; dg, digestive gland; do, distal oviduct; dv, dorsal vessel; ed, ejaculatory duct; ey, eye; f, foot; fgl, female gland; fgo, female gonopore; gog, gastro-oesophageal ganglion; i, intestine; ltn, labial tentacle nerve; nb, notum border; od, oviduct; oe, oesophagus; og, optic ganglion; on, optic nerve; ot, oral tube; ov, ovotestis; p, penis; pag, parietal ganglion; pc, pericardium; pcc, pedal commissure; pg, pedal ganglion; ph, pharynx; plg, pleural ganglion; pn, pedal nerve; pod, postampullary gonoduct; pr, prostate; prd, preampullary gonoduct; ps, penial sheath; r, radula; rpd, renopericardioduct; s, statocyst; sgd, salivary gland duct; sgl, salivary gland; sp, spicule cavity; subg, subintestinal ganglion; supg, supraintestinal ganglion; v, ventricle; vd, vas deferens; vg, visceral ganglion; vn, visceral nerve; *, aggregation of nerve cells. Scale bars: **A** = 700 μm; **B, E** = 500 μm; **C** = 300 μm; **D, H, I** = 200 μm; **F, G** = 600 μm.
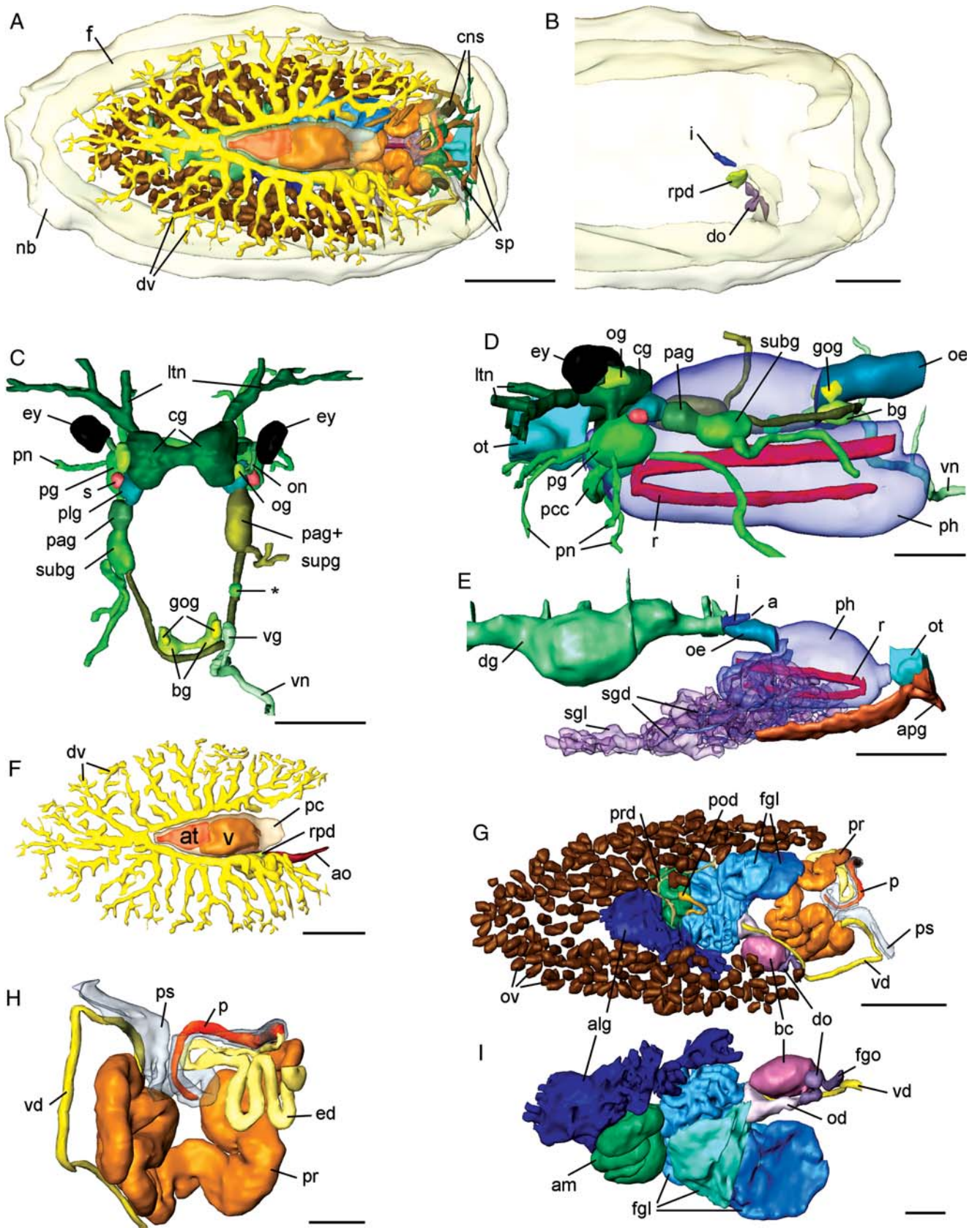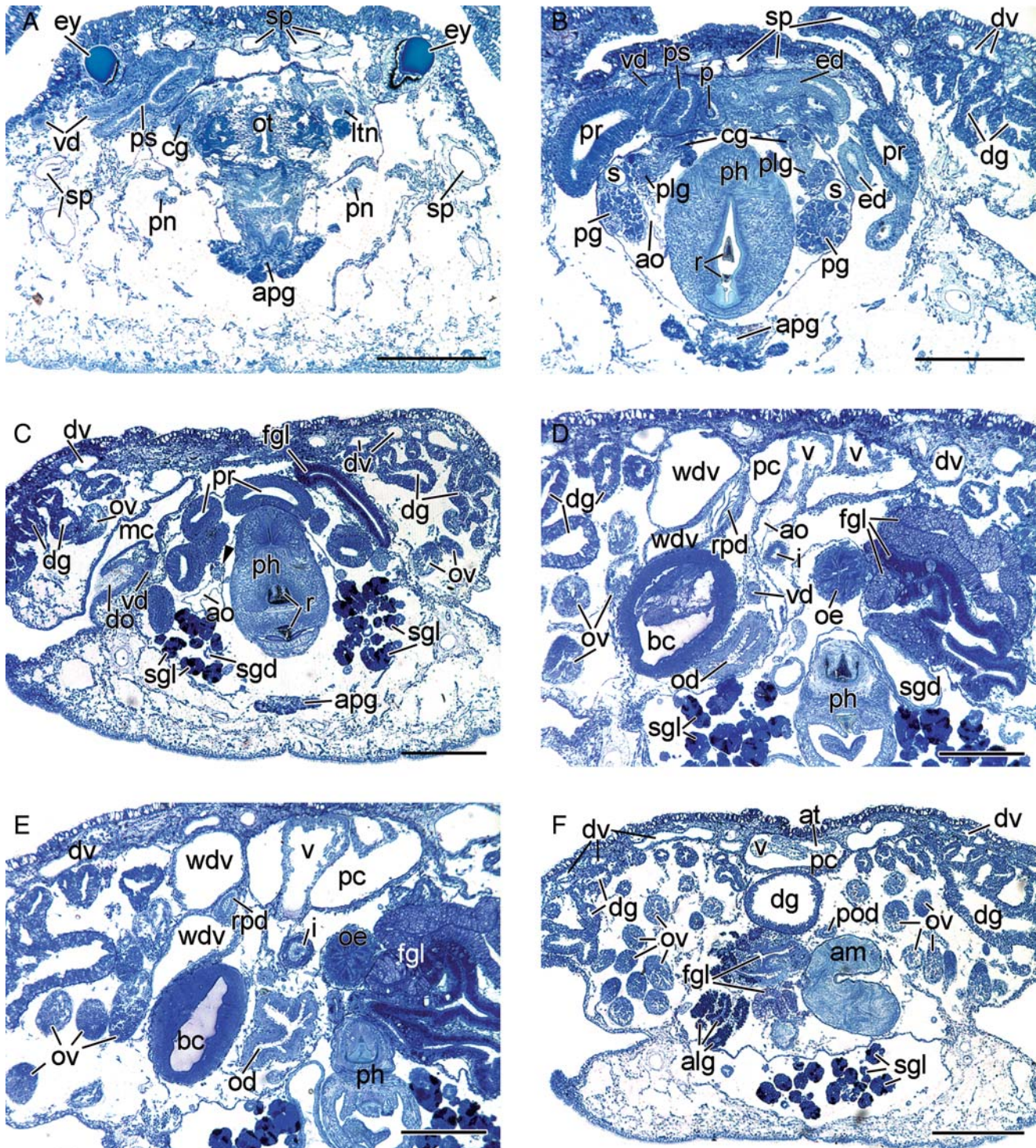
Figure 2. Histological cross-sections of *Aiteng ater*. **A.** Eyes, vas deferens and penial sheath. **B.** Ganglia, prostate. **C.** Mantle cavity. **D.** Dorsal vessels, renopericardioduct. **E.** Bursa copulatrix, ovotestis. **F.** Ampulla. Abbreviations: alg, albumen gland; am, ampulla; ao, aorta; apg, anterior pedal gland; at, atrium; bc, bursa copulatrix; cg, cerebral ganglion; dg, digestive gland; do, distal oviduct; dv, dorsal vessel; ed, ejaculatory duct; ey, eye; fgl, female gland; i, intestine; ltn, labial tentacle nerve; mc, mantle cavity; od, oviduct; oe, oesophagus; ot, oral tube; ov, ovotestis; p, penis; pc, pericardium; pg, pedal ganglion; ph, pharynx; plg, pleural ganglion; pn, pedal nerve; pod, postampullary gonoduct; pr, prostate; ps, penial sheath; r, radula; rpd, renopericardioduct; s, statocyst; sgd, salivary gland duct; sgl, salivary gland; sp, spicule cavity; v, ventricle; vd, vas deferens; wdv, wide lumen of dorsal vessel; arrowhead, aggregation of nerve cells on visceral nerve cord. Scale bars: **A, B** = 250 μm; **C** = 300 μm; **D, E** = 200 μm; **F** = 400 μm. This figure appears in colour in the online version of *Journal of Molluscan Studies*.

