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Article

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Two new sacoglossan sea slug species (Opisthobranchia, Gastropoda): *Ercolania annelyleorum* sp. nov. (Limapontioidea) and *Elysia asbecki* sp. nov. (Plakobranchoidea), with notes on anatomy, histology and biology

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Abstract

Two new sacoglossan species, belonging to the genus *Ercolania* Trinchese, 1872 (*Ercolania annelyleorum* **sp. nov.**) and the genus *Elysia* Risso, 1818 (*Elysia asbecki* **sp. nov.**) are described from Lizard Island, Great Barrier Reef, Australia. Anatomy of both species was reconstructed by analyzing histological serial sections. Radula morphology was investigated by using light microscopy and scanning electron microscopy. Sequence analyses (NeighborNet; sequence divergence) and tree reconstructions showed for both species their distinction from con-generic species, but also two distinct mitochondrial lines in the new *Ercolania* species.
Adults as well as freshly hatched juveniles of *E. annelyleorum* **sp. nov.** have been found in clusters of the ulvophycean alga *Boodlea* sp., which are sucked out by piercing the cell walls with their radular teeth. This new species differs from other, similar transparent, *Ercolania* species by its pattern of the green branches of the digestive gland and the presence of two distinct red patches, one in the anterior and the other in the posterior third of the dorsal body part. This coloration and furthermore the combination of following characters distinguishes the new species from all other described *Ercolania* species so far: rhinophores, elliptic in cross section, with one distinct branch of digestive gland running half way up; cerata not inflated; smooth cutting edge of sabot-shaped tooth; two-lobed prostate gland and presence of two allosperm receptacles with no re-opening of the receptaculum seminis to the outside. According to sequence divergence data of CO1, two mitochondrial lines seem to be present in the new species, which are clearly distinct from all other included *Ercolania* species.

Elysia asbecki **sp. nov.** differs from other *Elysia* species by its whitish coloration with orange and dark brown dots and a distinct lighter spot in the neck region of the head. The rhinophores exhibit a black and yellow ribbon at the tip. The species has distinct reddish patches at the anterior base of the parapodia (at the conjunction with the head), one along the middle part of the parapodial edge on both sides and very distinct lateral patches at the end of the foot. CO1 sequences clearly distinguish this species from all closely related *Elysia* species. The food source of *Elysia asbecki* **sp. nov.** could not be verified yet. Measurements of photosynthetic activity within these two new species indicate that *E. annelyleorum* **sp. nov.** digests chloroplasts immediately after sequestration, whereas *Elysia asbecki* **sp. nov.** shows high maximum quantum yield values, similar to *E. timida* (Risso, 1818) and *E. crispata* (Mørch, 1863), both known as long term retention forms.

Key words: Sacoglossa, new species, photosynthetic activity, DNA taxonomy, bar coding, phylogeny

Introduction

Only few gastropods have such a specialized feeding strategy like the opisthobranch taxon Sacoglossa. While feeding mainly on siphonous or siphonocladous algae, they pierce the algal cell wall and suck out their contents. Many of these slugs are cryptic by appearing as green as their algal food source, due to the sequestration of chloroplasts in the digestive gland system for some time. This probably has led to their

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oversight (see Jensen 2007) and only intensive research on this group now reveals more and more new species. Scientific publications or identification books dealing with records of opisthobranchs from various geographic areas usually list many undescribed sacoglossans (Carlson & Hoff 2003; Wägele *et al.* 2006b; Burghardt *et al.* 2006; Gosliner *et al.* 2008; Trowbridge *et al.* 2010). More than 40 undescribed sacoglossan species have been recorded on Rudman's website on Opisthobranchia (www.seaslugforum.net, last access 24th of September 2010), which is about 20 % of all sacoglossan species on this website. During a collecting trip to Guam in 2009, two of the authors have sampled nearly 20 undescribed sacoglossan species. At the same time, new descriptions augmented our number of described species: *Elysia clarki* Pierce, Curtis, Massey, Bass, Karl & Finney, 2006; *Alderia willowi* Krug, Ellingson, Burton &Valdés, 2007; *Ercolania kencolesi* Grzymbowski, Stemmer & Wägele, 2007.

Here we describe two new species of Sacoglossa from Lizard Island (Australia, Great Barrier Reef). One of the new species belongs to the genus *Ercolania*. This species was detected for the first time in 2006 in the same habitat as *E. kencolesi* and listed by Händeler & Wägele (2007) as *Ercolania* spec. 5. Further samples were taken in 2007 and 2008 at the same place. In contrast to the more common *Ercolania* species described here, the new species of the genus *Elysia* was found only twice on Lizard Island on the reef top in the intertidal zone (2004 and 2006) but also during a field trip of one of the authors to the Samoan Islands in 2005.

Cytochrome c oxidase subunit 1 (CO1) has been accepted as a practical, standardized species-level barcode for animals (see http://www.barcodeoflife.org, last access 15.09.2010 and few examples: Hebert *et al.* 2003, 2004; Smith *et al.* 2008; Kress & Erickson 2008: Skevington *et al.* 2007; Victor *et al.* 2009). Partial sequences of the CO1 gene were obtained for both species and analyzed separately with NeighborNet algorithms as well as compared statistically (sequence divergence) on species and/or higher taxa level. Additionally, a phylogenetic tree based on a concatenated data set of partial sequences retrieved from the CO1 (first and second positions only), 16S rDNA and 28S rDNA gene including available con-generic species and/or other related taxa is presented for the Limapontiidae, to provide further evidence of distinctiveness of the new *Ercolania* species described here.

For some sacoglossan species it is known that incorporated chloroplasts from the food algae remain photosynthetically active and contribute to their nutrition (e.g. Rumpho 2001; Evertsen *et al.* 2007; Händeler *et al.* 2009; see Wägele & Johnsen 2001 for older literature). Here we also describe photosynthetic abilities of the two new species which show completely different photosynthetic performances.

Methods

Histology. Animals that have been investigated for anatomy were preserved in formaldehyde/seawater (1/5). These animals were embedded in hydroxyethylmethacrylate for serial sectioning and sections (2.5 µm) were later stained with toluidine blue. Sections served for reconstruction of anatomy.

SEM. Two radulae of *Ercolania annelyleorum* **sp. nov.** and one of *Elysia asbecki* **sp. nov.** were investigated by light microscopy and additionally by scanning electron microscopy. Radulae were investigated under a ZEISS Imager Z2m and multidimensional pictures created with the program AxioVision 4.8. Analyses of the same radulae have been performed in a Hitachi SEM.

Molecular investigations. DNA was extracted from alcohol-preserved specimens by means of the DNeasy® Blood and Tissue Kit by Qiagen, following manufacturer's recommendations. Partial sequences of the nuclear 28S rDNA gene and partial sequence of mitochondrial CO1 were produced as described in Händeler *et al.* (2009) with primers 28SC1 and 28SD3 (Vonnemann *et al.* 2005), and LCO1490 and HCO2198 (Folmer *et al.* 1994; Bass & Karl 2006) respectively. PCR products were sequenced by Macrogen Inc. (Seoul, Korea). Accession numbers of all used sequences are listed in Table 1. Sequences were aligned using the web server of MAFFT (Katoh *et al.* 2002; Katoh & Toh 2008). NeighborNet analyses were performed only on CO1 sequences by using the program SplitsTree 4.10 (Huson & Bryant 2006). Sequence divergence data were based on CO1 only and were calculated by using uncorrected p-distances.

TABLE 1. Accession numbers of sequences used in the phylogenetic analyses. Different sequences of the three markers usually from the same specimen. * —sequence taken from GenBank and origin unknown. Accession numbers of new sequences are in bold.

Species	Origin	28S rDNA	16S rDNA	CO1
Ercolania				
Ercolania annelyleorum **sp. nov.** (internal number 787)	Australia: Lizard Island	HQ380188	EU140839	HQ380195
Ercolania annelyleorum **sp. nov.** (internal number 69)	Australia: Lizard Island	-	-	HQ380194
Ercolania annelyleorum **sp. nov.** (internal number 71)	Australia: Lizard Island	-	-	HQ380196
Ercolania annelyleorum **sp. nov.** (internal number 76)	Australia: Lizard Island	-	-	HQ380197
Ercolania sp.	USA: Guam, Apra harbour	-	-	HQ380198
Ercolania boodleae	Japan	GU191021	GU191050	-
Ercolania felina	New Zealand: Auckland	GU191022	GU191038	GU191060
Ercolania	Bermuda	GU191023	GU191039	GU191061
Ercolania kencolesi	Australia: Lizard Island	GQ996620	EU140840	GQ996660
Ercolania sp.	Japan: Sobe	GU191024	GU191051	GU191062
Ercolania viridis	France: Banyuls-sur-Mer	HQ380189	HQ380182	HQ380199
Alderia				
Alderia modesta	USA: California, Oregon, Alaska	GU191030	DQ364417	DQ364343
Alderia willowi	USA: California	GU191036	DQ364419	DQ364404
Limapontia				
Limapontia nigra		AY427465*	-	-
Limapontia senestra	Germany: Helgoland	HQ380190	HQ380183	HQ380200
Calliopea				
Calliopea bellula		AY427464*	-	-
Placida				
Placida cremoniana	USA: Guam	HQ380191	HQ380184	HQ380201
Placida dendritica	Spain: Tossa de Mar	GQ996616	EU140871	GQ996663
		GQ996617	EU140870	GQ996662
Placida kingstoni	USA: Florida: Key West	GU191028	GU191044	GU191063
Placida verticillata	Bahamas: San Salvador	GU191029	GU191045	GU191064
	Bolivarian Republic of Venezuela, Isla Margaritha	HQ380192	HQ380185	HQ380202
Stiliger				
Stiliger ornatus	Egypt: Dahab	-	HQ380186	HQ380203
		-	HQ380187	HQ380204
Mourgona				
Mourgona osumi	Australia: Lizard Island: North Point	GQ996646	EU140847	GQ996667
Cyerce				
Cyerce nigricans	Australia: Lizard Island: Channel	GQ996644	EU140843	GQ996658
Elysia				
Elysia amakusana	Australia: Lizard Island	GQ996621	EU140851	GQ996686

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TABLE 1. (continued)

Species	Origin	28S rDNA	16S rDNA	CO1
Elysia asbecki **sp. nov.**	Australia: Lizard Island	GQ996629	EU140856	GQ996690
Elysia asbecki **sp. nov.**	Australia: Lizard Island	-	-	HQ380193
Elysia bennettae	Samoa: Upolu Island Apia, harbour, ZSM20060293	GQ996637	EU140868	GQ996675
	Australia: Lizard Island	HM187619	HM187586	HM187621
Elysia chlorotica	USA: Massachusetts: Martha's Vineyard	GU191035	GU191054	GU191073
		HM187618	DQ480200	NC 010567
Elysia cornigera	USA: Florida: Summerland Key, Henry Street	GQ996624	HM187587	HM187622
	USA: Florida: Cudjoe Key	HM187617	HM187588	HM187623
Elysia crispata	USA: Florida: Summerland Key	GQ996634	HM187589	HM187624
	USA: Florida: Summerland Key	GQ996635	HM187590	DQ471225*
Elysia macnaei	Indonesia: Sulawesi: Bunaken nationalpark, Gangga, ZSM20033821	GQ996628	EU140854	GQ996689
Elysia marcusi	USA: Florida: Summerland Key	HM187616	HM187591	HM187625
		GQ996641	HM187592	HM187626
Elysia obtusa	Samoa: Savaii Island, Vaisala lagoon, ZSM20060257	GQ996627	EU140860	GQ996685
Elysia ornata	Australia: Lizard Island	GQ996622	EU140848	GQ996688
	Australia: Lizard Island: North Point	GQ996623	EU140850	GQ996687
Elysia papillosa	Bahamas: Sweetings Cay	GU191033	GU191049	GU191070
Plakobranchus				
Plakobranchus ocellatus	Australia: Lizard Island	GQ996619	EU140876	GQ996680
	Australia: Lizard Island	AY427459*	EU140875	GQ996679
Thuridilla				
Thuridilla carlsoni	Australia: Lizard Island	GQ996614	EU140878	GQ996681
Thuridilla hoffae	Samoa: Savaii Island, Vaisala lagoon, ZSM20060224	GQ996618	EU140880	GQ996670
Thuridilla kathae	Australia: Lizard Island	GQ996615	EU140879	GQ996676

For the phylogenetic analysis concatenated alignments were used. The Limapontiidae data set comprised an alignment with the length of 1953 bp: 28S rDNA gene, positions 1–1069, 16S rDNA gene, positions 1070–1517 and CO1 gene (first and second position), positions 1518-1953. The data set for *Elysia* had a length of 1967 bp: 28S rDNA gene, positions 1-1069, 16S rDNA gene, positions 1070–1531 and CO1 (first and second position), positions 1532–1967. Phylogenetic analysis (Maximum Likelihood approach) was performed with the help of the web server of RAxML (Stamatakis *et al.* 2008). Data set was partitioned according to the three different partial gene sequences. 100 bootstrap replicates were executed. Bootstrap support values are shown on the trees.

