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Article

Freshwater Slugs in the Caribbean: Rediscovery of Tantulidae (Acochlidimorpha, Panpulmonata) with the Description of *Potamohedyle espinosai* n. gen. n. sp. from Cuba

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Abstract: Freshwater slugs are scarce and belong exclusively to panpulmonate Acochlidimorpha. There is a radiation of eight species of large-sized slugs living benthically in rivers on tropical Indo-Pacific Islands. In the Western Atlantic, only one small interstitial slug, *Tantulum elegans* Rankin, 1979, is known from the Caribbean island of St. Vincent. We recently discovered a novel species of freshwater slugs in Cuba. Here, we describe *Potamohedyle espinosai* n. gen. n. sp., which is the first freshwater slug in the region of the Western Atlantic with a benthic lifestyle, in 3D-microanatomical and histological detail using light and scanning electron microscopy. It shows a mix of characters from different freshwater acochlidimorph genera, such as a medium body size, the presence of an osphradial ganglion, a distal gonoduct with a muscular sphincter, a penis with a solid thorn and cuticular comb, and a basal finger with a hollow stylet. Morphological adaptations to a life in freshwater include multiplicated renopericardioducts. The taxonomic character mix justifies the establishment of a novel genus within the herein diagnostically modified freshwater family Tantulidae. A molecular phylogenetic hypothesis of riverine slugs including the first Caribbean representatives suggests that the transition to freshwater occurred once along the stemline of limnic Acochlidiidae, secondarily marine Pseudunelidae and limnic Tantulidae.

Keywords: biodiversity; conservation; histology; identification key; integrative; Mollusca; scanning electron microscopy; 3D microanatomy

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1. Introduction

The Cuban archipelago is considered a biodiversity hotspot [1,2], with diversity expressed not only in terms of species richness but also in high endemism [3]. This exceptional biodiversity has been well-documented in many diverse taxa [4–6]. In contrast to Cuban terrestrial habitats, which have been in the focus of conservation and evolutionary studies [7], the Cuban freshwater fauna have only gained little attention recently [8].

Next to crustaceans and insects, molluscs belong to the comparably well-studied groups in Cuban freshwater [8,9]. In general, freshwater molluscs are of considerable significance in freshwater ecosystems, offering benefits such as nutrients in food webs [10] and ecosystem services like water filtration [11]. However, they can also have adverse effects as disease vectors [12] or invasive species [13,14]. Although they are frequent in ecological surveys [15], freshwater molluscs are understudied in comparison to marine or terrestrial species [16]. Currently, 6756 valid species of extant freshwater molluscs are known, among these are 1427 bivalve species and 5329 gastropods [17] with an overwhelming majority of freshwater snails. In Cuba, there are currently 44 species (36 gastropods and 8 bivalves) of freshwater molluscs recorded; among those, 10 are considered endemics and three are presumably extinct [18–20]. Yet, the Cuban freshwater malacofauna are far from being satisfactorily explored: molecular barcoding projects might reveal cryptic diversity, especially prone are complexes of several subspecies as, e.g., within *Biomphalaria havanensis* [21], and new records of (potentially introduced) gastropod species (see, e.g., Vázquez, et al. [22] on *Littoridinops monroensis*) are not unusual.

In our 2023 survey of Cuban freshwaters, we discovered a novel species of freshwater slugs, thereby indicating that the Cuban freshwater fauna still harbour malacological secrets and undescribed species, which contribute to a better understanding of the evolution of freshwater gastropods. With a total of only nine described species [23,24], true freshwater slugs (i.e., not amphibious) are rare, all belonging to panpulmonate Acochlidimorpha. This order of heterobranch gastropods shows remarkable diversity in body plans and habitats. Acochlidimorpha contains mostly marine mesopsammic species but also several that have colonized brackish, (semi)terrestrial and freshwater systems [25,26]. Most freshwater species are large-sized slugs (10 mm–35 mm) living benthically in coastal rivers on tropical Indo-Pacific Islands. The all belong to the family Acochlidiidae, which consists of eight species in the genera *Strubellia*, *Wallacellia*, *Palliohedyle* and *Acochlidium* [23,26]. From other parts of the world, only Tantulidae is known. This family was established based on a single species—*Tantulum elegans* Rankin, 1979—living interstitially in a mountain spring swamp on the Caribbean island of St. Vincent [27]. This enigmatic species shows unique character states, e.g., unarmed anterior male copulatory organs and salivary pumps [28], and forms a basal offshoot of marine mesopsammic Hedylopsacea in cladistic analysis based on biological and morphological characters [26]. Surprisingly, no other hedylopsacean species has yet been discovered in the entire Western Atlantic nor in the terrestrial or freshwater habitats of the Caribbean.