ganglion (Figs 1C, 3) attached laterally to each cerebral ganglion. Optic nerve (on) (Figs 1C, 3) emerges from optic ganglion innervating pigmented eye (ey) of 150 μm (Figs 1C,

D, 2A, 3). Precerebral accessory ganglia absent. Pedal commissure (Fig. 1D) longer than cerebral commissure. Statocyst (Figs 1C, D, 2B, 3) attached dorsally to each pedal ganglion
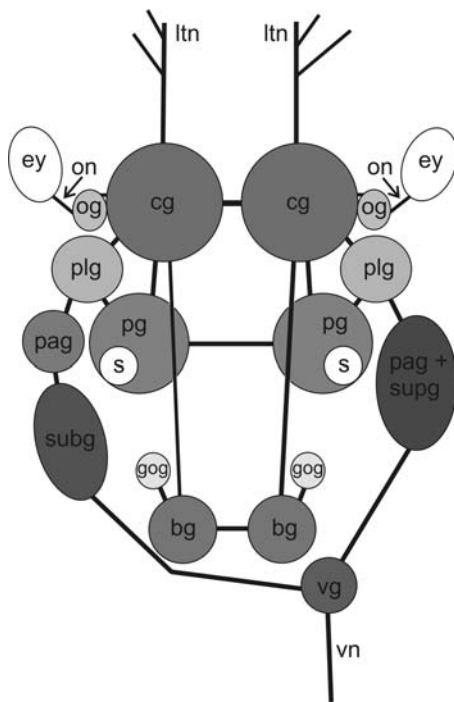
Figure 3. Schematic overview of the central nervous system of *Aiteng ater* (dorsal view). Abbreviations: bg, buccal ganglion; cg, cerebral ganglion; ey, eye; gog, gastro-oesophageal ganglion; ltn, labial tentacle nerve; og, optic ganglion; on, optic nerve; pag, parietal ganglion; pg, pedal ganglion; plg, pleural ganglion; s, statocyst; subg, subintestinal ganglion; supg, supraintestinal ganglion; vg, visceral ganglion; vn, visceral nerve. Not to scale.

(Figs 1D, 2B, 3). Pleural ganglion (Figs 1C, 3) connected to visceral nerve cord by very short connective. Four separate ganglia on visceral nerve cord (Figs 1C, 3): left parietal ganglion (pag), subintestinal ganglion (subg), small visceral ganglion (vg) and fused supraintestinal/right parietal ganglion (pag + supg). Aggregation of few cells on visceral nerve cord (Figs 1C, 2C) between visceral ganglion and fused supraintestinal/right parietal ganglion. No osphradial ganglion and no histologically differentiated osphradium detected. Paired buccal ganglia (Figs 1C, D, 3) posterior to pharynx, short buccal commissure ventrally to oesophagus. Small gastrooesophageal ganglion (Figs 1C, D, 3) dorsally to each buccal ganglion.

Digestive system: Anterior pedal gland (apg) (Figs 1E, 2A−C) discharging ventrally of mouth opening to exterior. Oral tube (ot) (Figs 1E, 2A) short. Radula (r) U-shaped (Figs 1D, E, 2B, C), 1−1.2 mm long, embedded within muscular pharynx (ph) (Fig. 1D, E, 2B−E). Ascending and descending limbs almost equally long (Fig. 1D), each terminating in muscular bulb. Radula formula 57 × 1.1.1, 33 rows of teeth on upper ramus, 24 rows of teeth on lower one. Each row consists of rhachidian tooth and one lateral tooth on each side. Lower ramus without any lateral teeth in oldest part, only *c*. 7 of youngest teeth of lower ramus with lateral teeth (Fig. 4A). Triangular rhachidian tooth (Fig. 4A−C) with one large, projecting central cusp (cc). Central cusp with up to 20 lateral denticles (ld) on each side (Fig. 4B, C). Distance between lateral denticles increasing towards tip of central cusp. Right lateral tooth (ltr) (Fig. 4B, D) plate-like with one pointed, well-developed denticle (d) (Fig. 4B, D) and 10−15 smaller denticles (sd) on anterior margin (Fig. 4D). Prominent notch (n) on posterior margin in which denticle of anterior lateral tooth fits. Posterior

margin with emargination on inner side of tooth. Left lateral tooth (ltl) (Fig. 4A, E) plate-like with two well-developed, pointed denticles on anterior margin, two prominent notches (n) on posterior one. Jaws absent. Oesophagus (oe) (Figs 1D, E, 2D, E) short, ciliated. One pair of large, folliculate salivary glands (sgl) (Figs 1E, 2C−F) connected via salivary gland ducts (sgd) (Figs 1E, 2C, D) at transition between pharynx and oesophagus. No distinct stomach detected. Digestive gland (dg) (Figs 1E, 2B−F) ramified, consisting of long main branch extending posteriorly and several smaller lateral branches only partly reconstructed. Intestine (i) (Figs 1E, 2D, E) densely ciliated, short. Anus (a) (Fig. 1E) opens on right side of body posterior to female gonopore into narrow and deep cavity (Fig. 1B).

Circulatory and excretory systems: Circulatory and excretory systems dorsal to digestive system. Circulatory system with wide, thin-walled pericardium (pc) surrounding large two-chambered heart (Figs 1F, 2D−F, 5A) with anterior ventricle and posterior atrium (Figs 1F, 2D−F, 5A). Aorta (Figs 1F, 2D, 5A) extending to head from anterior of ventricle. Renopericardioduct (rpd) (Figs 2D, E, 5A) well developed, densely ciliated, next to mantle cavity (Figs 1B, 2C); it connects to extensive system of ramified dorsal vessels (Figs 1A, F, 5A). The latter with very thin epithelium with minute vacuoles (Fig. 2C−F) inside cells extending to notum border. Part of dorsal vessels connected to renopericardioduct wider (wdv) than other branches of dorsal vessels (Figs 2D, E, 5A). However, histologically both parts look identical; distinct kidney with characteristic large, highly vacuolated cells absent. Nephroduct and nephropore not detected.

Reproductive system: Reproductive system ventral to digestive system, hermaphroditic and showing a special androdiaulic condition (Fig. 6). Ovotestis (ov) with follicles (Figs 1G, 2D−F, 6) located in semicircle over whole visceral sac. Tiny ducts emerge from follicles, joining in preampullary gonoduct (prd) (Fig. 6). Large tubular ampulla (am) (Figs 1I, 2F, 6) with autosperm in disorder. Sperm heads short. Receptaculum seminis absent or not developed in examined specimen. Four nidamental glands (Figs 1G, I, 2D−F, 6) from proximal to distal: ramified albumen gland (alg) discharges into postampullary gonoduct (Figs 1I, 2F, 6), followed by three glands with different histological and staining properties. Distal part of nidamental glands extends to right side of body where hermaphroditic duct bifurcates into internal vas deferens (vd) and short oviduct (od) (Figs 1I, 2D, 6). Bursa copulatrix (bc) large (Figs 1G, I, 2D,E, 6), splits off oviduct, without pronounced bursal stalk. Distal oviduct (do) opens through female gonopore (fgo) (Figs 1I, 2C, 6) at right side of body into narrow and deep cavity (Fig. 1B, 2C). Female gonopore considerably anterior to anus. Internal vas deferens (Figs 1G, H, 2A, 6) extends subepidermally up to head connecting to long, tubular prostate gland (pr) (Figs 1G, H, 2B, C, 6). Muscular ejaculatory duct (ed) (Figs 1H, 2B, 6) arises from prostate, discharges at top of penis (p) (Figs 1H, 2B, 6). Penis slender, without any stylet or spine, partially surrounded by thin-walled penial sheath (ps) (Figs 1H, 2A, B, 6).