PAM measurements. Analyses of photosynthetic activity were performed with a Pulse Amplitude Modulated Fluorometer (DIVING PAM, Walz, Germany) by measuring the maximum quantum yield of chlorophyll a fluorescence emitted by photosystem II ($\Phi_{IIe\text{-}max}$). The fiber optics of the PAM was usually placed 5 mm above the animal. Measurements were taken with chloroplasts acclimated to darkness for 15 minutes, thus with all reaction centres open and a minimal fluorescence emission. While measuring, an actinic

light flash is emitted which induces closure of reaction centres and yields maximum emission of fluorescence. Initial measurements were taken on the day of collection. For further details of the methods see Wägele and Johnsen (2001) and Burghardt and Wägele (2004).

Species description

Ercolania Trinchese, 1872

Type species: *Ercolania siotii* Trinchese, 1872, by subsequent designation (Iredale & O'Donoghue 1923).

Diagnosis. According to Baba and Hamatani (1970), Schmekel and Portmann (1982) and Jensen (1985, 1993), the genus *Ercolania* is characterized by the following features: Presence of cerata, which are fusiform or inflated; digitiform rhinophores, which are circular or slightly flattened in cross-section; elongate or inconspicuous reno-pericardial prominence; presence of sabot-shaped teeth; presence of a curved penial stylet; probably absence of albumen gland in the cerata.

Ercolania annelyleorum sp. nov.

Type material. All specimens were collected in front of Casuarina Beach, Lizard Island (North Queensland, Australia) in shallow water up to 1 m depth at high tide. Type material is deposited at the Australian Museum Sydney. Holotype (AM C.464067): 25th June 2007 (length of preserved specimen: 1.5 mm); three paratypes (AM C.464068, including SEM preparation of radula): 10th July 2006; 13th July 2006; 20th October 2008 (length of all paratypes between 1 and 2 mm). For further material collected and investigated see Table 2.

TABLE 2. Synopsis of investigated specimens and collecting data of *Ercolania annelyleorum* **sp. nov.** Material is listed according to collecting dates. FSW: preservation in formaldehyde/seawater; PAM: measurements of photosynthetic activity; LM: light microscopy; SEM: scanning electron microscopy; ZFMK Zoologisches Forschungsmuseum Alexander Koenig in Bonn, Germany; ZMS Zoologische Staatssammlung Munich.

Date of collection	Date and kind of preservation	PAM	Type of investigation
07.07.2006	08.07.06 FSW	Yes	Histology (ZSM 20100675)
07.07.2006	08.07.06 EtOH	Yes	Gene analyses: 28S, 16S, CO1 (internal number 5787) (publ. in Händeler *et al.* 2009 as *Ercolania* sp.)
10.07.2006	10.07.06 EtOH	Yes	Paratype AM C.464068
13.07.2006	01.08.06 FSW	Yes	Paratype AM C.464068
25.06.2007	28.06.07 FSW		Radula preparation (LM) (lost)
25.06.2007	01.07.07 FSW		Histology
25.06.2007	01.07.07 FSW		Histology
25.06.2007	01.07.07 EtOH		2 specimens not investigated
25.06.2007	02.07.07 EtOH		Holotype AM C.464067
20.10.2008	21.10.08 EtOH		Gene analysis: CO1 (internal number 71) Radula preparation (LM) (Fig. 3 E) Voucher material: ZFMK–DNA SacSti 0001
20.10.2008	21.10.08 EtOH	Yes	Paratype AM C.464068 (Radula only) Gene analysis: CO1 (internal number 76) Radula preparation (LM and SEM (Fig. 3 A–D)
20.10.2008	22.10.08 EtOH	-	Gene analysis: CO1 (internal number 69)

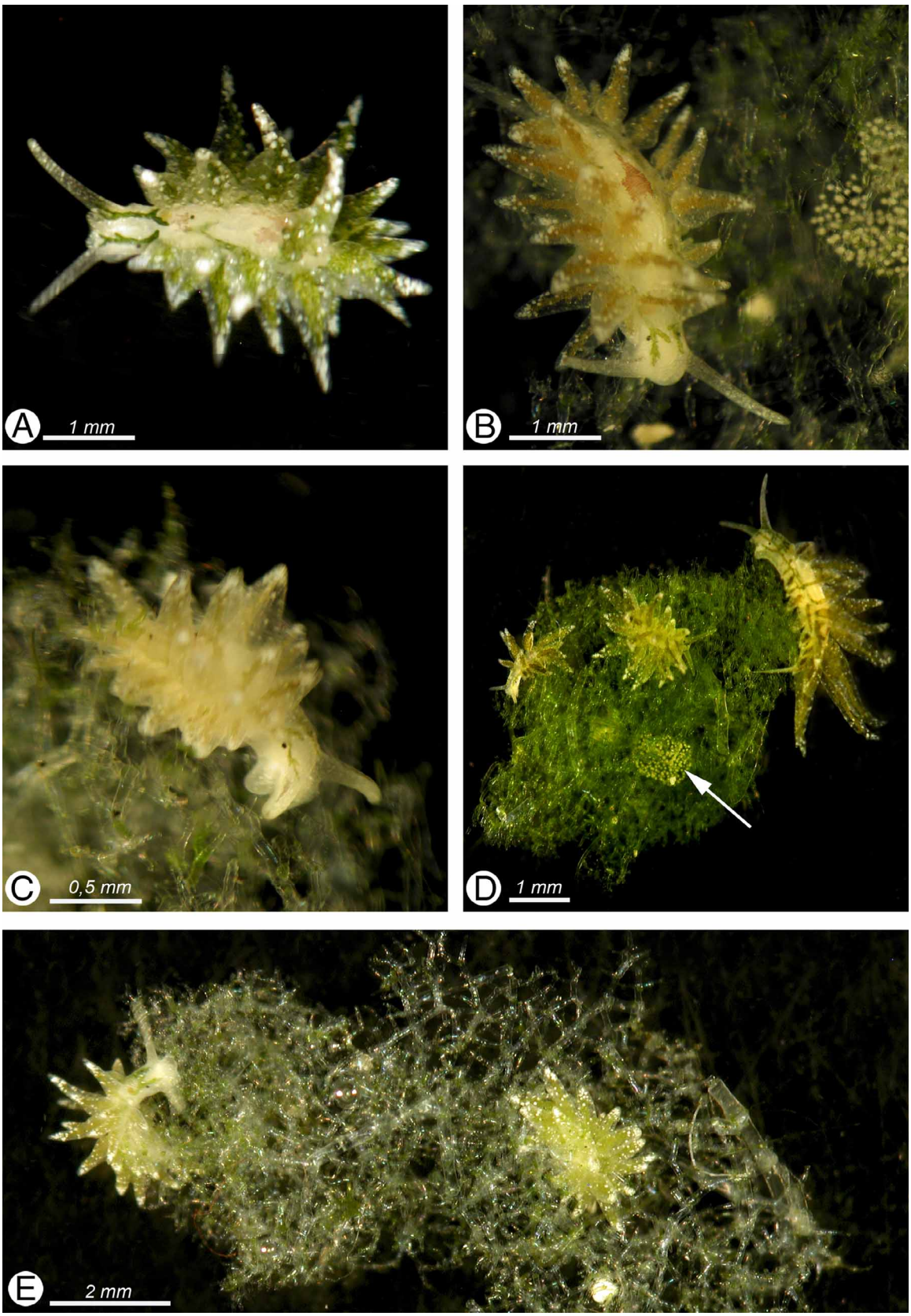

FIGURE 1. *Ercolania annelyleorum* **sp. nov.**: living animals from Lizard Island; (A) Animal freshly isolated from food algae; length about 5 mm. (B) Animal after 1 day starvation. Note the characteristic dorsal red mark in the posterior third; length about 5 mm. Egg clutch visible on right side. (C) Juvenile starved for four days and now back on *Boodlea*; length about 2 mm. (D) One adult and two juveniles feeding on *Boodlea*. Note the egg clutch (arrow). (E) Two animals feeding on *Boodlea*.

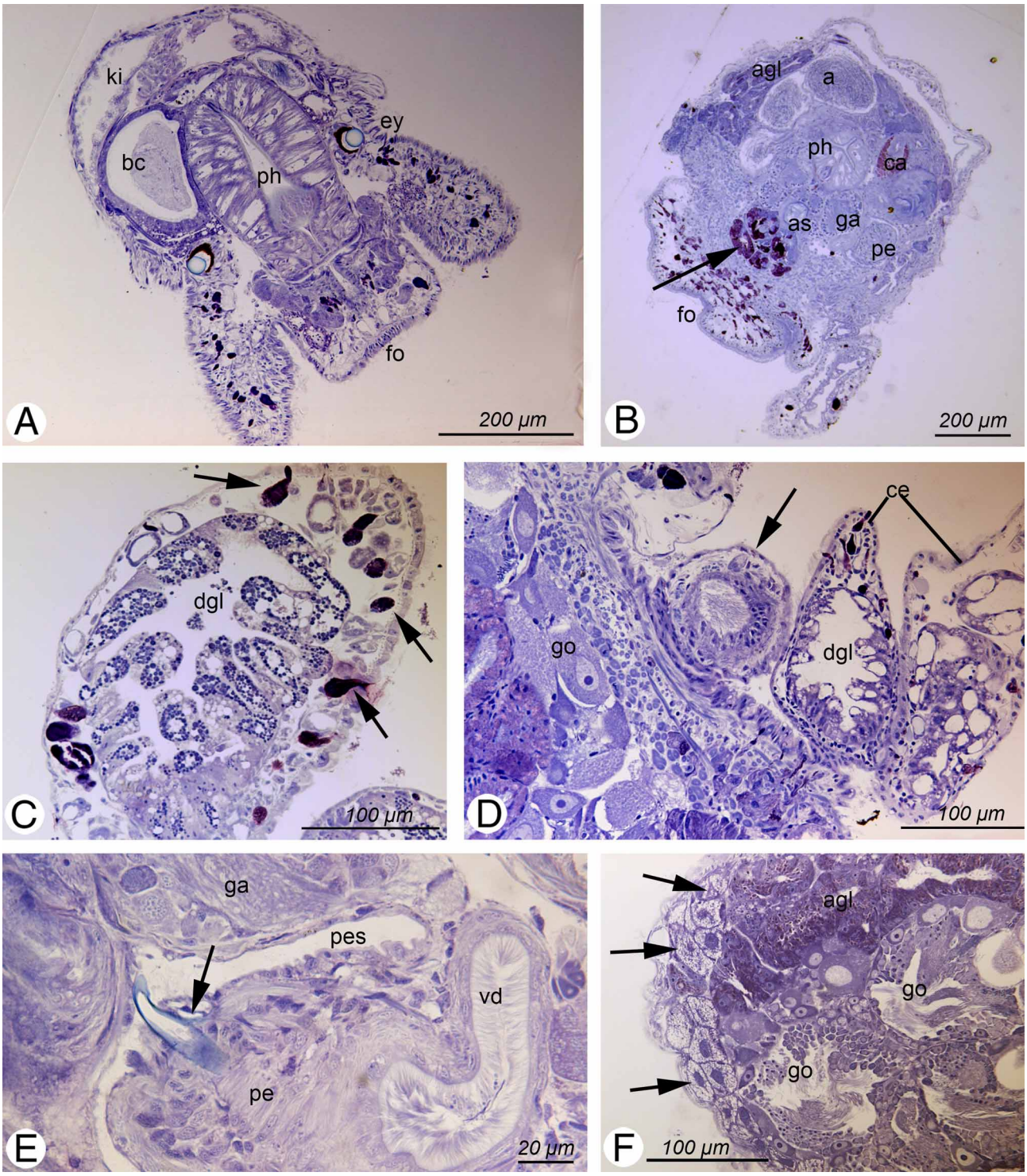

FIGURE 2. *Ercolania annelyleorum* **sp. nov.**: histology; (A) cross section in head area. Note the eyes laterally from the pharynx. (B) Cross section behind the head region. Note the large glandular structure below the ascus being part of the elaborate oral glands (arrow). (C) Longitudinal section of ceras with folded digestive gland. Note the dark dots representing chloroplasts in the epithelium of digestive gland. Large subepithelial glandular cells present, filled with acid mucopolysaccharides (arrows). (D) Detail of right lateral side with two cerata and one with an unidentified structure containing sperm (arrow). (E) Longitudinal section through penial sheath with muscular penis and ciliated vas deferens. Note insertion of hollow cuticular spine projecting into penial sheath (arrow). (F) Cross section in posterior half of body. Note the special glandular cells beneath the epidermis (arrows). Abbreviations: agl albumen gland, am ampulla, as ascus, bc bursa copulatrix, ca capsule gland, ce ceras, dgl digestive gland, fo foot, ga ganglion, go gonad, ki kidney, pe penis, pes penial sheath, ph pharynx, vd vas deferens.