Tantulum still awaits a placement in molecular phylogenetic analyses due to the lack of properly preserved type material for molecular analyses and the failure of recollection attempts at the type locality (likely due to land-use changes, pers. obs.). Almost 50 years after the discovery of *Tantulum* on the island of St. Vincent, we now found a second species of freshwater slugs in the Caribbean.

In the present study, we describe the novel genus and species *Potamohedyle espinosai* n. gen. n. sp. from Cuba in 3D-microanatomical detail and highlight its morphology in a phylogenetic context with special regard to adaptations to life in freshwater. We present a molecular phylogeny of riverine slugs including the first Caribbean representatives and discuss potential evolutionary scenarios. A genus-level identification key of freshwater slugs is provided to support future surveys.

2. Materials and Methods

2.1. Sampling and Fixation

Several living specimens of the freshwater slug were collected in the river Río Turquino (municipality Guamá, Santiago de Cuba Province) in the southeast of Cuba (Figure 1A) (19°56′50.971″ N, 76°45′32.81″ W) during the low rainy season in November 2023. From November to March, the river carries little water, but never dries up. Small lagoons persist next to the river mouth, even in the dry season, and are protected from saltwater by a narrow coastal strip. No salinity was detected during sampling. Individuals were sampled in a lagoon (Figure 1B,C) located near the mouth of the river and about 300 m upstream. They were collected at a 0.3–1 m depth by hand from the underside of small volcanic stones (Figure 1D) that are spread over the sandy and muddy ground of the riverbed and among the reeds (*Typha* sp.) on the riverside. The co-occurring malacofauna consist of the freshwater species *Gundlachia radiata* (Guilding, 1828), *Nereina punctulata* (Lamarck, 1816), and *Tarebia granifera* (Lamarck, 1816) and brackish species *Vitta virginea* (Linnaeus, 1758), *Neritilia succinea* (Récluz, 1841), and *Neritilia serrana* Espinosa, Ortea & Diez-García,

2017. The external morphology of living specimens was documented photographically in the field. Additionally, squash preparations of whole specimens were made in the laboratory and documented by light microscopy. Specimens were fixed with 96% ethanol for molecular analyses and, after anaesthetisation with menthol crystals, with 70% ethanol for microanatomical and histological analyses. An overview of the material used in this study is given in Table 1.