Remarks: Our microanatomical results substantially revise the original description of *A. ater*, with discrepancies related to all organ systems (summary in Table 5). The original description of the CNS of *A. ater* is limited to mentioning four prepharyngeal ganglia, two of them being the fused cerebro-pleural ganglia. Instead, our reconstruction clearly shows the cerebral and pleural ganglia being separated rather than fused. We supplement the original description with the presence of the paired optic, buccal and gastro-oesophageal ganglia and four
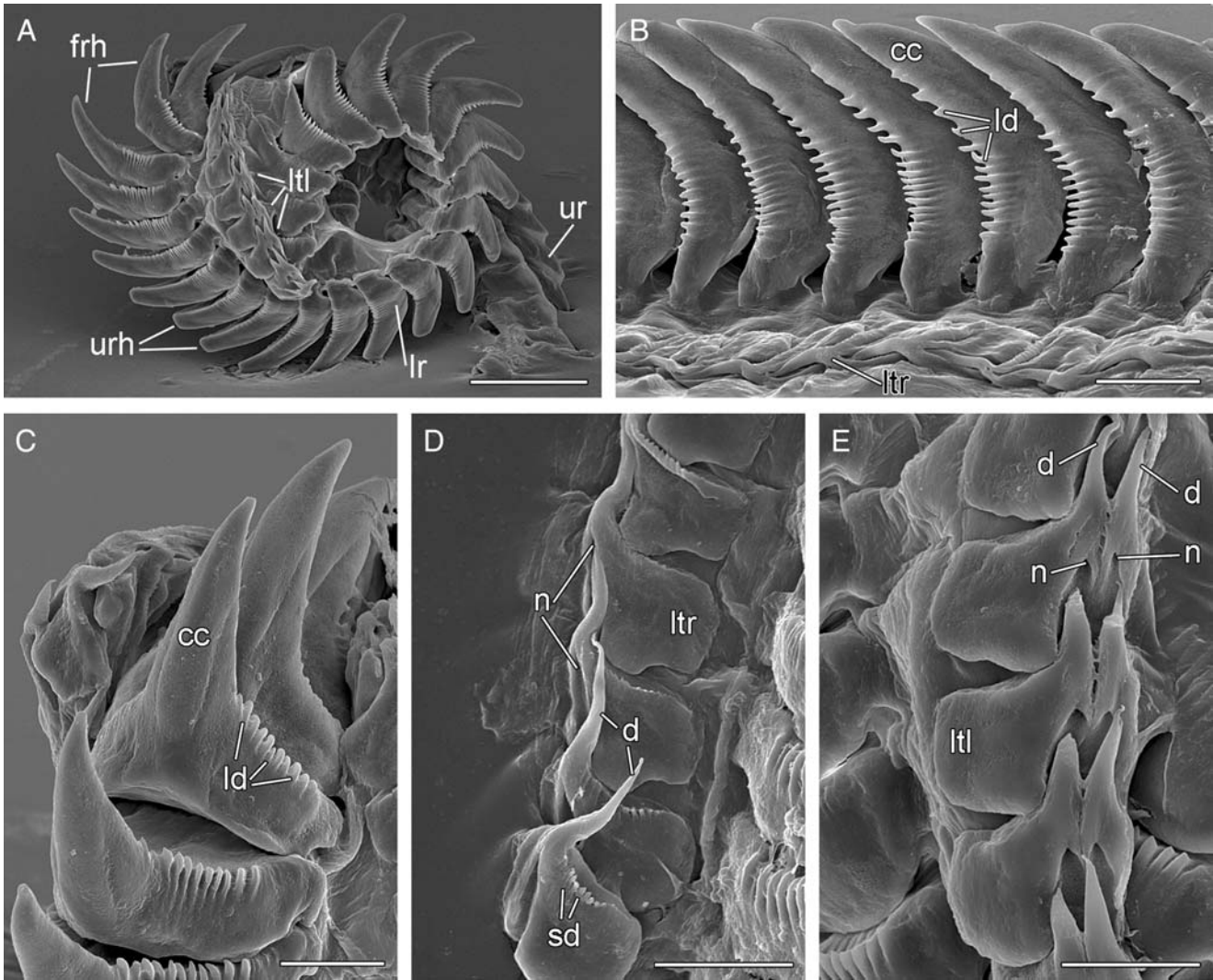
Figure 4. SEM micrographs of the radula of *Aiteng ater*. **A.** Radula, left view. **B.** Rhachidian teeth, right view. **C.** Rhachidian teeth, anterior view. **D.** Right lateral teeth. **E.** Left lateral teeth. Abbreviations: cc, central cusp; d, denticle; frh, functional rhachidian tooth; ld, lateral denticle; lr, lower ramus; ltl, left lateral tooth; ltr, right lateral tooth; n, notch; sd, small denticle; ur, upper ramus; urh, used rhachidian tooth. Scale bars: **A** = 60 μm; **B**–**E** = 20 μm.
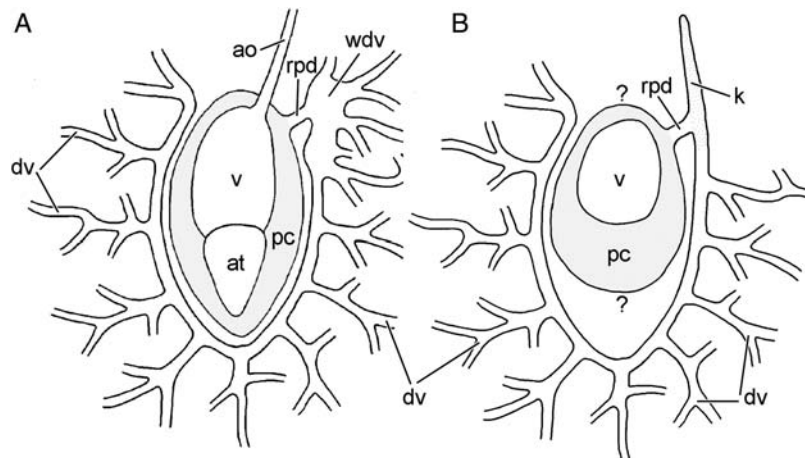
Figure 5. Schematic overview of the circulatory and excretory systems (dorsal view). **A.** *Aiteng ater*. **B.** *Aiteng mysticus* n. sp. Abbreviations: ao, aorta; at, atrium; dv, dorsal vessel; k, kidney; pc, pericardium; rpd, renopericardioduct; v, ventricle; wdv, wide lumen of dorsal vessel; ?, no data available. Not to scale.
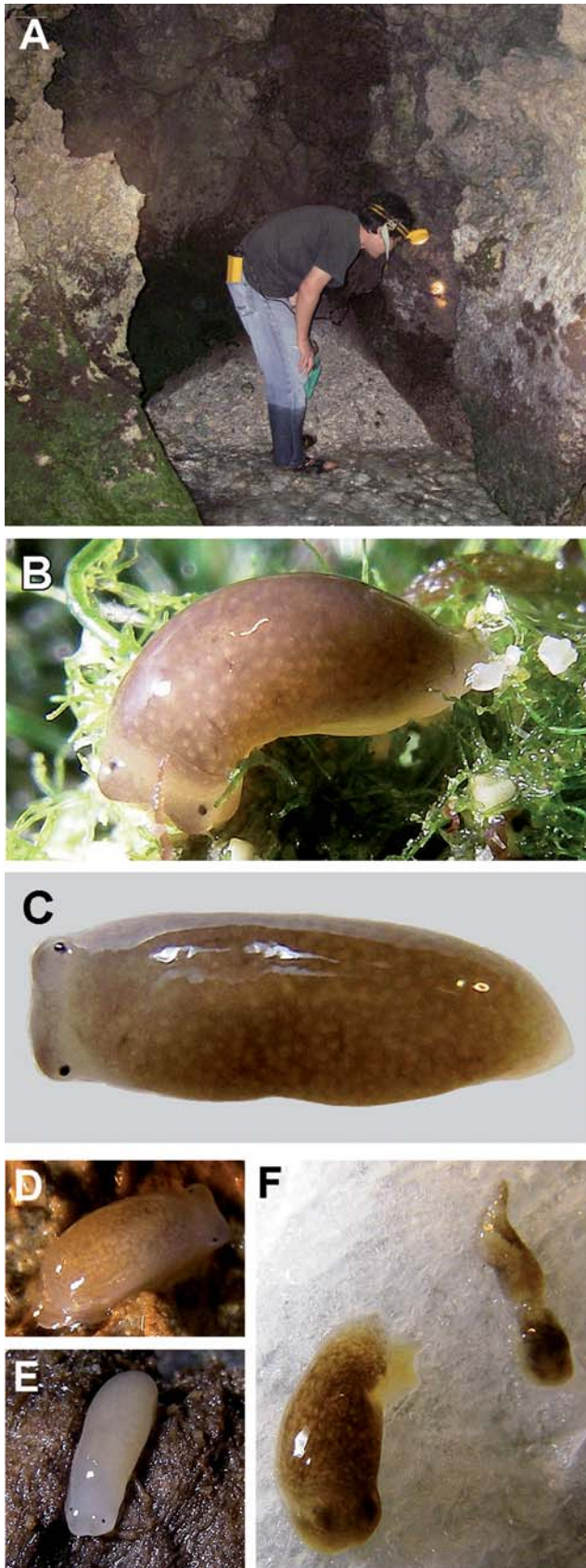
Figure 7. Habitat and external morphology of *Aiteng mysticus* n. sp. **A.** Coastal cavern on Miyako Island, Okinawa, Japan. **B–D, F.** Living specimens of *c.* 5 mm on Miyako Island. **B.** On algae. **C.** Brownish coloration. **D.** Pale coloration. **E.** Pale coloration (Yonaguni Island). **F.** Autotomy.

The radula in *A. ater* was reported as being uniseriate with only one rhachidian tooth per row, but our histological sections suggested the presence of one lateral tooth on each side. The examination by SEM clearly confirms the presence of a triseriate radula with a rhachidian tooth and one lateral tooth on each side (the latter of which is lacking in the oldest rows of the descending limb). In contrast to the original description we could not detect any sacoglossan-like ascus and there are no broken teeth at the posterior end of the descending limb in the pharynx. However, both radular limbs terminate in a separate muscular bulb.

Besides mentioning heart beats there are no more data about the circulatory system in the original description. Our reconstruction shows *A. ater* with a well-developed two-chambered heart, an aorta emerging from the ventricle, and the renopericardioduct connecting to a widened lumen of the dorsal vessel system. Our results for the reproductive system match well with the original data with one difference: whereas in the original description the postampullary hermaphroditic duct splits into vas deferens and oviduct, in our study the vas deferens splits off distal to the female glands, i.e. spermatocytes have to pass the female glands before entering the internal vas deferens and being transported to the male copulatory organs.