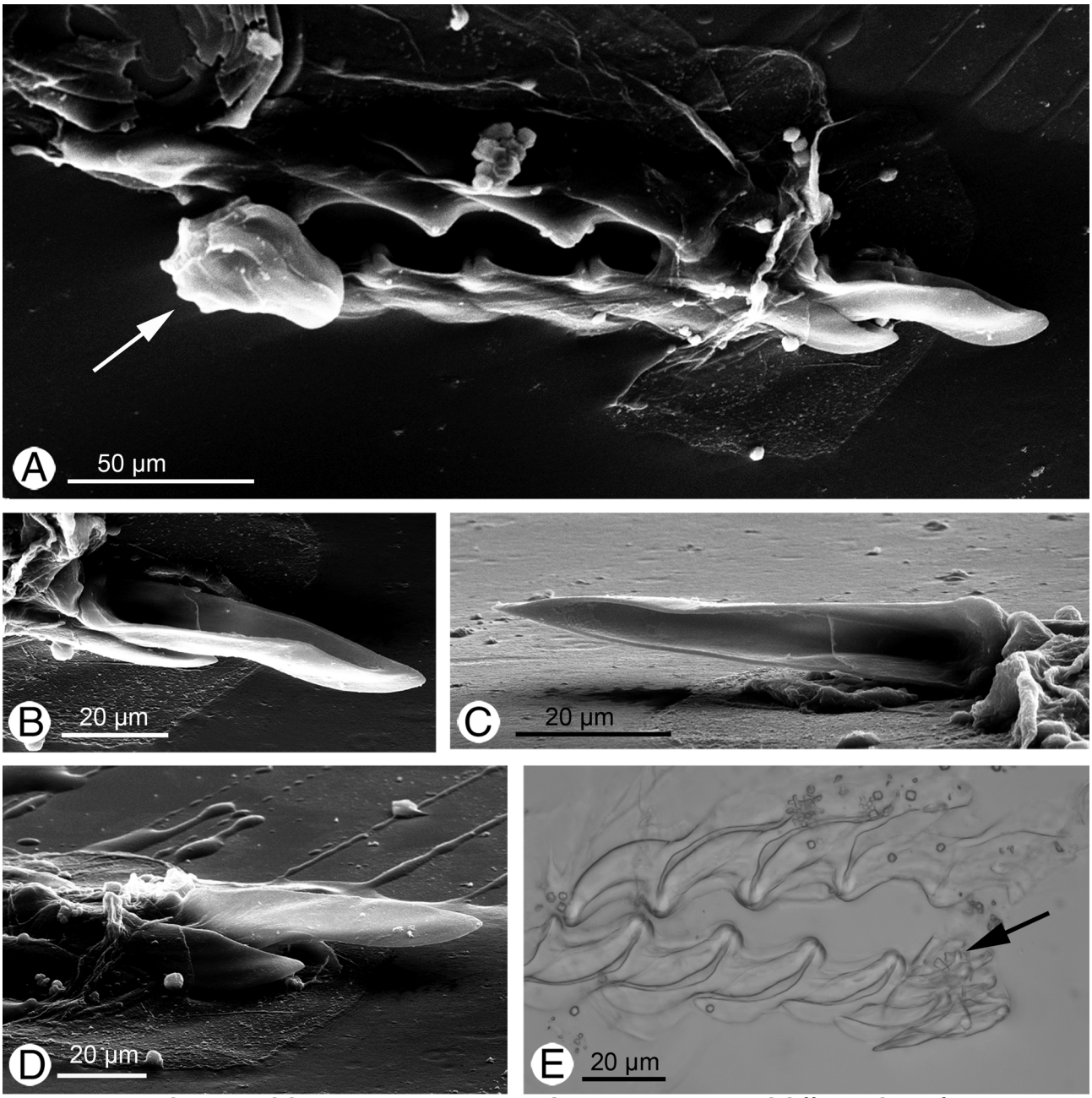

FIGURE 3. *Ercolania annelyleorum* **sp. nov.**: scanning electron microscopy and differential interference contrast microscopy of radula: (A) complete radula of specimen 76 with leading tooth to the right. Ascus (white arrow) covered by thin cuticle. (B) – (D) different views of leading tooth. (E) Radula of specimen 71. Note the small teeth and pre-radula teeth (black arrow) in ascus.

Distribution. Up to now this species has only been recorded from Casuarina Beach, in front of the Lizard Island Research Station, in clusters of the alga *Boodlea* sp. (Figs. 1 C–E, material studied here) and from the Mariana Islands (see discussion).

Etymology. This species is dedicated to Dr. Anne Hoggett and Dr. Lyle Vail, the directors of the Lizard Island Research Station (LIRS, Great Barrier Reef, Australia) for continuously supporting our projects.

Description. *External morphology and color of living specimens* (Fig.1). Size up to 4 mm; body elongate; foot tapering posteriorly; anterior foot slightly extended to lateral sides with small notch, but without any propodial tentacles; rhinophores long, solid and digitiform; eyes located behind rhinophores; cerata club-shaped to elongated, in two to three rows, with smaller ones on outer side; cerata standing close, those of similar size in opposite position; central part of dorsal surface free of any cerata, without a prominent pericardial hump (Figs. 1A–C).

Overall color of body translucent to whitish; green branches of digestive gland (color is due to undigested chloroplasts) shining through translucent body wall, especially in cerata and margin of the body. Two green lines beginning in posterior part of back and running parallel towards head; ending in anterior part of rhinophores, but not reaching into tips. These digestive gland ducts hardly branch. White dots spread over body, but more frequent and larger on distal parts of cerata and rhinophores. In posterior third of body red irregular formed patch visible dorsally between these two green lines, an additional red patch of different shape also visible in anterior third of dorsal surface of body. Branches of digestive gland brownish in animals starved for one day, their color therefore more translucent and light brown (Fig. 1B). After longer starvation periods due to physiological experiments most cultivated specimens lost their greenish appearance, looked rather pale and were colored whitish to slightly pinkish (Fig. 1C).

Description of anatomy and histology (Figs. 2–4). Three specimens investigated (see Table 2). Specimens were bent slightly to the ventral side in the longitudinal axis. This is considered (and corrected) in the description of the anatomy.

Digestive tract. Mouth opening between ventral part of head and anterior foot lip. Epithelium of oral tube with ciliated cells; subepithelial glandular follicles forming a layer around oral tube and entering the latter; cells with bluish to violet stained mucus. This glandular layer extends far back below pharynx and ascus (Fig. 2B, arrow). Anterior outer pharynx (labial disc) covered by thin unarmed cuticle (labial cuticle), continuing interiorly. Pharynx on dorsal part forming a longitudinal buccal pump with an internal thin cuticular layer. Ascus separate from pharynx, surrounded by thick muscle layer and with teeth present in lumen (Fig. 2B). Radula formula 0.1.0; at least four to five larger teeth in ascending limb and six to seven teeth in descending limb (Figs. 3A – E). Leading tooth of sabot-shaped type, with a smooth cutting edge (Figs. 3A, D). Several smaller teeth (six counted in one of the investigated radulae) and two pre-radular teeth in ascus present (Fig. 3 E). Salivary glands could not be found. Oesophagus originating from posterior pharynx, above ascus; here surrounded by nerve ring. Oesophageal epithelium composed of cylindrical ciliated cells, interspersed with few glandular cells, staining violet. Stomach lined by columnar, ciliated epithelium and hardly distinguishable from oesophagus. Digestive gland ramifying and running into cerata (Figs. 2C, D), as well as into rhinophores, forming one central tubular rugose structure; no ramifications in foot observed. Epithelium of digestive gland filled with globular chloroplasts (Fig. 2C). Intestine inconspicuous; anus lying dorsal in front of pericardium.

Genital system. A schematic outline of the distal genital system is given in Figure 4. Gonad partly lying lateral and dorsal, occupying most of body cavity in posterior third of animal. Gonad follicles with oogonia lying in periphery and spermatogonia in median part (Fig. 2F). Ampulla large and coiled, with flat epithelium; lumen filled with sperm exhibiting elongate heads (Fig. 2B). Bursa copulatrix extremely large, occupying most of space in head area to right of pharynx (Fig. 2A); bursa entering proximal oviduct directly before beginning of nidamental glands. Epithelium composed of large cuboidal ciliated and partly apocrine secreting cells. Lumen filled with disintegrating sperm. A saclike elongate receptaculum seminis filled with parallel lying sperm (Fig. 2D) present. Direct opening to outside not present, but connection to internal parts of genital system could not be observed. Epithelium of receptaculum consisting of flat to cuboidal cells. Nidamental glands comprising of three distinct areas: albumen, capsule and mucus gland. Albumen gland forming small tubes and running laterally into posterior part of body (Fig. 2F), but never into the cerata. Cells cuboidal, apical part filled with violet staining granules. Capsule gland tubular and mainly situated in central part of body. Capsule gland cells filled with smaller vacuoles staining bright red (Fig. 2B). Cells of medium size, i.e., smaller than mucus gland cells and larger than albumen gland cells. Mucus gland forming a thick tubular structure situated latero-dorsal and posterior of pharynx. Cells very large with red stained contents. Opening of nidamental glands in ciliated vestibule without any glandular structures.

Vas deferens with a separate prostate gland composed of two large limbs, each with a central duct surrounded by glandular cells, forming a subepithelial layer. Cell contents staining light bluish. Epithelium of distal muscular part of vas deferens (before entering penis) composed of ciliated cells (Fig. 2E). Vas deferens inside penis lined by epithelium with long cilia. Muscular penis lying within sheath; distal part of penis with one hollow stylet, which is lightly curved (Fig. 2E).

Excretory system. Kidney lying dorsal; forming two branches on lateral sides of pericardium which unite behind heart region into a dorsal sac-like structure (Fig. 2A). Left branch reaching further anteriorly than the right one. Epithelium slightly folded, with larger cells containing several non-staining vacuoles. Nephroproct close to anus.

Nervous system and sensory organs. Nervous system located at transition of pharynx into oesophagus, forming a ganglionic ring with cerebral and pleural ganglia lying closely annexed and only partly fused. Pedal ganglia completely separated from cerebropleural complex. Additional two to three small ganglia present below the oesophagus. Eyes with pigment cup and globular, homogeneously light blue-stained lense; orientation to laterodorsal side (Fig. 2A). Statocyst large, with one otolith.

Epithelia and glandular structures. Epidermis of cerata composed mainly of flat to cuboidal and ciliated cells. Large subepithelial glandular cells present, staining homogenously violet (Fig. 2C). Epidermis of body composed of flat cells, without any subepithelial glandular structures (Fig. 2A, B). A special glandular structure present mainly in posterior part of body; starting between albumen gland and epidermis in anterior half, then present on both lateral sides until posterior part of body. Glandular cells characterized by large nucleus and large non staining vacuoles (Fig. 2F arrows). Cells not connected by any ducts and without openings to outside.

Molecular investigations (Figs. 5–6). Partial CO1 sequences of four specimens were investigated. Three of them were collected from the same small algal clutch and at the same day (see Tab. 2). A fourth one was collected two years earlier at the same locality. The four sequences form two pairs, with a high sequence similarity within the pair (1.31 to 3.56%) and a high sequence divergence between the two pairs (17.07 to 17.64%) (Tab. 3). This is also visualized in the NeighborNet analysis, where the two pairs are separated by similar distances as to other members of the Limapontiidae (Fig. 5).