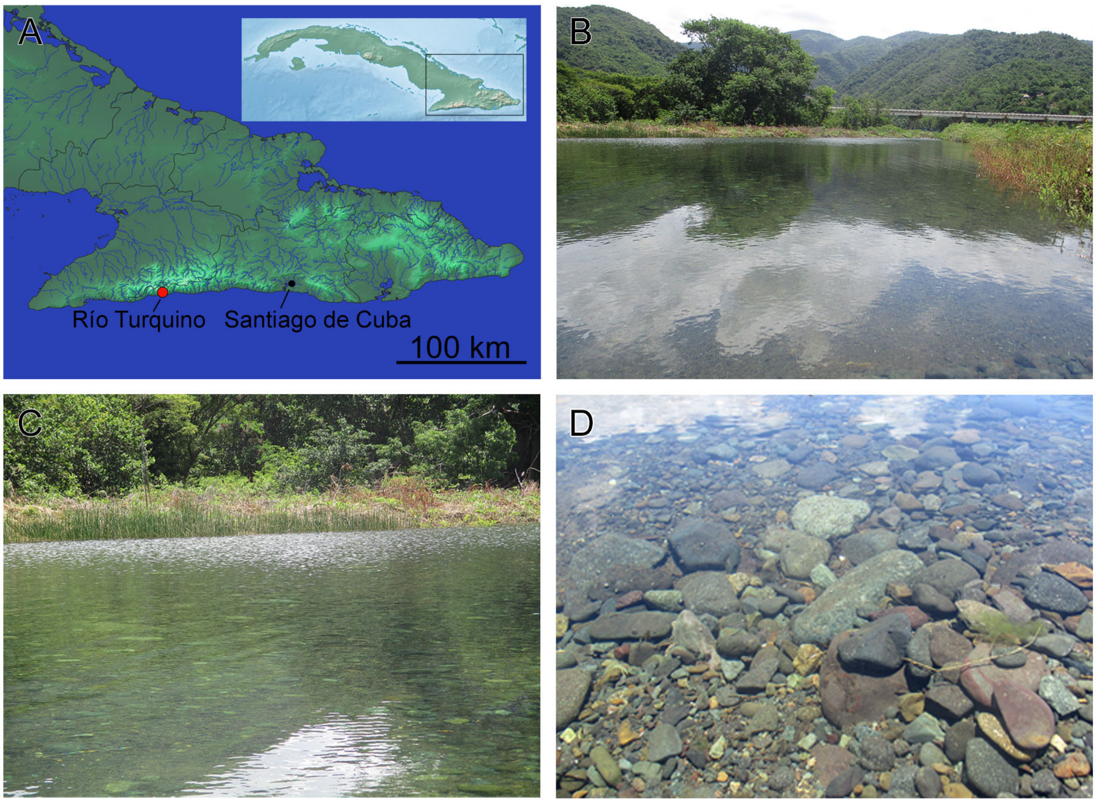

Figure 1. Map of Cuba, type locality, and habitat of *Potamohedyle espinosai* n. gen. n. sp. (**A**) Island of Cuba with the type locality (red mark) at Río Turquino; (**B**) lagoon at the mouth of Río Turquino; (**C**) habitat with reeds at the riverside; (**D**) substrate of the river bed.

Table 1. (Type) Material of *Potamohedyle espinosai* n. gen. n. sp. examined in the present study and deposited at the Bavarian State Collection of Zoology, Munich (ZSM).

ZSM Museum N°	Fixation	Type of Investigation	Storage and Remarks
Mol 20240444	96% eth	external morphology	holotype
Mol 20240445	96% eth	external morphology	paratypes (3 specimens)
Mol 20230584	70% eth	histology, 3D	ss, 1.0 µm; adult, mature
Mol 20230585	70% eth	histology, 3D	ss, 1.5 µm; juvenile
Mol 20230586	70% eth	histology	ss, 1.5 µm; subadult
Mol 20230587	96% eth	molecular analyses	DNA sample
Mol 20230588	96% eth	molecular analyses	DNA sample
Mol 20240446	70% eth	radula analysis by SEM	radula on SEM stub
Mol 20240447	70% eth	light microscopy	whole mounts, 5 slides

Abbreviations: eth, ethanol; ss, series of semithin sections; SEM, scanning electron microscopy.

2.2. Embedding and Semi-Thin Sectioning

We performed embedding with a Leica EM TP automatic tissue processor. First, individuals fixed with 70% ethanol were rehydrated in a descending ethanol series (70%, 50%, 30%, and 10%) and decalcified in ascorbic acid (1%) overnight to remove spicules. Afterwards, the samples were washed three times for 20 min with cacodylate buffer (0.1 mol). The specimens were post-fixed with 1% osmium tetroxide (in 0.1 mol of cacodylate buffer) for two hours, and after that, they were again washed three times for 20 min with cacodylate buffer (0.1 mol). We then dehydrated the samples in an ascending acetone series (30%, 50%, 70%, 90%, and 4 × 100%) for 15 to 20 min and embedded them overnight in Spurr's low-viscosity resin [29] mixed 1:1 with acetone. Finally, we transferred them to 100% Spurr's medium and polymerised them at 60 °C for 24 h. We prepared three series of ribboned semi-thin sections (1.5 µm) with a diamond knife (Histo Jumbo, Diatome, Biel Switzerland) following Ruthensteiner [30]. The ribbons were stained with methylene blue–azure II [31]. All section series are deposited at the ZSM (see Table 1).