Swennen & Buatip (2009) reported "white, cigar-shaped bodies of different sizes" distributed "under the skin and loose on other organs in some specimens" of *A. ater* and supposed these were endoparasites. We cannot confirm this finding; instead our histological sections indicate the presence of subepidermal spicules (Figs 1A, 2A, B), which are distributed over the whole body, but concentrate in the head. We suppose these spicules have been misinterpreted in the original description as the endoparasites, as the latter dissolved later in the laboratory in an acidic solution (C.K. Swennen, personal communication).

Aiteng mysticus new species
(Figs 5B, 7B–F, 8–10)

Type material: Holotype: in 75% ethanol, *c.* 3 mm (ZSM Mol 20110185). Type locality Shimozaki, Nikadori, Hirara, Miyako Island, Okinawa, Japan, 24°49′49″N, 125°16′42″E.

Paratypes: two section series (ZSM Mol 20110186, ZSM Mol 20110188), one radula on SEM stub (ZSM Mol 20110187), two specimens in 99% ethanol (NSMT Mo 77319, OKCAB M21473) and one in 5% formalin with radula on SEM stub (OKCAB M21474). For localities see Table 1.

Etymology: After the Japanese common name 'himitsu name-kuji' (English: secret slug), given to the specimens when they were found.

Material examined: See Table 2.

Distribution: Known from Miyako Island, Kuroshima Island and Yonaguni Island (Okinawa Prefecture, Ryukyu Islands, Japan).

Habitat: The specimens were found in two different habitats. In Nikadori, Miyako Island, the animals were found on the surface of notches and lateral walls of small caves formed by erosion caused by strong waves (Fig. 7A), on shores of white limestone facing the open sea. In the intertidal zone were many small crevices which were usually moist with seawater and covered with two algae, *Caulacanthus ustulatus* (Gigartinales: Caulacanthaceae) and *Cladophora herpestica* (Cladophorales: Cladophoraceae). The specimens were
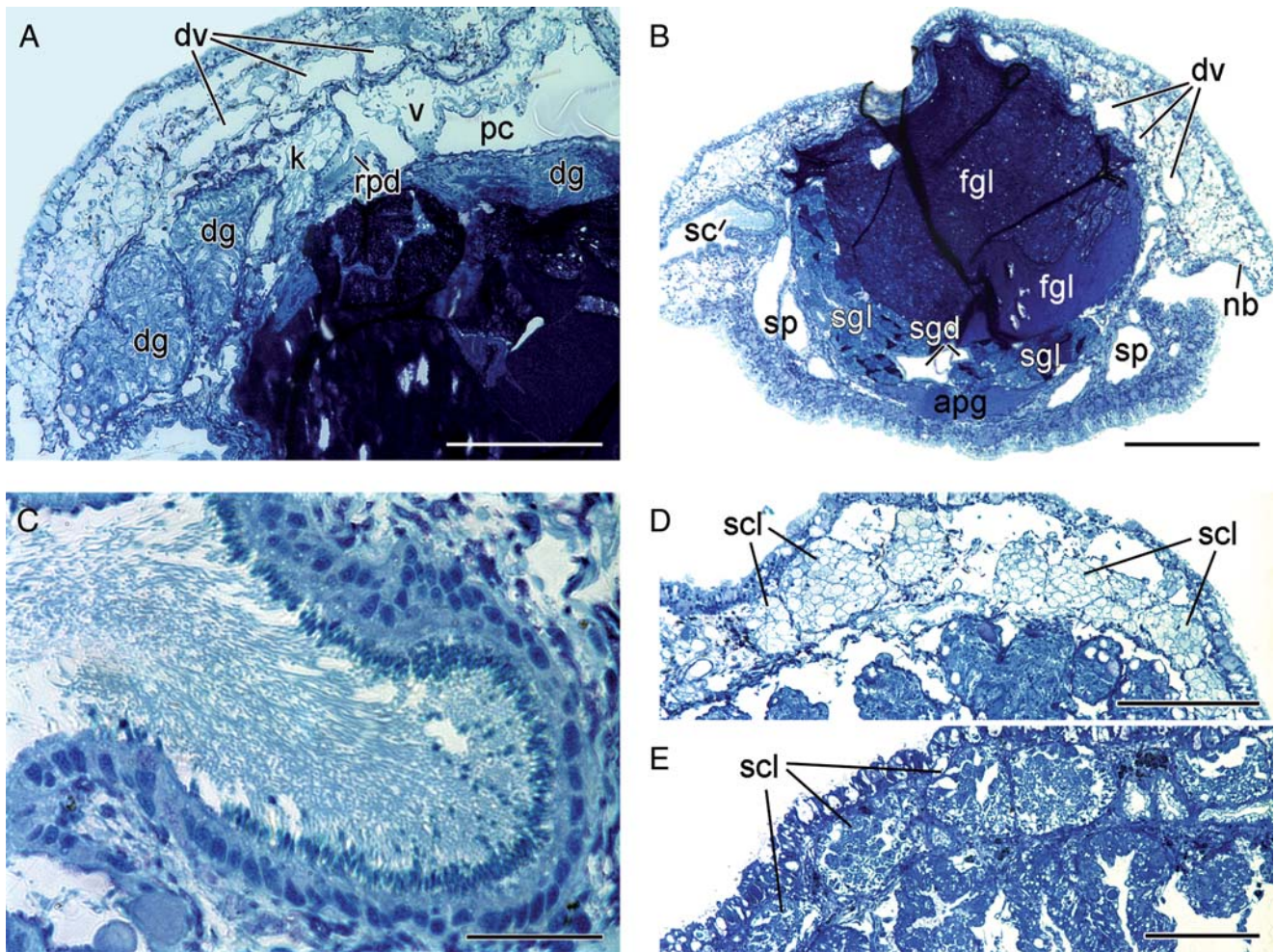
Figure 8. A–D. Histological cross-sections of *Aiteng mysticus* n. sp. **A.** Kidney, pericardium. **B.** Female glands, spermatocytes under notum border. **C.** Spermatocytes. **D.** Supporting cells. **E.** Supporting cells in *Aiteng ater*. Abbreviations: apg, anterior pedal gland; dg, digestive gland; dv, dorsal vessel; fgl, female gland; k, kidney; nb, notum border; pc, pericardium; rpd, renopericardioduct; sc, spermatocytes; scl, supporting cells; sgd, salivary gland duct; sgl, salivary gland; sp, spicule cavity; v, ventricle. Scale bars: **A** = 150 μm; **B** = 200 μm; **C** = 20 μm; **D, E** = 100 μm. This figure appears in colour in the online version of *Journal of Molluscan Studies*.

observed crawling just above the high tidal line at night from 11 p.m. to 5 a.m., together with *Paludinella* sp. and *Angustassiminea* sp. (both Assimineidae), *Pedipes jouani*, 'Allochroa' aff. *affinis* and *A. layardi* (all Ellobiidae). While the ellobiids occurred in high numbers, *A. mysticus* was rare and it was hard to find more than two individuals in the same locality in one night. As reported for most of the ellobiid species found in the same habitat (Fukuda, 1996), *A. mysticus* is truly nocturnal and rapidly disappears after sunrise. In the same habitat the large chiton *Acanthopleura spinosa* (Chitonidae) was often found alive at midnight. Sasaki, Hamaguchi & Nishihama (2006) reported the distribution and habitat of *Ac. spinosa* in Miyako Island, and *Ai. mysticus* was also collected from one of their localities. The habitat of *Ai. mysticus* in Kuroshima Island was similar to Nikadori, but *Ac. spinosa* was not found. In Yonaguni Island, *Ai. mysticus* was found in a narrow space among rocks at the innermost part of a spacious cave (about 10 m in width and length) similar to the Nikadori habitat. The inside of the cave was always dark and humid. The accompanying molluscan species were the same as those of Nikadori, with the addition of *Ditropisena* sp. (Assimineidae) and the ellobiid *Microtralia* sp.

Aiteng mysticus was also found in Matsubara, Miyako Island, however the habitats differ considerably. This site was a brackish area neighbouring a small mangrove swamp on a narrow (about 10 m) river estuary at the innermost part of a small bay. Many rocks of various sizes lay on flat, sandy-mud bottom in the intertidal. *Aiteng mysticus* was found alive beneath large rocks (30–50 cm diameter) deeply buried in mud in the upper intertidal zone, during daytime. The underside of these rocks was usually wet. *Angustassiminea* sp. and several other ellobiid species (e.g. *Blauneria quadrasi*, *Laemodonta monilifera*, *L.* aff. *minuta*, *L. octanflacta*, *L. typica*, *Melampus fasciatus*, *Me. granifer*, *Me. parvulus*, *Me. sculptus*, *Melampus* sp., *Microtralia* sp. and *Pedipes jouani*; see Fukuda, 1996) were also found.