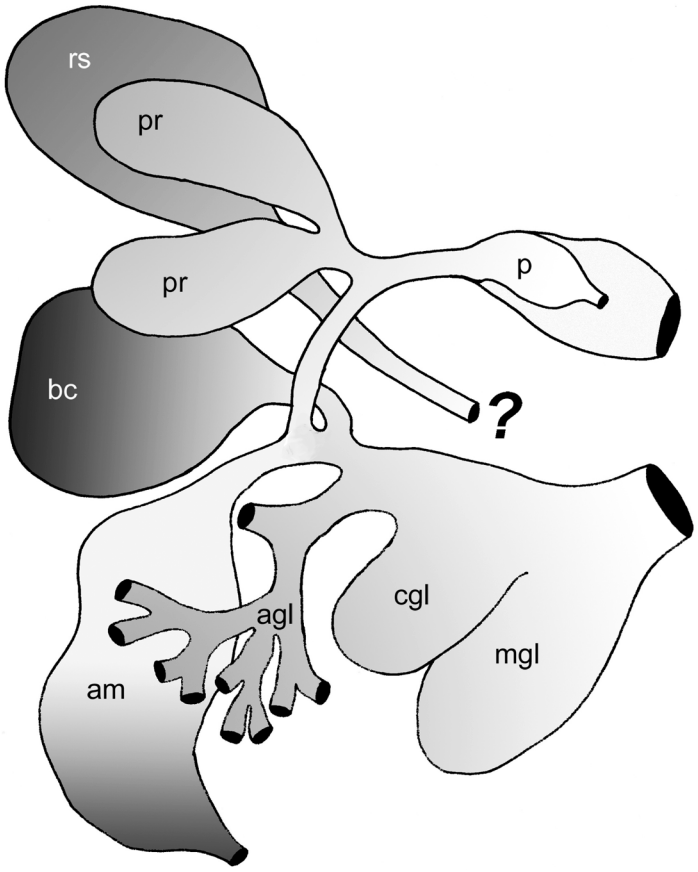

FIGURE 4. *Ercolania annelyleorum* sp. nov.: schematic outline of genital system, reconstructed from three different specimens. Agl albumen gland, am ampulla, bc bursa copulatrix, cgl capsule gland, mgl mucus gland, p penis, pr prostate gland, rs receptaculum seminis.

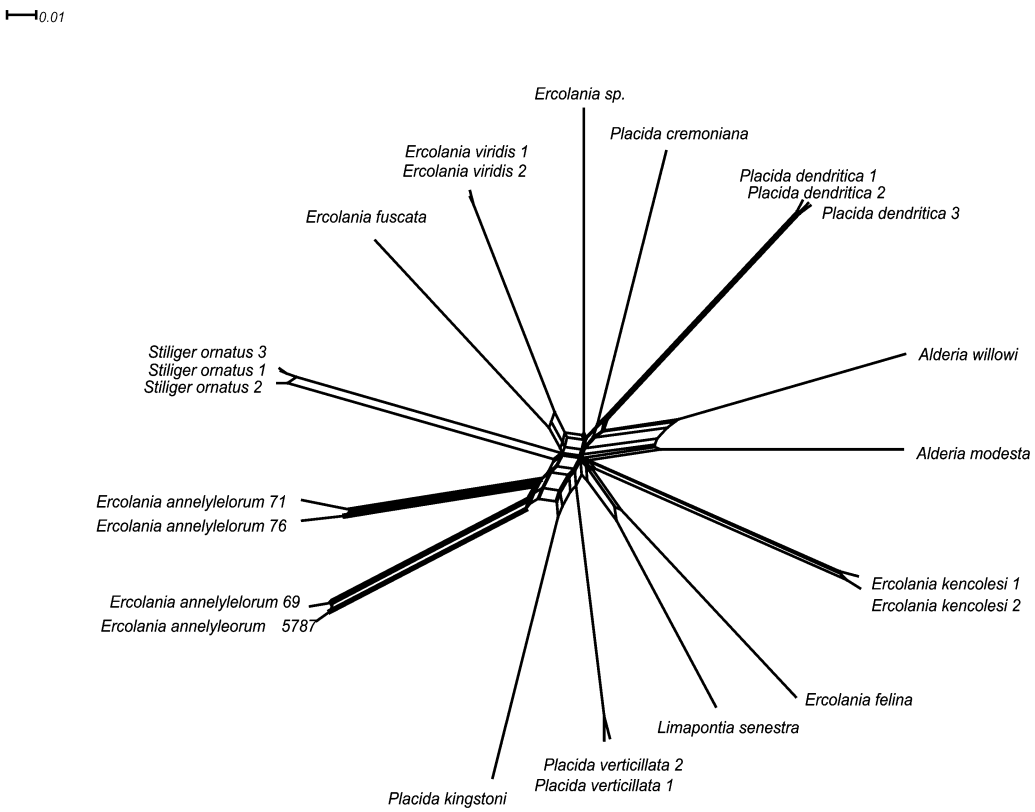

FIGURE 5. NeighborNet analysis of Limapontiidae based on mitochondrial CO1 sequences only without application of evolutionary models, performed with SplitsTree. Note the two distinct branches of *Ercolania annelyleorum* **sp. nov.**

The number of available sequences of other *Ercolania* species is still low, hence only five additional species out of the about 20 described ones (see Jensen 2007) could be included. Table 3 shows divergence of partial CO1 gene sequences of further three included *Ercolania* species, as well as limapontiid species against *Ercolania annelyleorum* **sp. nov.** The smallest sequence divergence of *E. annelyleorum* **sp. nov.** (69 and 5787) to any other limapontiid sequences included (uncorrected p-distances) is against the sequence of *Ercolania fuscata* (17.64 to 17.82%), and to *Placida verticillata* (71 and 76; 15.95%; see Table 3). Intraspecific variability of *E. viridis* (two sequences: 0.79%) and *E. kencolesi* (two specimens: 1.27%) is considerably lower and similar to the divergence observed within the two groups of *E. annelyleorum* **sp. nov.**. Intraspecific variability for three *Stiliger ornatus* sequences lies from 0.56% to 1.31 and 1.50%.

TABLE 3. Divergence of CO1 sequences between individuals of *Ercolania annelyleorum* **sp. nov.** (*Ea*) and related species (only species presenting lowest and highest values are listed). *Ercolania fuscata* showed lowest values of all *Ercolania* species and *Placida verticillata* lowest values in total against *E. annelyleorum* **sp. nov.**. *Placida dendritica* showed highest values against *E. annelyleorum* **sp. nov.**.

	Ea 71	*Ea* 69	*Ercolania fuscata*	*Placida verticillata*	*P. dentritica* 2
Ea 71	0.00	17.63	18.76	15.95	21.95
Ea 76	3.56	17.07	18.57	15.95	22.51
Ea 5787	17.64	1.31	17.82	19.32	23.45
Ea 69	17.63	0.00	17.64	19.70	23.26
Ercolania felina	21.39	22.51	19.70	20.45	21.95
Ercolania viridis	17.82	18.95	15.76	16.14	19.51

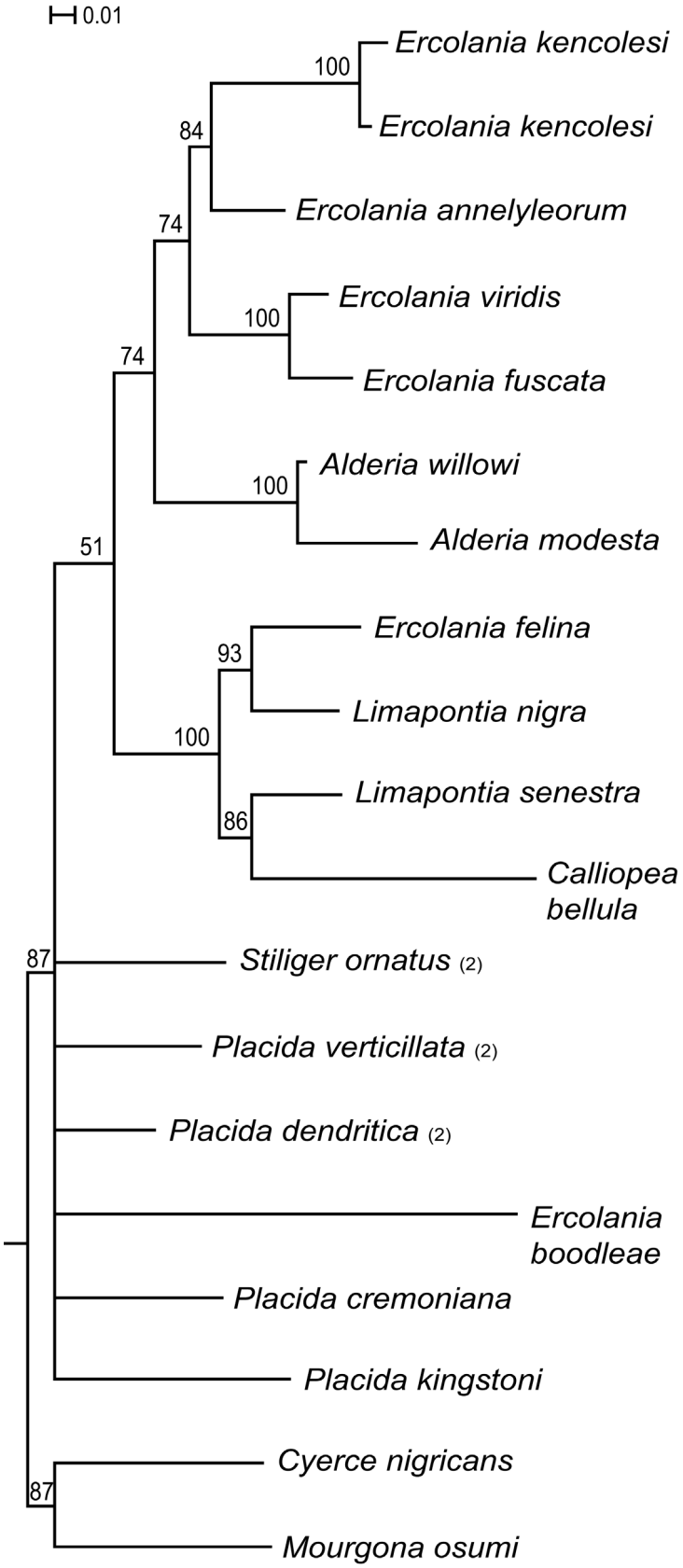

FIGURE 6. Phylogeny of the Limapontiidae. ML analysis was performed on concatenated partial gene sequences of the nuclear 28S rDNA, the mitochondrial 16S rDNA, and the mitochondrial CO1 (first and second position only) loci. Support values are given. Numbers behind names indicate number of individuals included.

Figure 6 shows the relationship of the new *Ercolania* species (only one specimen is included) within Limapontiidae. The genus *Ercolania* is not monophyletic, since *E. boodleae* and *E. felina* do not group with the other four *Ercolania* species. Sister taxon of *E. annelyleorum* **sp. nov.** is the endophytophageous species *E. kencolesi*. The specimen named *Ercolania* sp. in the analysis by Händeler *et al.* (2009) represents a further individual of the species *E. kencolesi* (here designated as *E. kencolesi* 2, since the overlapping parts of 28S rDNA are identical, sequence divergence between the 16S rDNA is lower than 0.5% (two of 413 positions) and overlapping parts of CO1 differ under 1.4% (three nucleotides in 1st position and six in 3rd position of 657) (see also Fig. 5).

Notes on biology and photosynthetic activity. *Ercolania annelyleorum* **sp. nov.** was observed crawling and feeding on the algal species *Boodlea sp.* (Chlorophyta, Cladophorales), which forms cushion-like patches. Branches of this alga are tough and the patches are rather stiff, forming compact shelters for the slugs (Figs. 1D, E). Juvenile animals appeared in a big number (up to 33 individuals per cluster) in the algal patches, suggesting that larvae stay close to *Boodlea* sp., where they hatch from egg clutches inside the algal patches. Feeding and crawling on this alga has been observed. It was not possible to rear the larvae.

PAM measurements were taken of ten specimens. Data are shown in Table 4. Mean values of maximum quantum yield measurements for single specimens do not exceed 0.14, the average of all specimens does not exceed 0.10. Standard deviation of mean yield values of specimens is low. These low maximum quantum yield values result from background noise and are no indication of photosynthetic activity. Up to three days starved and pale animals of *Ercolania annelyleorum* **sp. nov.** were allowed to feed again on *Boodlea* sp. for four days. Their green coloration returned, but values of their photosynthetic activity never exceeded 0.2, indicating direct break down and digestion of chloroplasts.

TABLE 4. PAM measurements (PSII maximum quantum yield, $\Phi_{IIe\text{-}max}$) of ten specimens of *Ercolania annelyleorum* **sp. nov.**. For specimens no. 7 and 8 a joined mean value is given. Mean value and standard deviation are given for days of starvation.