2.3. Digitalisation and 3D Reconstruction with Amira®

Two histological semi-thin series were digitalised using the virtual slide system dotSlide Olympus VS-ASW (FL) and an Olympus BX61VS microscope. We made a screenshot of every second section using the software packages OlyVIA 3.8 and ClipboardSaver and used the software Amira 6.0.1 for the generation of a 3D reconstruction largely following the procedure described by Ruthensteiner [30].

2.4. Radula Analyses by Light and Scanning Electron Microscopy (SEM)

We macerated two specimens in 10% KOH for half an hour, and after preparation from the pharynx, we rinsed the radula with distilled water. One radula was photographically documented by light microscopy; the other (ZSM Mol 20240446) was transferred to a SEM stub with a self-adhesive carbon sticker. We sputter-coated the radula with gold (SEM Coating System, Polaron SC510, BIO-RAD) in an Argon atmosphere for 160 s at 2.4 kV and 20 mA. The radula was analysed with a LEO 1430VP (18.52 kV) at the ZSM.

2.5. Molecular Analyses

Two specimens (ZSM Mol 20230587 and 20230588) were subsampled for molecular analyses by clipping off a piece of their foot. DNA was extracted from the dried tissue samples using a standard CTAB extraction protocol. We amplified four molecular standard markers, nuclear 18S and 28S rRNA, the mitochondrial large subunit ribosomal RNA gene (16S), and cytochrome c oxidase subunit I (COI), using the standard protocols and primers reported in previous studies [25].

Based on the morphological identification (see the Results below), the acochlidimorph from Cuba can be assigned to the family Tantulidae within the clade Hedylopsacea, which was also confirmed by the results of BLAST searches used to check the generated sequences for potential contamination against the GenBank database. Accordingly, we selected a taxon sample of Hedylopsacea for phylogenetic placement of the novel species, which includes at least one representative of all available hedylopsacean species in GenBank, representing all currently known families and genera, except the Caribbean *Tantulum*, which unfortunately lacks molecular data. Our taxon sample includes 31 species; three species of Microhedylacea were chosen as outgroups (see Table 2 for vouchers and GenBank accession numbers).

Table 2. List of included acochlidimorph taxa for phylogenetic analyses with museum numbers of the DNA voucher materials and GenBank accession numbers. Microhedylacea are selected as outgroup taxa.

Taxon	Museum Number	GenBank Accession Numbers of Sequences				Source
		18S rRNA	28S rRNA	16S rRNA	COI	
HEDYLOPSACEA						
Tantulidae						
Potamohedyle espinosai	ZSM Mol 20230588	PQ276770	PQ276771	PQ276769	PQ276046	present study
	ZSM Mol 20230587			PQ276768	PQ276045	present study