The two habitats mentioned above were rather different from each other, but *Angustassiminea* sp., *Pedipes jouani* and *Microtralia* sp. were observed in both. Among them, *P. jouani* was considered to be restricted to notches or caves in the rocks. Judged from the presence of *P. jouani* and *Aiteng mysticus*, the two habitats may share some environmental conditions that are suitable for these two species. Two specimens of *Ai. mysticus* from the two habitats were found to share exactly the same COI sequence (see below), supporting their conspecific status.
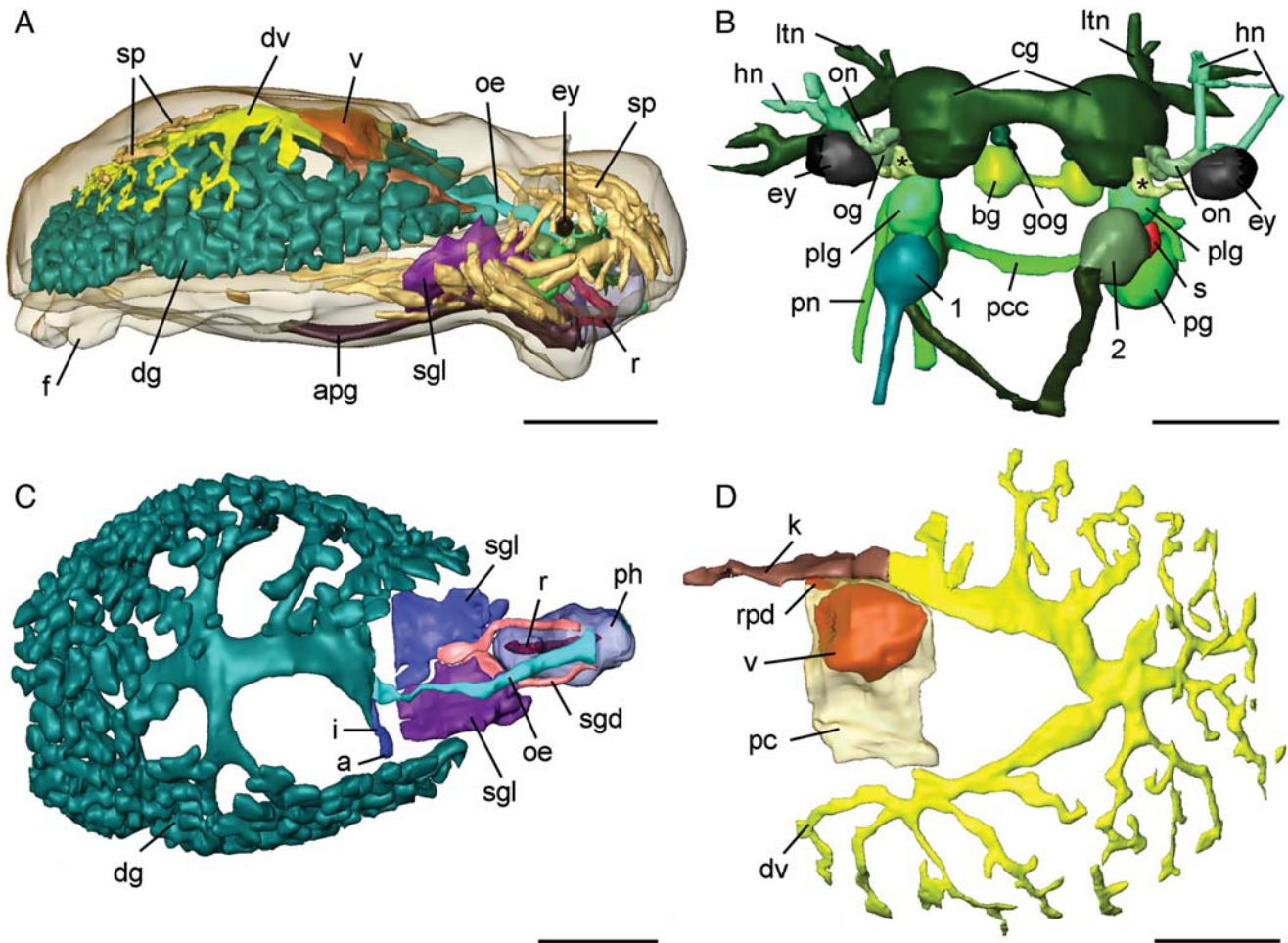
Figure 9. 3D reconstruction of *Aiteng mysticus* n. sp. **A.** General microanatomy, right view. **B.** Central nervous system, dorsal view. **C.** Digestive system, dorsal view. **D.** Circulatory and excretory systems, dorsal view. Abbreviations: a, anus; apg, anterior pedal gland; bg, buccal ganglion; cg, cerebral ganglion; dg, digestive gland; dv, dorsal vessel; ey, eye; f, foot; gog, gastro-oesophageal ganglion; hn, Hancock's nerve; i, intestine; k, kidney; ltn, labial tentacle nerve; oe, oesophagus; og, optic ganglion; on, optic nerve; pc, pericardium; pcc, pedal commissure; pg, pedal ganglion; ph, pharynx; plg, pleural ganglion; pn, pedal nerve; r, radula; rpd, renopericardioduct; s, statocyst; sgd, salivary gland duct; sgl, salivary gland; sp, spicule cavity; v, ventricle; 1,2, ganglia on the visceral nerve cord; *, ganglion attached to the cerebral ganglion. Scale bars: **A, C** = 400 µm; **B** = 150 µm; **D** = 300 µm.

External morphology of living specimens: Slug-like, lacking cephalic tentacles or other body processes (Fig. 7B, C). Length *c*. 5 mm. Dorsal surface glossy from copious mucus. Dorsal mantle pale to purplish brown. Brown coloration (Fig. 7B–D) variable in intensity, some individuals (e.g. from Yonaguni Island; Fig. 7E) paler than others. Large, vacuolated supporting cells visible as many distinct white granules through translucent skin of dorsal mantle (Figs 7, 8D). Head with pair of short, round bulges with distinct black eyes at postero-lateral corners. Head colour almost same as on dorsal mantle. Dorsal foot around head with thin pigment of same colour as dorsal mantle. Shallow transverse groove across anterior part of foot (uncertain whether or not this is an artefact by contraction). Sole flat, elongate oval, pale beige, without pigmentation. It consists of propodium and rest of foot: propodium occupies anterior 1/6 of whole foot; weak constriction on both sides at posterior end of propodium. Indistinct longitudinal groove on centre from portion just posterior to propodium to posterior end of foot. Foot simple, round. Lateral sides of foot pale beige without pigments.

Possible autotomy observed in one individual from Nikadori (Fig. 7F). While kept alive in small container, posterior edge of mantle and foot suddenly separated from rest of animal. This happened automatically without disturbance, but might have been a reaction to change of environmental condition from field to laboratory. The individual was still alive and crawled after this.

Central nervous system: CNS of *Aiteng mysticus* euthyneurous, pre-pharyngeal (Fig. 9B); arrangement of ganglia mainly as in *A. ater* (Fig. 3). Paired cerebral ganglia (cg) connected by short cerebral commissure. Labiotentacular nerve (ltn) (Fig. 9B) emerges from cerebral ganglion anteriorly. Optic ganglion (Fig. 9B) attached laterally to each cerebral ganglion; connective not detected. Optic nerve (on) arises from optic ganglion innervating pigmented eye (ey) of 100 µm (Fig. 9A, B). Hancock's nerve (Fig. 9B) splits off optic nerve innervating Hancock's organ. Small ganglion (Fig. 9B) attached to cerebral ganglion posterior to optic ganglion with unknown function. Precerebral accessory ganglia absent. Paired pedal ganglia (pg) ventral to cerebral ganglia; pedal commissure (Fig. 9B) considerably longer than in *A. ater*. Statocyst small, attached to each pedal ganglion. Pleural ganglion (plg) smaller than cerebral and pedal ganglia, posterior to both;
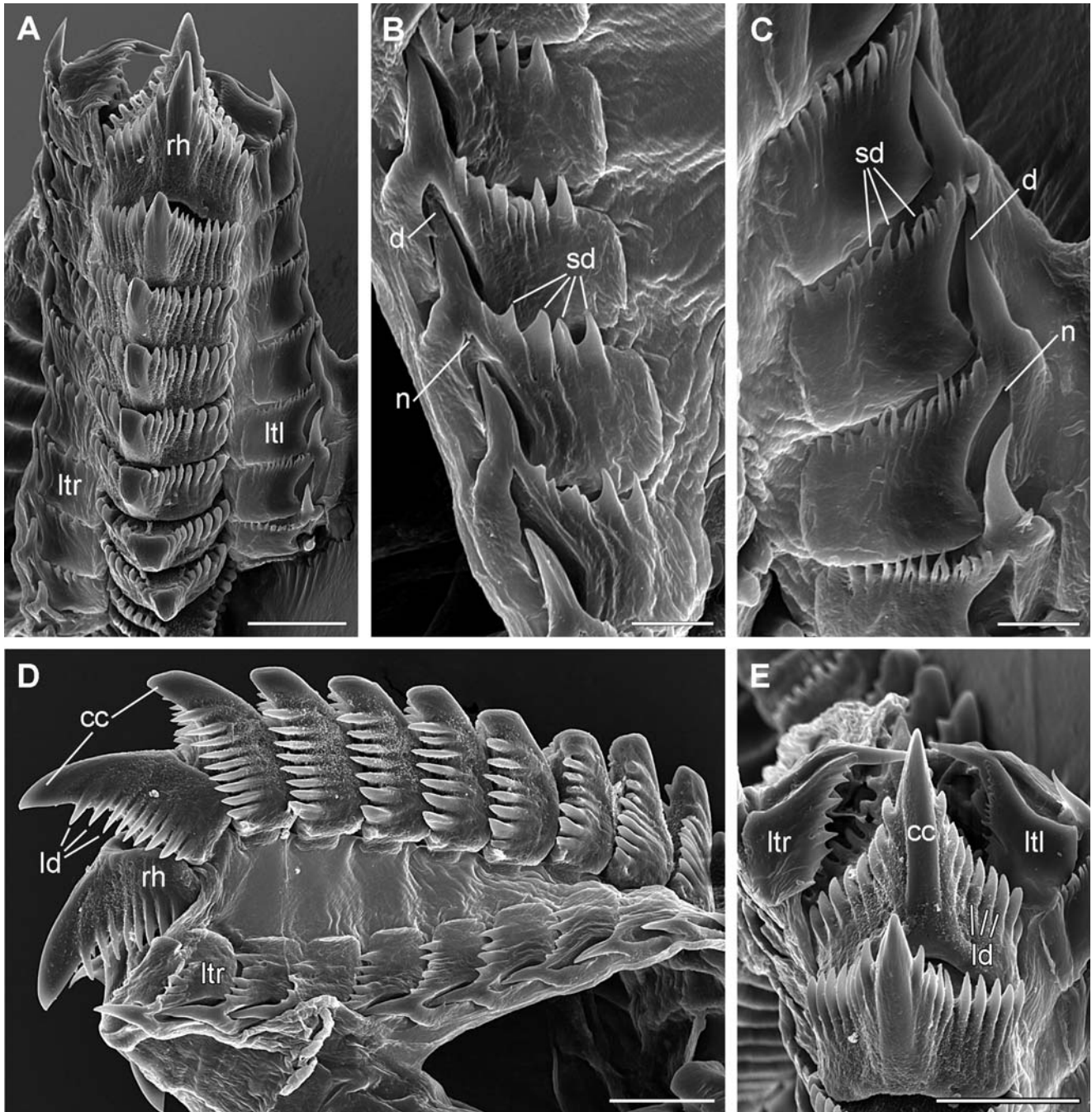
Figure 10. SEM micrographs of the radula of *Aiteng mysticus* n. sp. **A.** Rows of radular teeth (anterior view). **B.** Right lateral teeth. **C.** Left lateral teeth. **D.** Rhachidian teeth, right view; **E.** Rhachidian teeth, anterior view. Abbreviations: cc, central cusp; d, denticle; ld, lateral denticle; ltl, left lateral tooth; ltr, right lateral tooth; n, notch; rh, rhachidian tooth; sd, small denticle. Scale bars: **A, D, E** = 20 μm; **B, C** = 6 μm.