Day of starvation	1	2	3	4	5	6	7 + 8	9	10	$\Phi_{IIe\text{-}max}$ Mean value	Standard deviation
0	0.13	0.00	0.05	0.03	0.06	0.00	0.14	-	-	0.06	0.06
1	0.12		0.14					0.03	0.09	0.10	0.05
2	0.10		0.00					0.08		0.06	0.05

Discussion. Taxonomy of *Ercolania annelyleorum* sp. nov.. Systematics of Limapontiidae has been under debate for long time (see Jensen 1993). According to the phylogenetic analyses of Sacoglossa (Händeler *et al.* 2009), the validity of different genera of the Limapontiidae (*Limapontia, Alderia, Placida* and *Ercolania*) seem to be solved, but validity of the limapontiid genus *Stiliger* still needs to be clarified. According to the present phylogenetic analysis of the Limapontiidae including more sequences from GenBank, validity of the genus *Ercolania* may also have to be reconsidered. Although distinct characters for the genus *Ercolania* were mentioned by Baba and Hamatani (1970), Marcus d. B.-R. (1982) rejected the fact of genus-specific characters for *Stiliger* or *Ercolania* and pointed out the high degree of variation in other genera. Until these taxonomic problems are solved, we follow Schmekel and Portmann (1982), Jensen (1985, 1993) and Grzymbowski *et al.* (2007) and assign the new species to the genus *Ercolania* due to the presence of digitiform rhinophores, the presence of smooth sabot shaped teeth, the presence of a curved penial stylet and the absence of the albumen gland in the cerata. The very similar *Placida* differs from *Stiliger* by the auriculate rhinophores, which are rolled or folded in *Stiliger* (Jensen 1993). *Placida* has an oesophageal crop, which could not be found in the specimens investigated here, and *Stiliger* lacks a penial stylet (Jensen 1993). Future studies additionally based on molecular markers will reveal validity of these three different genera.

About 20 valid species of *Ercolania* are recognized. Few of them have been described from the Mediterranean Sea and the Atlantic (Jensen 2007). Additional to the locality, these species (*E. fuscata* (Gould, 1870), *E. lozanoi* Ortea, 1981 and *E. nigra* (Lemche, 1935) are distinguished by their different coloration. One species, *Ercolania coerulea* Trinchese, 1892, has been recorded from the Atlantic as well as the Pacific

Ocean. Due to the inflated numerous cerata and the darker coloration, partly due to the much higher branching of the green digestive gland, that species differs considerably from the new species described here. Additionally, Sanders-Esser (1984) described the receptaculum seminis with a distinct opening to the outside, which is not present in our new species. Several species described from the Pacific Ocean (see Jensen 2007), like *E. erbsa* (Marcus & Marcus, 1970), *E. felina* (Hutton, 1882), *E. gopalai* (Rao, 1937) and *E. margaritae* Burn, 1974, are distinguished by their differing coloration. *Ercolania irregularis* (Eliot, 1904) is described by the author with posterior cerata which are twice as long as the front ones.

In the following, those species are discussed, which show a similar coloration to our newly described species. *Ercolania boodleae* (Baba, 1938) is recorded to feed on the tubular alga *Boodlea,* similar to the new species described here. *Ercolania boodleae* is darkly green colored, nearly blackish, with distinct white stripes along the head. The eyes are situated within these stripes (Baba & Hamatani 1970). Due to this coloration, it cannot be confounded with *E. annelyleorum* **sp. nov.**. *Ercolania emarginata* Jensen, 1985 is very similar in coloration to *E. boodleae* and can also not be confounded with the new *Ercolania* species. Besides, the radula teeth of *E. emarginata* show a distinct prominence and there is only one prostate lobe (Jensen 1985). A very similar color and body shape to the new described sea slug can be observed in *E. subviridis* (Baba, 1959). The body of this species is translucent yellowish with fine green branches of digestive gland shining through the body wall. Within the cerata, several green branches of the digestive gland are visible as longitudinal lines, giving each ceras a characteristic green-lined appearance. Baba (1959) described a red marking at the anterior end of pericardial prominence which is also found on the new species. However, specimens of our species always exhibit an additional red patch in the posterior part of the body. Furthermore, only one central channel of digestive gland in each ceras with short pustule-like branches is present, and never several longitudinal stripes. The new species shows two distinct green branches on both sides of the head, which are missing in *E. subviridis*. *Ercolania viridis* (Costa, 1866) usually shows much more inflated cerata and the digestive gland branches up to tertiary degree within the cerata, but does not reach into the rhinophores (unpublished results based on histological investigations of a Mediterranean specimen). *Ercolania endophytophaga* and *E. kencolesi* are also differentiated by their inflated cerata, the former also show peculiar teeth with knoblike apices (Jensen 1999). Both can be separated also from the new species by their life style: they penetrate into algae and spend at least some time of their life cycle inside the algal tube. *Ercolania translucens* Jensen, 1993 has inflated cerata, a distinct pericardial prominence and the digestive gland does not reach into the rhinophores (Jensen 1993). Although Jensen (1993) mentioned a reddish sphere in the neck region, this species lacks the second patch, but exhibits reddish-brown pigment stripes along the sides of the body and is therefore quite distinct from *E. annelyleorum* **sp. nov.** *Ercolania nigrovittata* (Rao & Rao, 1963) is also described with only one pale-pinkish patch and not a second one in the rear. In contrast to our species, the digestive gland reaches far into the rhinophores, starting from a distinct patch over the head. The overall color is described as “faintly speckled grey over a faint orange background" (Rao & Rao 1963: 233). *Ercolania varians* (Eliot, 1904) from Zanzibar was described as “brilliant green”, although the color may vary. This coloration is due to “numerous lines of deeper color”, which can be interpreted as many digestive gland branches reaching throughout most parts of the body (“except at the sides of the body and in the centre of the back”) (Eliot 1904: 290). Although he also mentioned crimson patches in some of his animals, it is not clear, whether there is only one patch or more on the dorsal body. Because of its general green color and a more elongate tooth, it is not considered to be the same species as the one described here. *Ercolania gopalai* (Rao, 1937) can be excluded due to the lack of a bursa copulatrix in the genital system and the presence of a ventral oesophageal pouch (Jensen 1985).

On the sea slug forum of Bill Rudman (www.seaslugforum.net), pictures are available of undescribed *Ercolania* species which show a similar color pattern to our specimens: Trowbridge (2005) depicted one specimen and several further ones are described in their appearance. According to the descriptions, the numbers of the red patches vary from zero to two, a variety that was not observed in our specimens. The digestive gland ducts in the cerata seem to branch more often than in our specimens and they are more inflated. The radula is very similar. Although these specimens are recorded from Okinawa, Japan, it can not be excluded that this is the species we describe here.

Tani (2006 a, b) followed with pictures of a similar specimen on the sea slug forum. The specimen was recorded from Saipan, Northern Mariana Islands on *Boodlea coacta*. The animal shows a distinct red patch in the median dorsal part of the body (Tani 2006 b). Fujie (2007) depicted further specimens from the same locality and he also mentioned only one brown mark. Therefore an assignment of their specimens to our new species is difficult, although the overall appearance is certainly similar to our specimens. The same applies to a specimen depicted in Gosliner *et al.* (2008) under the designation *Stiliger* sp. 6, which might represent a member of the new species, but coloration has to be verified first.

Only few species are investigated by histological means. It is of interest that the new species described here and *E. kencolesi* exhibit a special gland (Fig. 2F, this study; Fig. 2D in Grzymbowski *et al.* 2007) with undetermined function. The lack of any ducts leading to the outside indicates an endocrine function. Thorough investigation of *Alderia modesta* (Lovén, 1844) and *A. willowi* Krug, Ellingson, Burton, and Valdés, 2007 (Krug &Wägele, unpublished results) did not reveal similar glandular structures. Further histological studies on other *Ercolania* species, and also related genera, will show, whether this gland is genus-specific or only present in few species.

Molecular characters. *CO1* is one of the major barcoding genes to identify or characterize metazoan species (see http://www.barcodeoflife.org) and has been successfully applied for detection of cryptic speciation (e.g. Burns *et al.* 2008; Hajibabaei *et al.* 2006; Vaglia *et al.* 2008). Krug *et al.* (2007) recently discovered cryptic speciation in sacoglossan members of the genus *Alderia* by analysing CO1 data. The new species *A. willowi* is distinguishable from the other species involved (*A. modesta*) in ontogeny, morphology and geography (see Krug *et al.* 2007). The detection of two distinct clades in *E. annelyleorum* **sp. nov.** with a similar sequence divergence as is observed between limapontiid species (see Tab. 3, Fig. 5) in general allows several interpretations: 1. There are pseudogenes present in the species. This would result in different CO1 sequences in the same individual. Usually pseudogenes evolve quicker and since they are not translated, there should be mutations not only in the silent third position, but also in the first and second positions. The translation into aminoacid sequences showed only completely conserved aminoacids, mutations occurred exclusively as silent mutations. 2. There is a cryptic speciation ongoing in this *Ercolania* species, which shows no differentiation on morphology level yet. The two genetic distinct populations would then co-exist sympatrically in one and the same algal clutch. 3. There are two different mitochondrial lineages coexisting in the species, or even within one and the same individual. We do not know anything on population structures of this new species. Incomplete lineage sorting, as was recently shown for a butterfly family Lycaenidae (Wiemers & Fiedler 2007), and occasional introgressive hybridisation as a still ongoing genetic exchange, e.g., described in fish populations in ancient lakes (see e.g., Herder *et al.* 2006), are factors that certainly needs further investigation.

In the phylogenetic reconstruction based on a concatenated alignment of all available Limapontiidae sequences, *Ercolania annelyleorum* has a closer relationship to a species which penetrates into algal cells (*E. kencolesi*), but the position is uncertain as nodal support is not high (bootstrap value: 84) and many *Ercolania* species were not included. The genus *Ercolania* is polyphyletic. We do not consider the phylogeny of the Limapontiidae resolved, nevertheless, the molecular analyses revealed the distinctiveness of this new species and separation from all other included species.

Photosynthetic activity. Photosynthetic activity of *Ercolania annelyleorum* **sp. nov.** was analysed by PAM measurements under starving conditions. Maximum quantum yield values ($\Phi_{IIe\text{-}max}$) of the alga *Boodlea* sp. range between 0.6 and 0.7 indicating a "healthy" photosynthetic activity. The maximum quantum yield values ($\Phi_{IIe\text{-}max}$) of *E. annelyleorum* **sp. nov.** are very low from the very beginning of the experiments. Feeding experiments with *Boodlea* sp. and subsequent starving experiments clearly show that digestion of chloroplasts occurs within few hours. This is also recognizable by the loss of the green color within the first days of starvation. Similar to the results on *E. kencolesi* (see Grzymbowski *et al.* 2007), it can be concluded that chloroplasts are not retained in the new species described here. Clark *et al.* (1990) reported that freshly collected animals of *E. coerulea* retain plastids in digestive gland diverticula for at least two hours of starvation, but no photosynthate was detectable by isotope tracer techniques in that other species, supporting the hypothesis of direct digestion.

Elysia Risso, 1818

Type species: *Notarchus timidus* Risso, 1818, by monotypy.

Comments and diagnosis. In spite of several older diagnoses of the genus, Jensen (1996) could not identify any apomorphic characters which are shared by all species referred to the genus *Elysia*, and this is still valid. Nevertheless, recent phylogenetic analyses based on several molecular markers (Händeler *et al*. 2009) indicate the monophyly of the genus *Elysia*. We refer here to one of the most recent descriptions (Jensen 1993) indicating the most prominent character for *Elysia*: pharynx without pharyngeal pouches (although this is shared by many sacoglossan genera, except within the Plakobranchoidea). Furthermore *Elysia* species are described with long blade-shaped radular teeth with a median denticulate cutting edge (Bouchet 1984; Thompson & Jaklin, 1988; Gosliner 1995) and broad parapodia (Thompson 1973).

Elysia asbecki sp. nov.

Type material. All specimens were collected in the intertidal reef flat of South Island, Lizard Island, North Queensland, Australia. Animals have been discovered in trays after collecting algae and coral rubble from these reef flats. Type material is deposited at the Australian Museum Sydney. Holotype (AM C.464069): 11th July 2006 (length of preserved specimen 3 mm); one paratype partly dissected (AM C.464070, including SEM preparation of radula): 11th July 2006 (length of preserved specimen 2 mm). For further material see Table 5.

TABLE 5. Synopsis of investigated specimens and collecting data of *Elysia asbecki* **sp. nov.** FSW: preservation in formaldehyde/seawater; PAM indicates measurements of photosynthetic activity, LM light microscopy, SEM scanning electron microscopy. ZMS Zoologische Staatssammlung Munich.

Date of collection	Date and kind of preservation	Type of investigation
11.07.2006	12.07.06 EtOH	Holotype AM C.464069
11.07.2006	12.07.06 EtOH	Paratype AM C.464070 Gene analysis: CO1 Radula preparation (LM, SEM)
11.07.2006	12.07.06 EtOH	Gene analyses (partial 28S, 16S and CO1 gene sequences) (publ. in Händeler *et al.* 2009 as *Elysia* spec. 1)
11.07.2006	12.07.06 FSW	Histology (ZSM 20100676)
11.07.2006	Died after PAM measurement	PAM (9 days; see Fig. 12)
13.09.2004	13.09.2004 FSW	animal lost during radula preparation

Distribution. Up to now this species has been recorded from South Island, Lizard Island on top of the reef flat in the intertidal zone. A few specimens of this species have also been found by one of the authors (IB) in the Samoan Islands in 2005 (unpublished data). Gosliner *et al.* (2008) spotted this species in different places in the Indopacific (Papua New Guinea, Indonesia, Philippines, Japan, Guam and the Hawai'ian Islands) and figured it under the designation *Elysia* sp. 16.