Table 2. *Cont.*

Taxon	**Museum Number**	**GenBank Accession Numbers of Sequences**				**Source**
		18S rRNA	**28S rRNA**	**16S rRNA**	**COI**	
Hedylopsidae						
Hedylopsis spiculifera	ZSM Mol 20080951	HQ168430	HQ168443	no data	HQ168455	[32]
	ZSM Mol 20080389	no data	KF709319	KF709245	KF709351	[25]
	ZSM Mol 20081016	KF709275	no data	KF709246	KF709353	[25]
Hedylopsis ballantinei	ZSM Mol 20090244	HQ168429	HQ168442	HQ168416	HQ168454	[32]
Hedylopsis MOTU Moorea	AM C. 476056.001	KF709276	no data	KF709247	KF709354	[25]
Hedylopsis sp. 1 Azores	ZSM Mol 20202407	no data	no data	MW684374	MW596409	[33]
Hedylopsis sp. 2 Azores	ZSM Mol 20130993A	no data	no data	MW684373	MW596408	[33]
Hedylopsacea indet.						
Hedylopsacea MOTU Moorea	AM C. 476059.001	KF709277	KF709320	no data	KF709355	[25]
Pseudunelidae						
Pseudunela cornuta	ZSM Mol 20071809	JF819754	KF709321	JF819748	JF819774	[34]
Pseudunela viatoris	ZSM Mol 20080020	JF819751	no data	JF819741	JF819766	[34]
	ZSM Mol 20070953	no data	KF709322	JF819745	JF819770	[34]
Pseudunela marteli	ZSM Mol 20080022	JF819753	no data	JF819746	JF819771	[34]
	ZSM Mol 20080393	HQ168431	HQ168444	HQ168418	HQ168456	[32]
	ZSM Mol 20100381	KF709278	KF709323	KF709248	KF709356	[25]
Pseudunela MOTU Maledives	ZSM Mol 20110029	KF709279	KF709324	KF709249	KF709357	[25]
Pseudunela espiritusanta	ZSM Mol 20080117	JF819755	KF709325	JF819749	JF819775	[34]
Acochlidiidae						
Strubellia paradoxa	ZMB Moll. 193.944	HQ168432	HQ168445	HQ168419	HQ168457	[32]
Strubellia wawrai	ZSM Mol 20080016	KF709280	no data	JF819730	JF819758	[35]
	ZSM Mol 20071810	no data	KF709326	JF819734	JF819762	[35]
	ZSM Mol 20080150	KF709281	KF709327	JF819736	JF819764	[35]
Strubellia MOTU Sulawesi	ZSM Mol 20100339	KF709282	KF709328	JF819740	JF819765	[35]
Wallacellia siputbiru	ZMB Moll. 193.966	KF709283	KF709329	KF709250	KF709358	[25]
Palliohedyle MOTU Sulawesi	ZSM Mol 20100356	KF709284	JF828039	JF828040	JF828032	[25]
Acochlidium fijense	ZSM Mol 20080063	HQ168433	HQ168446	HQ168420	HQ168458	[32]
Acochlidium amboinense	ZMB Moll. 193.942a	KF709285	KF709330	KF709251	KF709359	[25]
Acochlidium bayerfehlmanni	ZSM Mol 20080384	KF709286	no data	KF709252	KF709360	[25]
Acochlidium sutteri	ZSM Mol 20080911	KF709287	KF709331	KF709253	KF709361	[25]
Acochlidium MOTU Flores	ZSM Mol 20080897	KF709288	KF709332	KF709254	KF709362	[25]
Acochlidium MOTU Sulawesi	ZSM Mol 20100341	KF709289	KF709333	KF709255	KF709363	[25]
Acochlidium MOTU Solomons	ZSM Mol 20080159	KF709290	KF709334	KF709257	KF709365	[25]
Bathyhedylidae						
Bathyhedyle boucheti	MNHN_IM-2000-27917	KX721049	KX721050	KX721048	no data	[36]
Aitengidae						
Aiteng ater		JF828036	JF828037	JF828038	JF828031	[25]
Aiteng marefugitus		AB914671	AB914672	AB914673	AB914674	[37]
Aiteng mysticus		HQ168428	HQ168441	HQ168415	HQ168453	[32]
MICROHEDYLACEA						
Asperspina brambelli	ZSM Mol 20100576	no data	JQ410991	JQ410990	JQ410924	[25]
Microhedyle glandulifera	ZSM Mol 20081019	HQ168437	HQ168449	HQ168424	HQ168461	[32]
Ganitus evelinae	ZSM Mol 20100328	KF709312	JF828044	JF828045	JF828034	[25]

Abbreviations: AM, Australian Museum, Sydney/Australia; MNHN, Muséum National d'Histoire Naturelle, Paris/France; ZMB, Museum für Naturkunde, Berlin/Germany; ZSM, Bavarian State Collection of Zoology, Munich/Germany.