pleural ganglion (Fig. 9B) clearly separated from cerebral ganglion. Visceral nerve cord with only two large ganglia (Fig. 9B), both at ends of visceral nerve cord next to pleural ganglia. In one specimen three ganglia on visceral nerve cord. No osphradial ganglion, no histologically differentiated osphradium detected. Buccal ganglion (bg) just posterior to pharynx; however, in 3D reconstruction shifted more anteriorly because buccal apparatus was somewhat withdrawn in this specimen. Small gastro-oesophageal ganglion (gog) dorsal to each buccal ganglion.

Digestive system: Digestive system closely resembles that of *A. ater*. Anterior pedal gland (apg) (Figs 8B, 9A) discharges ventrally of mouth to exterior. Oral tube (ot) very short. Radula (r) U-shaped (Fig. 9A, C), 900 μm long, within muscular pharynx (ph) (Fig. 9C). Ascending and descending limbs almost equally long, each terminating in muscular bulb. Radula formula 70 × 1.1.1, 26 rows of teeth on upper ramus, 44 rows on lower one. Each radular row with triangular rhachidian tooth and one lateral tooth on each side (Fig. 10A). Lower ramus without any lateral teeth in oldest

part, only *c*. 16 of youngest teeth of lower ramus bear lateral teeth. Rhachidian tooth (Fig. 10D, E) with one large central cusp (cc) with 7–9 thinner, pointed lateral denticles (ld) on each side (Fig. 10D, E). All lateral denticles of almost same size. Right lateral tooth (ltr) (Fig. 10B, D) elongated plate-like with one prominent, pointed denticle (d) on anterior margin and well-developed notch (n) on posterior one, in which denticle of anterior lateral tooth fits. Additionally, 4–6 small denticles (sd) (Fig. 10B) on inner side of right lateral tooth. Left lateral tooth (ltl) (Fig. 1C) with same shape as right one with one large denticle and well-developed notch, but anterior margin with 12 or 13 small denticles (Fig. 1C) which look smaller and thinner than on right side. Jaws absent. Oesophagus (oe) (Fig. 9C) long, ciliated. Paired salivary glands (sgl) large (Figs 8B, 9A, C) with numerous small follicles reconstructed only in part. Follicles connected by small ductules before uniting in broad salivary gland ducts (sgd) (Figs 8B, 9C) that discharge at posterior of pharynx. Digestive gland (dg) (Figs 8A, 9A, C) ramified, extending to posterior end of visceral sac, as in *A. ater*. Intestine (i) (Fig. 9C) densely ciliated, short. Anus opens on right side of body posterior to female gonopore into small mantle cavity.

Circulatory and excretory systems: Circulatory and excretory systems dorsal to digestive system (Fig. 9A). Circulatory system with one-chambered heart surrounded by thin-walled pericardium (Figs 5B, 8A, 9A, D). Aorta and atrium not detected. Renopericardioduct (rpd) (Figs 5B, 8A, 9D) well developed, densely ciliated, connected to kidney (Figs 5B, 9D) with highly vacuolated cells (Fig. 8A). Kidney is one anterior branch of ramified dorsal vessel system (Fig. 5B); can be distinguished only histologically; whereas dorsal vessels have very thin epithelium (Fig. 8A) with minute vacuoles inside cells, kidney is characterized by highly vacuolated tissue with large vacuoles. Nephroduct and nephropore not detected.

Reproductive system: Reproductive system of *A. mysticus* not reconstructed in 3D due to very compressed tissue; general anatomy as in *A. ater* (Fig. 6). Reproductive system hermaphroditic, special androdiaulic, ventral to digestive system. Ovotestis (ov) with follicles united by small ductules discharging into preampullary gonoduct. Ampulla large, tubular. Sperm heads short. Receptaculum seminis absent or not developed in examined specimen. Albumen gland with follicles, discharges into postampullary gonoduct. Other nidamental glands very compressed in examined specimens, cannot be distinguished clearly from each other. Hermaphroditic duct bifurcates into internal vas deferens and short oviduct. Bursa copulatrix large, splits off oviduct. Bursal stalk connects to distal oviduct which opens through female gonopore into small mantle cavity at right side of body. Internal vas deferens subepidermally on right side of body wall up to head, connects to glandular prostate; prostate tubular, coiled. Ejaculatory duct muscular, arises anteriorly from prostate, connects to slender penis lacking any armature. Penis surrounded by thin-walled penial sheath. Male gonopore opens to exterior on right side of body near eye. In one examined specimen spermatocytes (Fig. 8B, C) under notum on right body side. Spermatocytes all directed with their heads to body wall filling notum rim from head up to female gonopore.

Remarks: Autotomy is known from several nudibranch species which detach their cerata, e.g. in *Janolus* (Schrödl, 1996), and parts of their mantle (e.g. *Discodoris* sp.; Fukuda, 1994: pl. 40, fig. 793) or even their whole mantle as in *Berthella martensi* (see Rudman, 1998). However, autotomy of the foot as in *A. mysticus* is only known from a few gastropods, such as the vetigastropod *Stomatella varia* (see Taki, 1930) or the sacoglossans *Oxynoe panamensis* and *Lobiger serradifalci* (see Lewin, 1970).

Noteworthy is the triseriate radula of *A. mysticus* (and *A. ater*) in which the lateral teeth are not present over the whole length of the descending limb and only the youngest rows of the lower ramus and the whole upper ramus bear lateral teeth. The oldest, i.e. no more functional rows of the lower ramus consist only of the rhachidian tooth. This phenomenon is unknown to us and is not observed in any sacoglossan or acochlidian species. The triseriate radula of the Acochlidia bears lateral teeth in all tooth rows, although the lower limb is usually considerably shorter than the upper limb (Schrödl & Neusser, 2010). If we imagine the oldest teeth rows (without lateral teeth) eliminated in the aitengid species, the radula could be perfectly an acochlidian one. On the other hand, nonshelled sacoglossan species have smaller, preradular teeth in front of the normal teeth rows (Jensen, 1996). However, the presence of such preradular teeth in Aitengidae is not likely as the teeth on the lower limb have the same appearance as the younger teeth, only the central cusps are used and more worn.

Our observation of the spermatocytes situated in the notum rim with their heads directed to the body wall in *A. mysticus* is peculiar. This specimen had mature female glands and a filled ampulla could not be detected, thus autosperm might have been just released. If these spermatocytes were autosperm, the question arises why they are situated under the notum rim; perhaps autosperm were released accidentally when the animal was disturbed, but in this case we would expect the spermatocytes unorientated rather than directing their heads to the wall. Thus, it is probable that these spermatocytes are allosperm. As there is a penis in *A. mysticus*, sperm are perhaps transferred by the copulatory organ and attached to the body and not near or directly inside the genital pore by copulation. Similarly, in the nudibranch *Aeolidiella glauca* a spermatophore is attached to the mate's body and sperm migrate externally towards the gonopore (Haase & Karlsson, 2000; Karlsson & Haase, 2002).