Etymology. This species is dedicated to Dr. Frank Asbeck (SolarWorld AG, Bonn) for his continuous sponsorship to the Zoologisches Forschungsmuseum Alexander Koenig, Bonn, Germany.

Description. *External morphology and color of living specimens* (Fig.7). Size up to 8 mm. Body elongate when crawling (Fig. 7B) or more compact when resting (Figs. 7 A, C, D). Prominent parapodia not fused anteriorly; usually covered with tiny tubercles, which change in height within the same animal (compare Figs. 7B, C, D). Margin of parapodium forming rather irregular lobes. Rhinophores rolled and rather short. No

distinct propodial tentacles at anterior foot present. Pericardial prominence oval with narrow posterior elongation (Fig. 7A). Dorsal vessels indistinct.

Background color of animals appearing whitish, due to accumulation of white dots; large amounts of yellow to orange spots scattered over body, sometimes densely arranged in stripe-like patterns (Fig. 7D). Tiny black spots evenly distributed on outer side of parapodia. Inner surface of parapodia and foot-sole translucent, with less numerous white spots, no black spots and with green digestive gland shining through. Head with similar coloration as body, in the neck region with a lighter spot. Eyes clearly visible behind rhinophores. Rhinophores translucent whitish with darker ring, followed by a yellow ring towards the tip. Two pink elongate patches on both sides of tail (Fig. 7C), as well as at frontal base of parapodia at the conjunction with head (Figs. 7B, C). Similar patches present at margin of parapodia, mainly in median part.

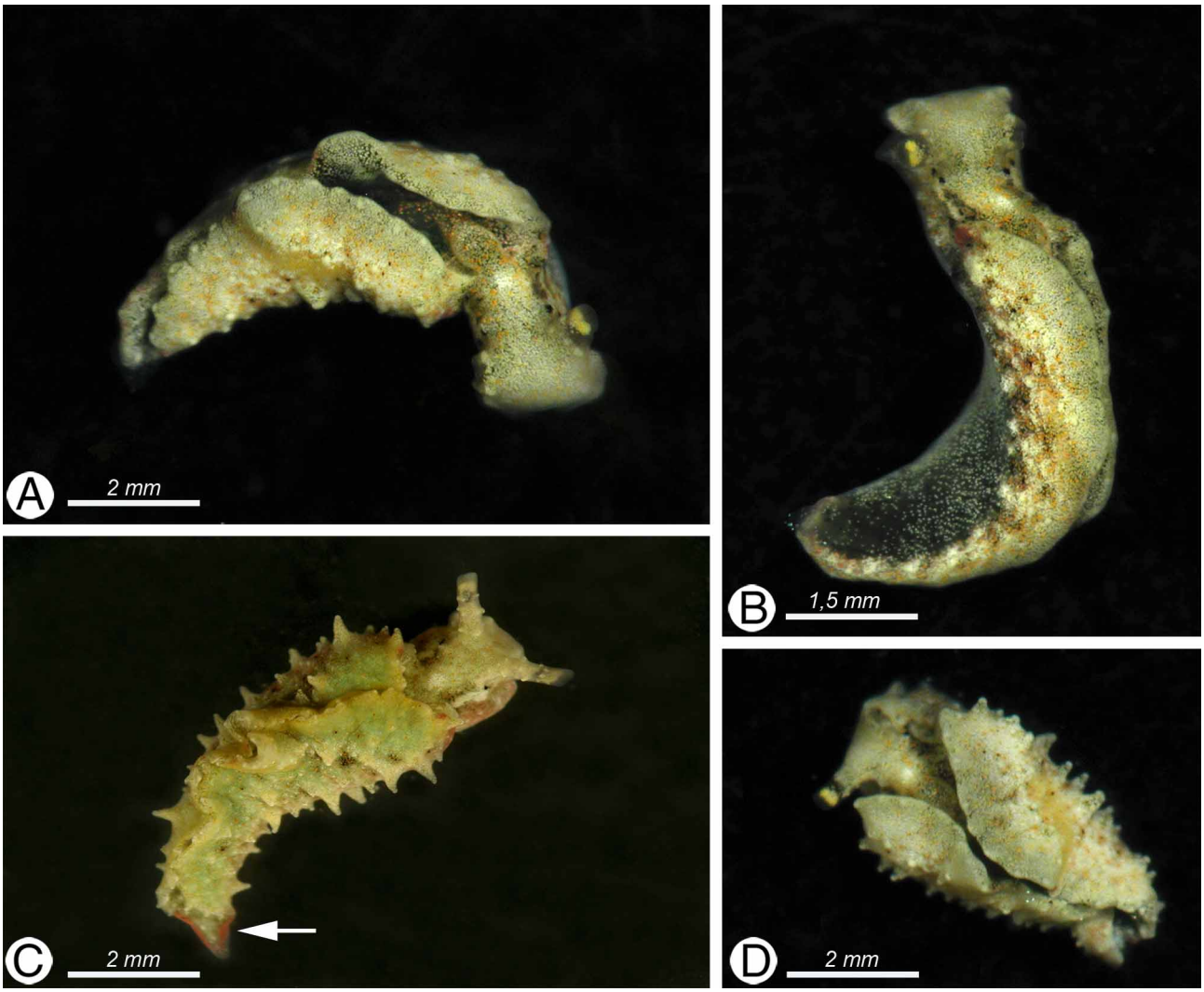

FIGURE 7. *Elysia asbecki* **sp. nov.**: living animals from Lizard Island; (A) Animal collected in 2006, resting with parapodia slightly opened. Note the pericardial hump. The right rhinophore is damaged. (B) Same animal with foot region exposed. Note the lack of white and orange pigment on foot. (C) Animal collected in 2002 and only documented by digital camera. Note the pronounced tubercles and the distinct red patches along the tail (arrow). (D) Same animal as in A and B after a few hours, with more pronounced tubercles, but still less than the one shown in (C).

Description of anatomy and histology of preserved specimen (Figs. 8–10). *Digestive tract*. Oral tube in the beginning without any subepithelial glands, but with ciliated cells. More posteriorly, oral tube surrounded by subepithelial glands with acid mucopolysaccharides (oral glandular layer) (Fig. 8A). Pharynx muscular, with a dorsal muscular pump. Radula with five teeth in descending limb and nine in ascending limb (Figs. 9A, F). Four smaller teeth and two pre-radula teeth in ascus present (Fig. 9F). Teeth with a denticulate cutting edge (Figs. 9B, D, E). Salivary glands lobate, located postero-ventrally to pharynx, as well as lateral and ventral to

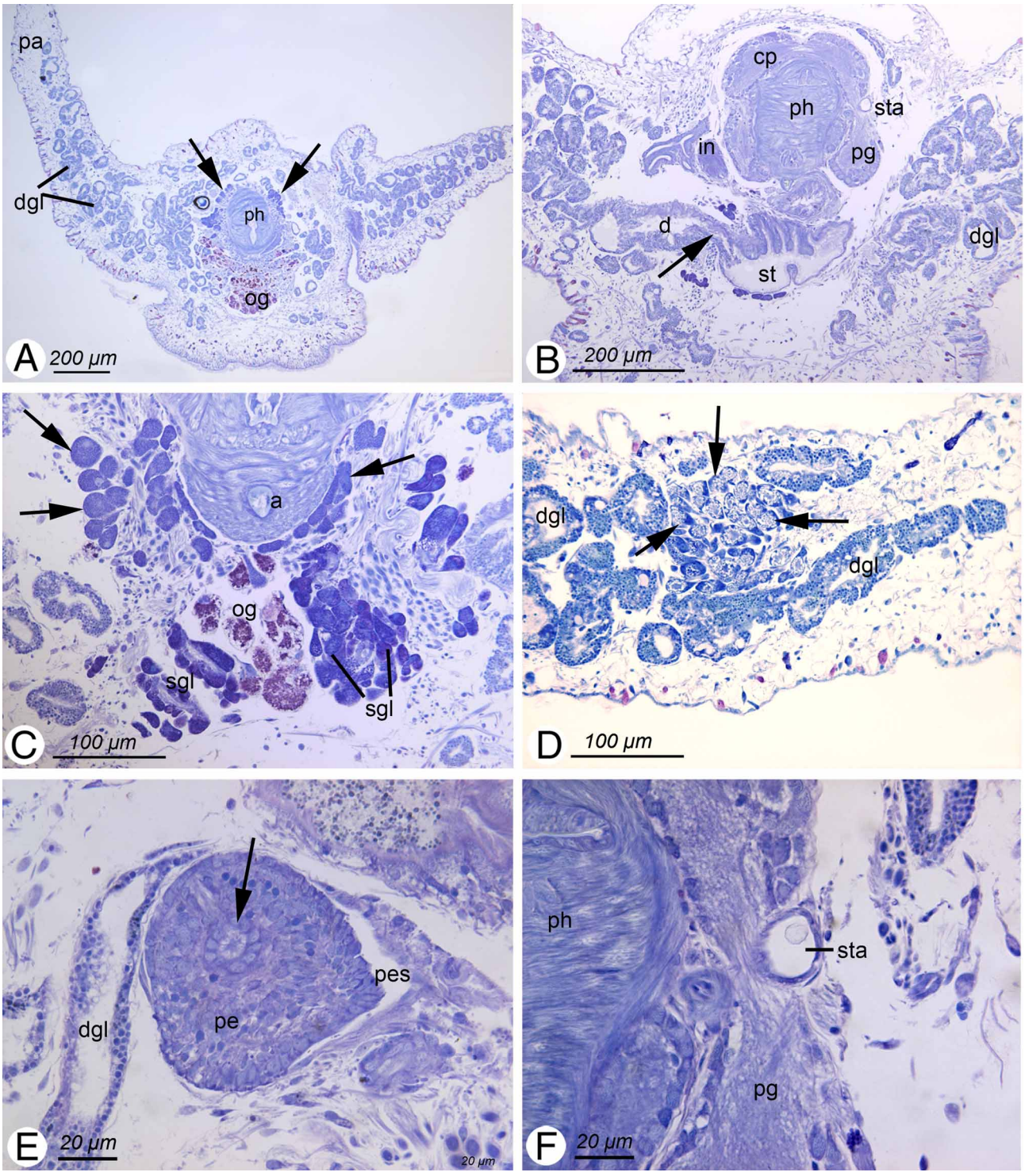

FIGURE 8. *Elysia asbecki* **sp. nov.**: histology; (A) Cross section near head area. Many branches of digestive gland reach into the lateral parapodia. Note the special glands (arrows) close to dorsal pharynx, which are not connected to salivary glands or any other part of the digestive system. (B) Cross section behind head. Nerve ring surrounds posterior part of pharynx. Posterior part of oesophagus surrounded by thick layer of muscles. Note entrance of stomach into digestive gland (arrow). (C) Cross section near head, somewhat posterior than (A). Salivary glands situated ventrally of pharynx. Note the special glands (arrows) now close to lateral parts of pharynx, which are not connected to salivary glands or any other part of the digestive system. (D) Cross section of parapodium exhibiting special glandular structures (arrows). Note the branches of digestive gland with an epithelium filled with chloroplasts (blue dots). (E) Penial sheath with muscular penis. Note the vas deferens (arrow) without any cuticular structures. (F) Detail of statocyst with one otolith. Abbreviations: a ascus, cpg cerebropleural ganglion, d duct into digestive gland, dgl digestive gland, in intestine, og oral gland, pe penis, pes penial sheath, pg pedal ganglion, sgl salivary glands, st stomach, sta statocyst.

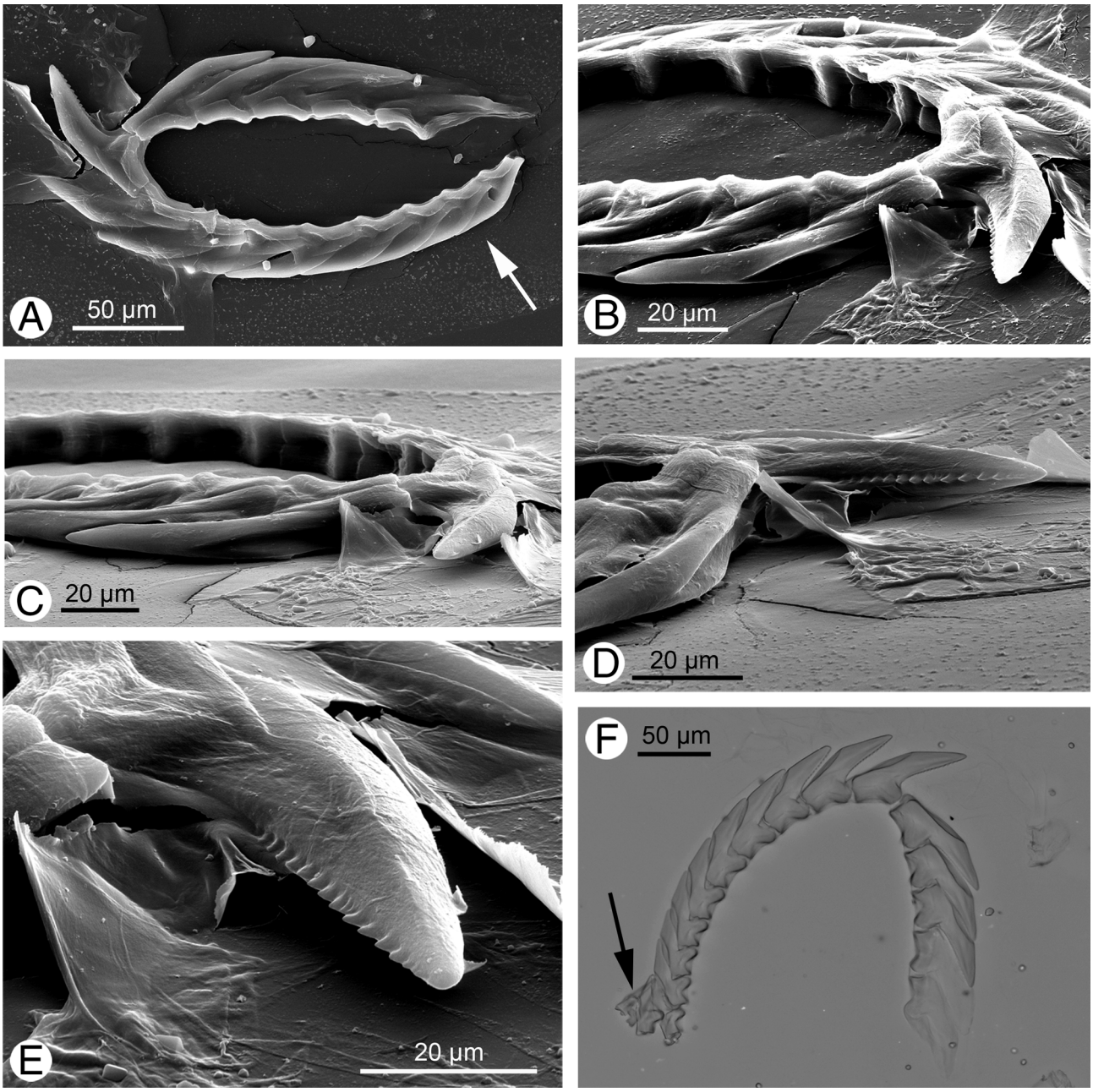

FIGURE 9. *Elysia asbecki* **sp. nov.**: scanning electron microscopy and differential interference contrast microscopy of radula: (A) complete radula with leading tooth to the right. Ascus (lower limb, white arrow) lost during preparation. (B) – (D) different views of leading tooth; note the denticles. (E) Radula of same specimen. Note the small teeth and preradula teeth (black arrow) in ascus.

oesophagus and stomach (Fig. 8C, Fig. 10). Gland composed of large secreting cells with granules staining bluish to dark violet and surrounding a tiny duct. Duct running anterior along pharynx, but entrance into pharynx not verified. Oesophagus starting from posterior part of pharynx and entering stomach a short distance behind on dorsal side. Epithelium of oesophagus highly folded and heavily ciliated (Fig. 8B). Few violet stained glandular cells (acid mucopolysaccharides) interspersed. Posterior part of oesophagus surrounded by thick muscle layer, but not differentiated into distinct bulb.

Stomach large, lined by ciliated epithelium. Transition into right and left digestive gland facing each other on lateral sides of stomach (Fig. 8B, Fig. 10). Digestive gland ramifying heavily, with branches reaching into lateral parapodia as well as into foot area (Fig. 8A). One tiny branch reaching half way into the rhinophores. Cells of these branches filled with chloroplasts. Intestine originating dorsally from stomach and opening to

outside behind right rhinophore, between lateral foot side and parapodium. No typhlosole present in proximal part of intestine. Epithelium folded, with ciliated cells, but without any glandular cells. In some areas, chloroplasts present in lumen of intestine.

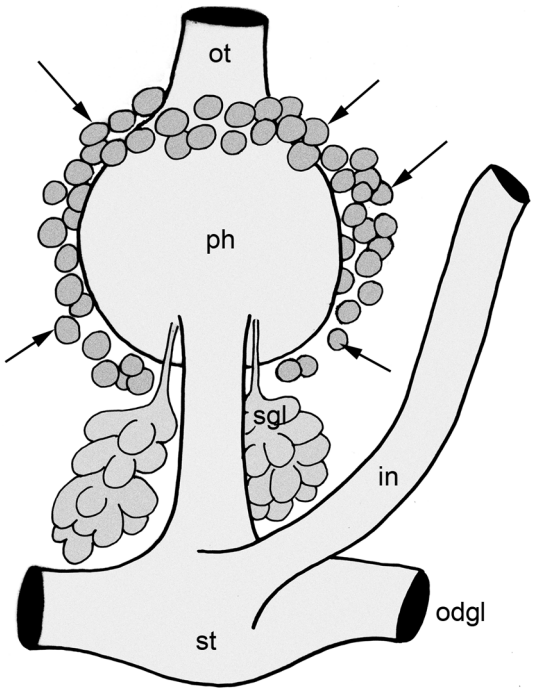

FIGURE 10. *Elysia asbecki* **sp. nov.**: anatomy: Schematic drawing of digestive system and position of special glands (arrows). Abbreviations: in intestine, odgl opening into digestive gland, ot oral tube, ph pharynx, sgl salivary glands, st stomach.

Genital system. The histologically investigated specimen was juvenile. Few gonad follicles present in parapodia, located dorsally in outstretched parapodia. Only spermatogonia in early stages recognizable. Female part of system not developed yet. A small penis present without cuticular structures (Fig. 8E); epithelium of vas deferens inside penis formed by cuboidal cells with light bluish contents indicating secretory function.

Excretory and circulatory systems. Pericardial region in anterior third of body forming distinct hump lying above pharynx. Ventricle inside pericardium muscular. Kidney forming sac-like structure, starting within this hump and reaching into posterior part of body.

Sensory organs. Eye with homogeneously stained globular lens (Fig. 8A); pigment cup of eye orientated to dorsolateral side. Statocysts large, lying between cerebral and pedal ganglion, containing one large otolith (Fig. 8F).

Epithelia and glandular structures. Epidermis composed of flat cells (Fig. 8B); few subepidermal glandular cells present only above pericardium and excretory system (dorsal hump) (Fig. 8A). Foot characterized by a loose layer of subepidermal mucus glands similar to those of dorsal area and some regions of parapodia. In few areas of parapodia, agglomerations of specialized cells visible. Form and shape of these cells differ, in some with star-like contents, in others with a vacuole that is filled with particulate-stained contents (Fig. 8D).

A special glandular layer present, starting at transition of oral tube into pharynx on dorsal and lateral sides (Fig. 8A arrows, Fig. 10). Further to the posterior, these glands are located only at lateral side, ending on ventral side near transition from pharynx into oesophagus (Fig. 8C, Fig. 10). This layer consisting of large drop-like glandular cells characterized by bluish granules; no connections to oral tube, pharynx or other parts of digestive system visible.

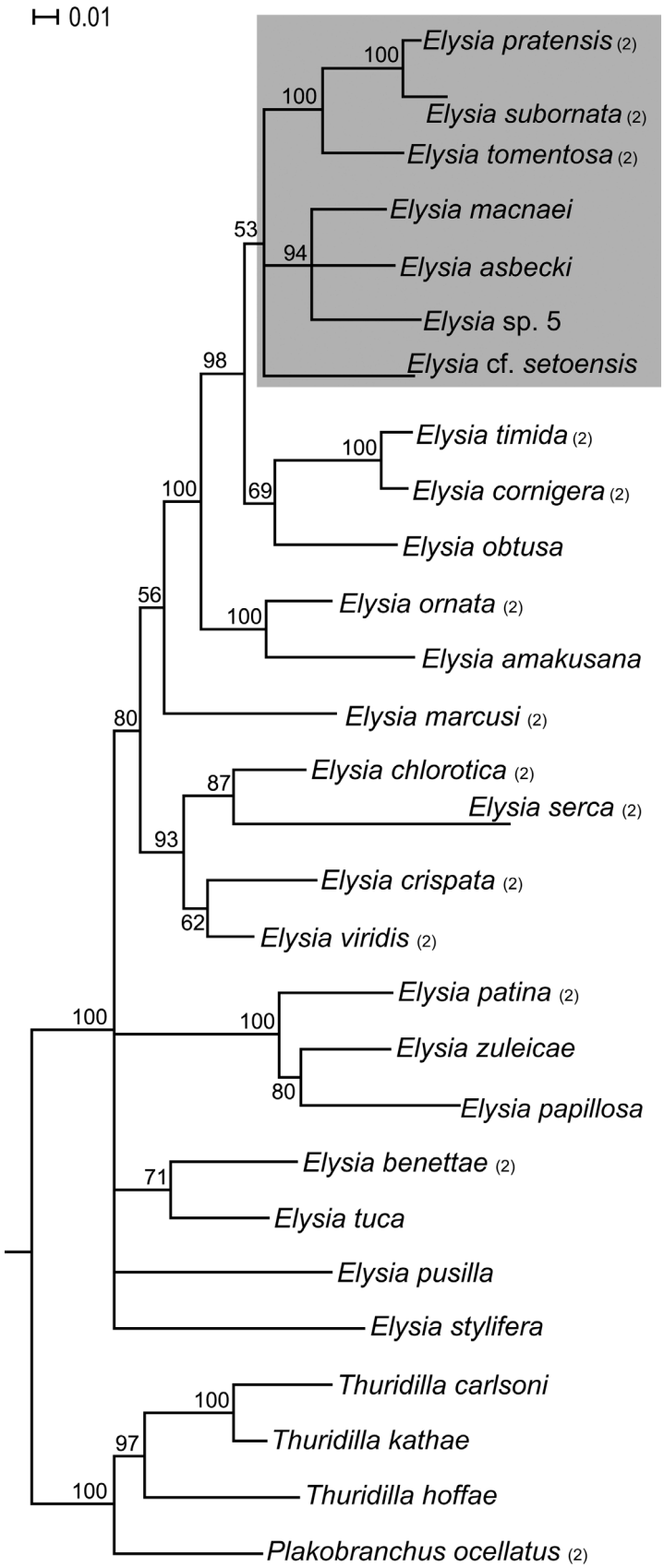

FIGURE 11. Phylogeny of the genus *Elysia* within Plakobranchidae. ML analysis was performed on concatenated partial gene sequences of the nuclear 28S rDNA, the mitochondrial 16S rDNA, and the mitochondrial CO1 (first and second position only) loci. Bootstrap support values are given. Numbers behind names indicate number of individuals included. Grey box comprise those species included in the sequence divergence analysis (see Table 6).