Molecular phylogeny: Two specimens of *Aiteng mysticus* from different habitats on Miyako Island (Table 3) were found to share the same COI sequence, supporting their conspecificity. Independent of the combination of molecular markers *A. ater* and *A. mysticus* always cluster together in a highly supported Aitengidae clade (see Fig. 11 for ML tree based on the 28S + COI + 16S dataset; trees from other gene combinations not shown). In all analyses Aitengidae cluster outside of the well-supported monophyletic Sacoglossa and within acochlidian Hedylopsacea. Their position within Hedylopsacea, however, varies according to the different genes combined for analysis: in 18S + 28S and 18S + 28S + COI trees Aitengidae form the sister group to a clade uniting marine and brackish Pseudunelidae with limnic Acochlidiidae (trees not shown). When 16S is included in the dataset Aitengidae form the sister group to all remaining Hedylopsacea (Hedylopsidae, Pseudunelidae and Acochlidiidae). Monophyly of Acochlidia (uniting Microhedylacea and Hedylopsacea) is poorly supported and in some analyses not recovered at all due to pulmonate taxa separating both clades (e.g. *Glacidorbis* or *Hygrophila*). This may be a result of the taxon set that was selected to cover acochlidian and sacoglossan families, rather than to comprehensively represent all other major euthyneuran groups, as done by Jörger *et al*. (2010). Acochlidian relationships recovered in the present study are congruent with a previous morphology-based hypothesis (Schrödl & Neusser, 2010), only the paraphyly of Ganitidae is surprising. The Sacoglossa form a well-supported clade in all analyses, with a division into shell-bearing Oxynoacea (including *Cylindrobulla*) and shell-less Plakobranchacea, with Platyhedylidae as most basal offshoot. Internal sacoglossan
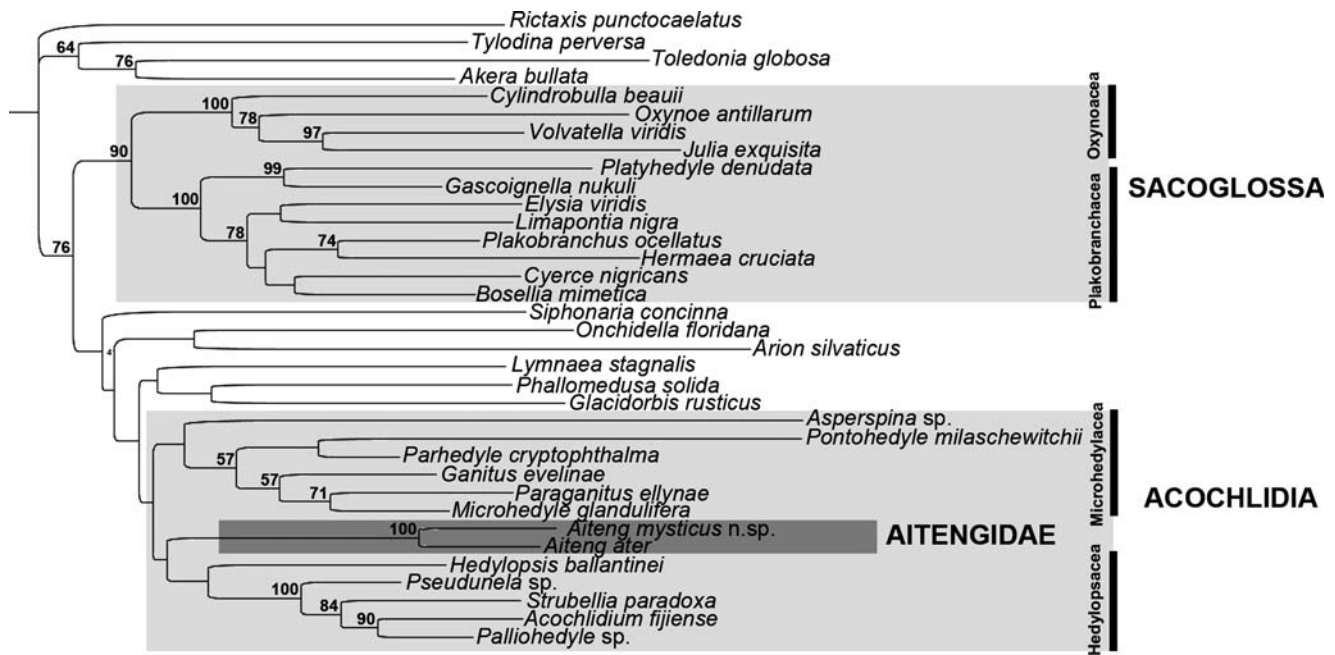
Figure 11. Maximum-likelihood tree generated with RAxML based on the concatenated 28S + COI + 16S dataset, clustering monophyletic Aitengidae basal within Hedylopsacea (bootstrap values >50% given above nodes) *Pseudunela* sp. = *P. marteli* Neusser *et al.*, 2011.

Table 6. Comparison of characteristic sacoglossan and acochlidian features with those of Aitengidae.

	Sacoglossa	Acochlidia	Aitengidae
Retractibility of the head	−	+	+
Calcareous spicules	−	+	+
CNS	Postpharyngeal	Prepharyngeal	Prepharyngeal
Cerebral and pleural ganglia separated	−	+	+
Radula	Uniseriate	Triseriate	Triseriate
Ascending and descending limb	+/−	+	+
Ascus	+	−	−
Branched digestive gland	+/−	+/−	+
Cephalic tentacles	−	+	−
Dorsal vessel system	+/−	−(+)	+
Albumen gland follicled	+	−	+

+, present; −, absent.

relationships slightly differ between the different analyses and resolved clades within Plakobranchacea are not entirely congruent with previous morphological analyses (Jensen, 1996).

DISCUSSION

Aitengid taxonomy

Our specimens from Japan can be clearly distinguished from *Aiteng ater* from Thailand by the habitat, the external morphology, the internal anatomy and perhaps by their feeding habits. *Aiteng ater* inhabits a dense mangrove forest high in the intertidal, which is not covered by the sea during high tides

(Swennen & Buatip, 2009), but the specimens are always associated with small pools of water in the mud. In contrast, *Aiteng mysticus* n. sp. from Japan is found on rocky shores in the upper intertidal in tiny crevices of small sea caves that are usually wet by sea water; or, it is found in a brackish area neighbouring a mangrove swamp on the underside of large, wet rocks deeply embedded in mud in the upper intertidal zone. Although these various habitats are quite different, they all provide a wet and shaded environment without direct exposure to sunlight. Furthermore, both species show a higher activity during the night.

The external morphology of *A. ater* is quite different from that of *A. mysticus*: the body size of *A. ater* is 8–12 mm (Swennen & Buatip, 2009) whereas mature specimens of *A. mysticus* are smaller with a body length of 4–6 mm. The living coloration of *A. ater* is grey-black (Swennen & Buatip, 2009), but brownish or pale in *A. mysticus*.

The internal anatomy is different in nearly all organ systems. At the present stage of knowledge we do not consider the absence/presence of the tiny Hancock's nerve or the small additional ganglion attached to the cerebral ganglion as suitable for species identification, as these tiny structures can be easily overlooked. However, the number of ganglia on the visceral nerve cord differs more clearly between the species: two or three in *A. mysticus*, but (at least) four in *A. ater*. The digestive system is very similar in both aitengid species, but with great differences in radular structure: whereas the rhachidian tooth in *A. ater* has one large, projecting central cusp with up to 20 lateral denticles on each side, in *A. mysticus* there is one large central cusp with 7–9 thinner, pointed lateral denticles on each side. Furthermore, the lateral denticles are smaller in the *A. ater* and the distance between them increases towards the tip of the central cusp, whereas in *A. mysticus* they are larger and evenly spaced. The right lateral teeth in both species bear one pointed, well-developed denticle; in *A. ater* there are 10–15 very small denticles on the anterior margin, whereas *A. mysticus* has only 4–6 small denticles, which are considerably stronger than those of the species from Thailand. Additionally, there is an emargination on the posterior margin of the inner side of

the right lateral teeth in *A. ater*, which is absent in the Japanese species. There are great differences in the left lateral teeth: whereas there are two well-developed, pointed denticles without small denticles on the anterior margin in *A. ater*, there is only one large denticle but accompanied by 12 or 13 small denticles in *A. mysticus*.

The circulatory and excretory systems show major differences between the two species. Whereas a well-developed two-chambered heart is present in *A. ater*, we could only detect a one-chambered heart in *A. mysticus*; however, the epithelium of the pericardium and the atrium is very thin and both organs may collapse artificially. Thus, we do not consider the absence of an atrium as species-specific yet. The thin epithelium of the dorsal vessel system with small vacuoles looks histologically similar in both species. However, in *A. ater* the renopericardio-duct connects to a widened lumen of the dorsal vessels, while in *A. mysticus* it is connected to a kidney. The latter is an anterior branch of the dorsal vessel system, but looks histologically very different and shows the characteristic tissue of the kidney with large vacuoles. Concerning the reproductive system we could not detect major differences between the two aitengid species.

The morphological and anatomical differences found in our study are paralleled by the molecular results, which show that our Japanese specimens belong to the family Aitengidae, but are distinct from *A. ater*. In all analyses *A. ater* and *A. mysticus* formed a highly supported clade (bootstrap 100%). Genetic similarities between the two *Aiteng* species are 89% in 16S rRNA and 85% in COI sequences.

Sacoglossa or Acochlidia?

Aiteng ater was described with an unusual mix of sacoglossan and acochlidian characters and the authors doubtfully suggested a sacoglossan relationship. A comparison of sacoglossan and acochlidian features is given in Table 6. Our results show that only a few characters remain that indicate a closer relationship to Sacoglossa: (1) the absence of any cephalic tentacles similar to e.g. the semi-terrestrial *Gascoignella aprica* (Jensen, 1985) or *Platyhedyle denudata* (Rückert, Altnöder & Schrödl, 2008); (2) the presence of an elysiid-like system of dorsal vessels, as in *Elysia* (Marcus, 1982; Jensen, 1996); (3) the albumen gland consisting of follicles as e.g. in the limapontioid *Hermaea* (Jensen, 1996). There are two ambiguous characters that are characteristic of at least some sacoglossan and acochlidian species: (1) the radula with an ascending and a descending limb present in all acochlidian species known in detail (Neusser *et al.*, 2006, 2009a, b; Neusser & Schrödl, 2007, 2009; Jörger *et al.*, 2008; Brenzinger *et al.*, 2010) and e.g. in the sacoglossan *Ascobulla* (Jensen, 1996); (2) the branched digestive gland which has been reported from the limnic *Acochlidium fijiense*, *A. amboinense* and *Palliohedyle weberi* (Bergh, 1895; Bücking, 1933; Haynes & Kenchington, 1991) and which is present e.g. in the sacoglossan *Limapontia* and *Hermaea* (Jensen, 1996).