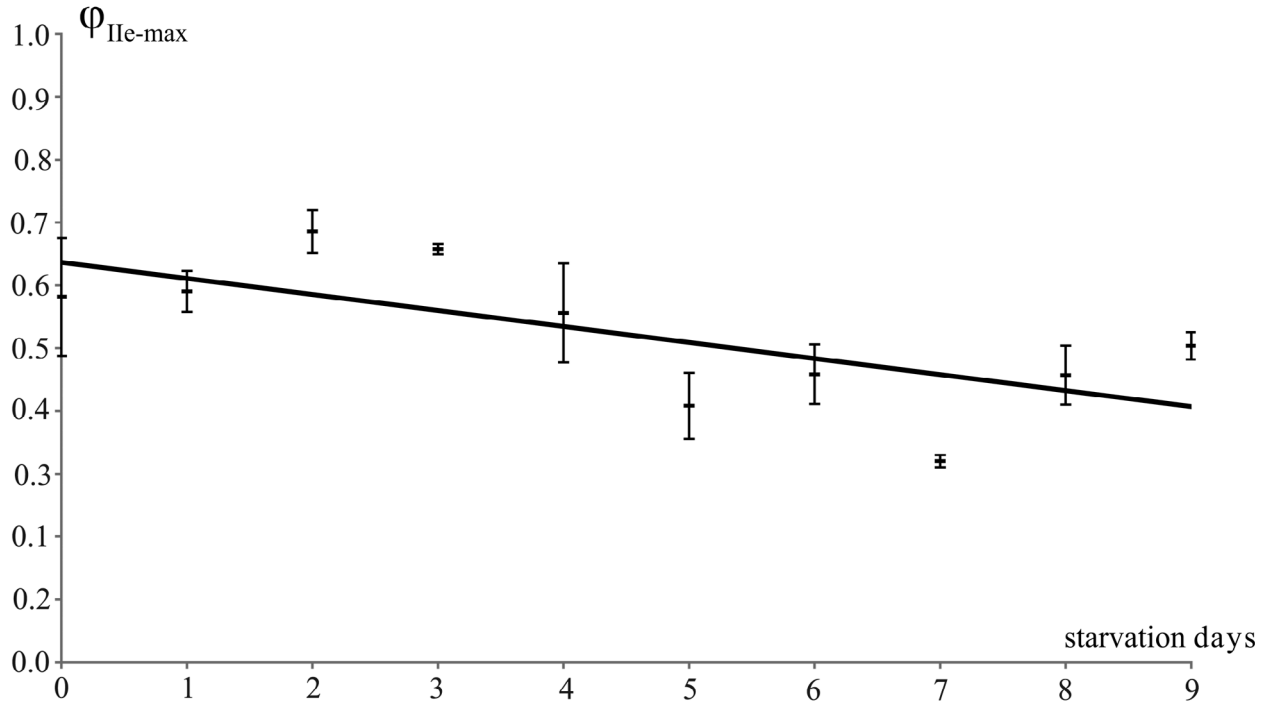

FIGURE 12. *Elysia asbecki* **sp. nov.**: PAM measurements (PSII maximum quantum yield, $\Phi_{IIe\text{-}max}$) of one specimen of *Elysia asbecki* **sp. nov.** plotted against starvation days. Two to four measurements were taken per day. Mean value of yield values and standard deviation is given (see also Table 7). The line represents the trend line as calculated in Excel.

Molecular investigation. Partial CO1 gene sequences were analysed for two specimens (see Tab. 6) in comparison with closely related *Elysia* species, according to the results based on the phylogenetic analyses (see Fig. 11, grey box). Two analysed *Elysia asbecki* **sp. nov.** sequences differ only in one nucleotide (sequence divergence 0.15%). The lowest interspecific divergence was found towards an undescribed species from Lizard Island (*Elysia* sp. 5) with 10.5% (uncorrected distance), followed by *E. macnaei* Marcus, 1982 with 11.6% (uncorrected distance). The phylogenetic analysis based on three genes also reflects the close relationship of these three species, but also their distinctiveness.

TABLE 6. Divergence of CO1 sequences between two individuals of *Elysia asbecki* **sp. nov.** and closely related species according to the results on the phylogenetic analysis (see grey box in Fig. 11). Two specimens of *E. pratensis* and *E. tomentosa* were included.

	E. asbecki **sp. nov.** (paratype)	*Elysia* sp. 5	*Elysia macnaei*	*Elysia subornata*	*Elysia pratensis*	*Elysia tomentosa*
Elysia asbecki **sp. nov.**	0.15	10.52	11.65	13.53	14.66–14.85	15.22–15.41

Notes on biology and photosynthetic activity. Figure 12 shows measurements of photosynthetic activity plotted against starvation days. Data are shown in Table 7. Maximum quantum yield values ($\Phi_{IIe\text{-}max}$) on the day of collecting (but after several hours of starving) start on a higher level of 0.6 and decrease to around 0.5 after nine days of starving. No information on algal food or development is available yet.

Discussion. Taxonomy of *Elysia asbecki* sp. nov.. The new species described here as *Elysia asbecki* **sp. nov.** was already illustrated in two varieties in Wägele *et al.* 2006b (Figs. 4C and D) as *Elysia* sp.. This material has not been preserved at that time, but was only documented with a digital camera (see also Fig. 7C this study). *Elysia asbecki* **sp. nov.** was also recorded by Gosliner *et al.* (2008) as *Elysia* sp. 16. The animal depicted in the photograph clearly shows the black and white ribbon around the upper part of the rhinophores,

the red patches along the edges of the parapodia in the middle part, the light stained dot in the neck area and the whitish appearance with the tiny yellow dots.

TABLE 7. PAM measurements (PSII maximum quantum yield, $\Phi_{IIe\text{-}max}$) of one specimen of *Elysia asbecki* **sp. nov.** during nine starvation days. Two to four measurements were taken per day. Mean value of yield values (see also Händeler *et al.* 2009) and standard deviation is given. For graphic illustration of the data see Figure 12.

Day of starvation	$\Phi_{IIe\text{-}max}$ (mean value)	standard deviation
0	0.58	0.09
1	0.59	0.03
2	0.69	0.03
3	0.66	0.01
4	0.56	0.08
5	0.41	0.05
6	0.46	0.05
7	0.32	0.01
8	0.46	0.05
9	0.50	0.02

The family Plakobranchidae are shell-less sacoglossans with a flattened body, with leaf like lateral expansions (parapodia), which are usually folded up on the dorsal side. The pericardium is located medio-dorsally just behind the head. A number of pericardial vessels are found branching from the pericardium along the dorsal surface of the body and parapodia. Marcus d. B.-R. (1980) described the branching patterns of the vessels as species-specific. According to anatomical data, we include the new species to the genus, since the presence of blade-shaped radula teeth with a median denticulate cutting edge is confirmed, and no pharyngeal pouch was found. Molecular data also give evidence for this assignment.

Roughly 80 species of *Elysia* are described worldwide (Jensen 2007), more than half of them from the Indopacific region. Additionally, many undescribed species are recorded in this region as well. Around 30 species have been recorded from the Mediterranean Sea and the Atlantic Ocean (Jensen 2007). Except of *E. timida* and *E. verrucosa* Jensen, 1985, none of these are similar in their external features and coloration to the here newly described *Elysia asbecki* **sp. nov.**. *Elysia timida* differs by the distinct red dots, which are missing in *E. asbecki* **sp. nov.**, and never shows the distinct red patches at the junction of the parapodia, along the edges of the parapodia and along the end of the foot (pers. observation). The leading tooth is more elongate in *E. timida*, than in *E. asbecki* **sp. nov.**. *Elysia verrucosa* shows white patches and black spots abundant on the entire body surface (Jensen 1985). Living animals can be easily distinguished by the green and white irregular patterns in *E. verrucosa*, as well as the lack of the distinct dark and orange ring on the rhinophores in the latter. The leading radula tooth in *E. verrucosa* is roundish, where as it is acute in our new species. *Elysia asbecki* **sp. nov.** clearly shows short u-shaped rhinophores, similar to those in *E. trisinuata* Baba, 1949 and *E. pusilla* (Bergh, 1872), but not in *E. timida* or in *E. verrucosa*.

The new species will be discussed with the following whitish colored and similar shaped species known from the Pacific Ocean: *Elysia mercieri* (Pruvot-Fol, 1930), *E. tomentosa* Jensen, 1997, *E. trisinuata* and *E. pusilla*. In terms of color, the new *E. asbecki* **sp. nov.** can be distinguished from other species by its whitish appearance due to many tiny white dots, the larger orange spots and tiny dark dots covering mainly the outer parapodia and dorsal body parts. *Elysia pusilla* differs by the color pattern, the reduced parapodia and the cryptic appearance on their food source *Halimeda* sp. (Jensen 1992). *E. trisinuata* appears similar in shape but the plain green color and the specific three raised folds along the parapodial edge distinguish it from our new species here. Also the radula is very similar, but the teeth in *E. trisinuata* appear more elongate (Jensen 1992) than those of the new species described here. *E. mercieri* is distinguished by the elaborate parapodial margin with structures similar to branched papillae, and the rhinophores show several brownish patches or bands. *Elysia tomentosa* has distinct papillae, which may even form branched processes. That species never exhibit

the typical color patterns of the rhinophores or the red markings of our new species. Furthermore, the radula teeth appear more elongate in *E. tomentosa* than in *E. asbecki* **sp. nov.** (Jensen 1997).

Comparison of histological results on several sacoglossan species shows that the special glands described here for the first time for *Elysia asbecki* **sp. nov.** are typical for members of the Plakobranchidae. A re-investigation of *E. crispata, E. ornata* (Swainson, 1840), *E. timida, E. viridis* (Montagu, 1804), as well as *Plakobranchus ocellatus* van Hasselt, 1824 and two *Thuridilla* species (*T. carlsoni* Gosliner, 1995, and *T. hopei* (Verany, 1853)) revealed similar glands in the dorso-anterior to lateral parts of the pharynx, additionally to separate salivary glands, which usually lie ventrolaterally and posterior to the pharynx. So far, these glands are absent in non-plakobranchoidean species investigated up to now (*Oxynoe viridis* (Pease 1861), *Alderia modesta, Ercolania annelyleorum* **sp. nov.** and *E. kencolesi*) (unpublished results of HW).

Wägele *et al.* (2006a) mentioned special glandular structures in the parapodia of *Elysia ornata*. These differ to the cells mentioned here in the parapodia of *E. asbecki* **sp. nov.** in so far as there seems to be a dense core composed of several cells in the former. The histologically investigated specimen of *E. asbecki* **sp. nov.** was a juvenile and nearly no female structures were formed yet. Comparisons with adult members of other *Elysia* species clearly show that these special cells in *E. asbecki* **sp. nov.** are not part of the albumen gland or even prostate gland, both usually ramifying within the parapodia. The function of these special cell structures in our new species is not known or investigated yet, but needs further analyses.

Molecular characters. Sequence divergence of the partial CO1 gene between the two investigated specimens collected at the same locality and same time is extremely low (0.15%) and lies within the normal range of intraspecific variability as was also observed for two sequences of *E. pratensis* Ortea and Espinosa, 1996 (0.18%) and of *E. tomentosa* (0.37%). Values of sequence divergence between *Elysia* species closely related to *E. asbecki* **sp. nov.** are lower, as is observed for the Limapontiidae data set discussed above. They range from about 10% *(E. pratensis/E. subornata* Verrill, 1901) to a maximum of 15,5% (*E. asbecki* **sp. nov.**/ *E. tomentosa*). Nevertheless, these species are clearly separated, especially when considering the very low intraspecific variability.

The sequences of the three different molecular markers used in the phylogenetic analysis of *Elysia* species within Plakobranchidae are already published (see Table 5): 16S rDNA as *Elysia spec.* (accession number EU140856 in Händeler & Wägele 2007), CO1 and 28S rDNA as *Elysia* spec. 1 (accession numbers GQ996690 and GQ996629 respectively in Händeler *et al.* 2009). The phylogenetic analysis including these three genes unambiguously revealed the assignment of the new species to the genus *Elysia*, but also clearly showed its distinctiveness to all other included 23 *Elysia* species (Händeler *et al.* 2009, Fig. 11).

Photosynthetic activity. There are three different categories of photosynthetic activity in Sacoglossa: no functional retention, short-term retention and long-term retention (Händeler *et al.* 2009). *Elysia asbecki* **sp. nov.** shows retention with a high starting maximum quantum yield ($\Phi_{\text{IIe-max}}$) that decreases only slightly within about ten days. Since retention behaviour can vary on situation and specimen (see Händeler *et al.* 2009 and references therein) and just one specimen has been investigated concerning photosynthetic activity, it can not be ruled out that *Elysia asbecki* **sp. nov.** is a long-term retention form with similar photosynthetic performance as is described for *E. timida* from the Mediterranean Sea and *E. crispata* from the Caribbean Sea. This would also render *E. asbecki* **sp. nov.** the second long-term retention form in the Pacific, along with *Plakobranchus ocellatus*. Recently Wägele *et al.* (2010) were able to reject the hypothesis that lateral gene transfer from the algal nuclear genome to the slugs' nuclear genome is responsible for maintenance of chloroplasts over weeks to months. Their findings are based on genome expression data of starved *Plakobranchus ocellatus* and *Elysia timida* (both long term retention forms with photosynthetic activity over several weeks to months). This is in contrast to findings in *E. chlorotica* Gould, 1870 based on single PCR gene fragment analysis (Pierce *et al.* 2007: Rumpho *et al.* 2008; Schwartz *et al.* 2010). *Elysia asbecki* **sp. nov.** is more closely related to *E. timida* than to *E. chlorotica* (Händeler *et al.* 2009). Hence we consider the properties of the chloroplasts as the main factor for photosynthetic activity. Chloroplasts sequestered by *E. asbecki* **sp. nov.** originate from at least three different species of ulvophycean algae (Händeler *et al.* 2010; as *Elysia* sp. 1). Unfortunately, these cannot be identified yet to species level, due to lack of reference algal sequences.

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