Finally, aitengids resemble acochlidians by (1) retractibility of the head; (2) presence of calcareous spicules; (3) prepharyngeal nervous system; (4) separated cerebral and pleural ganglia; (5) triseriate radula; (6) absence of a sacoglossan-like ascus; and (7) the "special androdiaulic reproductive system" (Schrödl, *et al.*, 2011) as present in *Tantulum elegans*, *Pseudunela cornuta* and *P. espiritusanta* (Neusser & Schrödl, 2007, 2009; Neusser *et al.*, 2009a). Furthermore, the large, laterally situated eyes of Aitengidae closely resemble the anatomy in members of the large, limnic acochlidian family Acochlidiidae (e.g. in *Strubellia paradoxa*) (Brenzinger *et al.*, 2010); as well as the prominent rhachidian tooth of members of Aitengidae, which is used to pierce insects and pupae in *A. ater* and for piercing neritid egg capsules in *Strubellia* (Brenzinger *et al.*, 2011). The

case for the originally suspected sacoglossan relationship of *Aiteng* is clearly weakened and, based on our morphological results, the affinity to Acochlidia, in particular to limnic Acochlidiidae, is more evident. Morphological features alone, however, might not be sufficient to reveal correctly the systematic relationships of aberrant species inhabiting special habitats (see e.g. Schrödl & Neusser, 2010). Thus, supporting molecular evidence is needed.

In a recent multilocus molecular analysis, *A. mysticus* (as Aitengidae sp.) also clusters within hedylopsacean Acochlidia (Jörger *et al.*, 2010); however, only a single aitengid species and single representatives of acochlidian families were included. Here we present a focused taxon sampling for Acochlidia and Sacoglossa and new sequence data for *A. ater*. Acochlidian rather than sacoglossan relationships for Aitengidae are again supported. Their position within Hedylopsacea, however, cannot be ascertained at the present stage of knowledge, differing depending on the molecular markers included: they are sister to a clade of marine/brackish Pseudunelidae and limnic Acochlidiidae in analysis of 18S + 28S (with or without COI); but sister to all remaining Hedylopsacea when 16S is included (see Fig. 11). A hedylopsacean origin of Aitengidae reflects morphological similarities discussed above. Any inner acochlidian origin would, however, imply that Aitengidae have lost the most characteristic acochlidian apomorphy (Sommerfeldt & Schrödl, 2005; Schrödl & Neusser, 2010), which is the subdivision of the body into a headfoot complex and a free, elongated visceral hump. Furthermore, the absence of cephalic tentacles gives the Aitengidae a compact external appearance that is very different from other marine or limnic Acochlidia.

Habitat shift

The question is whether or not these external differences between Aitengidae and other Acochlidia, and perhaps also some peculiar anatomical features, might be evolutionarily related to the habitat shift from an ancestrally aquatic to an amphibious lifestyle.

The cephalic head appendages and the free, elongated visceral sac of 'normal' aquatic acochlidian species are supported in shape while under water, but in air, e.g. during collecting, they collapse to an amorphous mass. Obviously, elongate head appendages on land should be hydrostatic and/or provided with muscles as in terrestrial stylommatophoran pulmonates, or must be reduced. Following the putative acochlidian relationship of Aitengidae, this implies that in *Aiteng* the ancestral rhinophores (as e.g. in the marine acochlidians *Pontohedyle milaschewitchii* and *Ganitus evelinae*; Marcus, 1953; Jörger *et al.*, 2008) were lost, and labial tentacles became short lobes that fused to a velum. The compact body shape of aitengids with a short stout head might be also interpreted as an adaptation to an amphibious lifestyle, with the visceral hump connected to the foot on all its length guaranteeing better stability and minimizing the body surface.

Calcareous spicules in the connective tissue are already present in aquatic acochlidians, and in aitengids spicules are present but do not build an elaborate skeleton. However, the notum of aitengids shows a unique layer of large, vacuolated supporting cells. This layer almost certainly contributes to a more stable and robust body shape in Aitengidae. Probably the notal layer also provides some mechanical protection as well as protection from desiccation. By analogy, the sea slug *Corambe* shows a thickened protective notum that, however, hinders the diffusion of oxygen through the notal tissue and thus likely induced the multiplication of hyponotal gills (Martynov *et al.*, 2011; Martynov & Schrödl, 2011). Despite the presence of the special notal supporting cell layer in *Aiteng*, the diffusion of oxygen is probably sufficient when animals are

exposed to air. If submerged for a long period, the compact gill-less animals may have a problem. Under any conditions, cells of the body wall need to be supplied with oxygen and other substances, and waste removed. We speculate that these and perhaps other functions might be carried out by the dorsal vessel system lying directly below the supporting cell layer, extending in fine ramifications to the notum border. Thus, the presence of the thin-walled dorsal vessel system of the Aitengidae, which is a modified portion of the kidney, is assumed to enhance respiratory, secretory and excretory processes in a secondarily amphibious lineage and, as such, might also be explained by the habitat shift.

Similar dorsal vessels exist in elysiid and some other non-shelled sacoglossans. Jensen (1992) assumed an excretory or osmoregulatory function, but also discussed a possible homology with the gills of the shelled sacoglossan species; so far neither cellular structures of sacoglossan dorsal vessels, nor the connections to the circulatory or excretory system, nor homologies with e.g. atrial, pericardial or renal tissue have been sufficiently explored. Accepting the phylogenetic distance between aitengids and elysiids, these vessel systems evolved convergently. Dorsal vessels have been discussed earlier as a 'negative gill' in sacoglossan species having functional kleptoplasts, i.e. species in which an excess of the oxygen produced must be transported away from the tissue (Jensen, 1996, and references therein). However, Aitengidae do not incorporate and maintain active plastids as do some sacoglossan species (Wägele *et al.*, 2011) and therefore such a function is not imaginable in *Aiteng*.

The dark body coloration of aitengid species might be a protection from UV radiation to which these species could be exposed, in contrast to other acochlidian species which live hidden in sand or under stones. This coincides with the mostly nocturnal activity of Aitengidae preventing an excessive exposure to sunlight.

Regarding acochlidians, Bücking (1933) reported vessels emerging from the heart bulb and extending over the whole dorsal surface of the visceral sac in the limnic *Acochlidium amboinense* and suggested a respiratory function. Wawra (1979) observed vessel-like structures in *Palliohedyle sutteri*. However, both observations were based on preserved specimens only. Other limnic Acochlidiidae, such as *A. fijiense* and *A. bayerfehlmanni* were described to lack any vessels (Wawra, 1980; Haynes & Kenchington, 1991). Preliminary re-examinations of *A. amboinense* and *A. bayerfehlmanni* show both species to possess a dorsal vessel system that is, however, less ramified than in aitengids (own unpublished data). Thus a histological survey on all known Acochlidiidae is necessary to confirm the presence or absence of dorsal vessels and to clarify the homology and the function of such vessels in the large limnic Acochlidiidae. Only if they are part of the excretory rather than circulatory system, could acochlidiid and aitengid dorsal vessels be synapomorphic and thus support a sistergroup relationship, as suggested by further potential morphological apomorphies and some molecular analyses discussed above.

Finally, the habitat shift might induce a change in the feeding habits. While the prominent rhachidian tooth in *Strubellia* is used to feed on neritid egg capsules (Brenzinger *et al.*, 2011), other molluscan eggs might not be available in the new habitat outside the water, but instead insects and pupae as in the case of *Aiteng ater*. The food source of *Aiteng mysticus* was not observed in the field. This species can be found frequently on intertidal algae, but shows no sign of feeding on algae. Furthermore, its pale coloration argues against any food containing plastids. Although the rhachidian tooth of *A. mysticus* is not as prominent as in *A. ater*, a grazing feeding habit is not likely. We assume that the food resource of *A. mysticus* is present on the algae and might consist of animal eggs or pupae similar to its congener from Thailand.

Conclusion

Aitengidae are small but highly specialized amphibious slugs, now known from two species from the Indian and Pacific Oceans. Traditional morphological means such as dissections and light microscopy gave a glimpse of the acochlidian relationship of *Aiteng ater*. Applying 3D-reconstruction methods to soft parts and SEM radula examinations substantially supplement and refine the original description of *A. ater* and reveal several putative apomorphies indicating the acochlidian nature of Aitengidae. Molecular data additionally support Aitengidae clustering within Acochlidia as a more or less basal offshoot of Hedylopsacea, implying a switch from aquatic to amphibious lifestyle. Considerable external dissimilarities and even aberrant anatomical structures such as the layer of vacuolated notal cells and the kidney that is modified into a highly ramified system of dorsal vessels can be explained as aitengid autapomorphies that evolved (or further elaborated) during that habitat shift. Surveying tropical slug diversity in different, not only aquatic, habitats may reveal further and perhaps even more specialized and aberrant creatures. Integrating biological observations such as 'bug-eating' with (micro)morphological and genetic data allows us to reconstruct an evolutionary scenario that turns a 'mysterious slug' into an instructive and amazing example of animal evolution.

AUTHORS' CONTRIBUTIONS

ACKNOWLEDGEMENTS

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