Sequences of each single marker were aligned using MUSCLE [38], as implemented in Geneious Prime. We removed ambiguous parts of the alignments with Gblocks [39,40] via the http://phylogeny.lirmm.fr webserver, accessed on 3 April 2024, using the options for a less stringent selection. We analysed single gene trees via the RaxML tree building method implemented in Geneious to check for potential contaminations within the dataset. The four alignments were then concatenated via Geneious Prime and analysed via the IQtree-webserver (using ultrafast bootstrap) http://iqtree.cibiv.univie.ac.at, accessed on 10 April 2024, [41–44] with or without partitioning according to the four markers. In parallel, we conducted RaxML analyses via the Cipres Portal [45] using RaxML-HPC v.8 on ACCESS [46] to be able to define the three Microhedylacea as outgroups.

2.6. Nomenclatural Acts

This published work and the nomenclatural act it contains have been registered in ZooBank, the proposed online registration system for the International Code of Zoological Nomenclature. The LSID for this publication is urn:lsid:zoobank.org:pub:E255F25A-C151-4E21-8F09-723E24204BDD.

3. Results

3.1. Systematics

Subclass Heterobranchia sensu Haszprunar, 1985
Subcohort Panpulmonata Jörger et al., 2010
Order Acochlidimorpha sensu Bouchet et al., 2017
SPF Acochlidioidea Küthe, 1935
Family Tantulidae, Rankin 1979

Diagnosis herein modified after Rankin [27]: Small (2–3 mm) interstitial to medium-sized (5–12 mm) benthic freshwater slugs, body colour translucent–whitish, cephalic tentacles digitiform, foot as broad as the body without propodial tentacles, eyes and heart bulb not visible externally, visceral sac elongated and round in cross-section, eyes unpigmented, osphradial ganglion present, salivary gland ducts with muscular salivary pumps, digestive gland sac-like, sequential protandric hermaphrodites with internal vas deferens.

Potamohedyle n. gen.

ZooBank registration (LSID): urn:lsid:zoobank.org:act:382F2FAD-C1DD-40DB-A532-B45ACDEFD091.

Type species: *Potamohedyle espinosai* n. sp., by monotypy.

Diagnosis: Benthic freshwater slugs with characteristics of family Tantulidae. Rhinophores longer than labial tentacles, multiplication of renopericardioducts, penial apical cuticle present, basal finger with hollow stylet.

Etymology: From Greek, potamos, meaning 'river' and –hedyle, a common component for acochlidimorph names based on the later synonymised genus name *Hedyle*.

Potamohedyle espinosai n. sp.

ZooBank registration (LSID): urn:lsid:zoobank.org:act:E512B6DF-50FF-4F57-93B0-F9E-BDCE66EFA.

Type material: Holotype (ZSM Mol 20240444), complete specimen preserved and stored in 96% ethanol. Paratypes (ZSM Mol 20240445), a lot of three complete specimens preserved and stored in 96% ethanol. The type material is deposited at the Bavarian State Collection of Zoology, Munich, Germany.

Type locality: Río Turquino, municipality Guamá, Santiago de Cuba Province, south-eastern Cuba, 19°56′50.971″ N, 76°45′32.81″ W.

Diagnosis: Body size 5–12 mm, one to three renopericardioducts, distal gonoduct with muscular sphincter, solid penial thorn of 40 µm, cuticular penial comb of 85 µm, basal finger stylet of 170–210 µm.

Etymology: The species epithet honours the Cuban malacologist José Espinosa Sáez for his extraordinary contribution to the study of Cuban molluscs. He has described more than 250 species of mainly marine molluscs from Cuba, the Caribbean, and Macaronesia.

3.2. External Morphology

The size of *Potamohedyle espinosai* n. gen n. sp. ranges from 5 to 12 mm in living specimens. The body consists of an anterior head–foot complex and a posterior elongated, free visceral sac (Figure 2A), into which the head can be fully retracted (Figure 2E,F). Anteriorly, the foot is nearly as broad as the body and tapered at its end (Figure 2A,D). Propodial tentacles are absent. The body colour is translucent–whitish, and the digestive gland (Figure 2A) shines yellowish to brownish through the epidermis. No eyes are visible externally. The head bears two pairs of appendages: one pair of slightly flattened labial tentacles with a wide base and a thinner tip and, more posteriorly, one pair of longer, digitiform rhinophores (Figure 2A–C). Elongated or slightly curved, calcareous subepidermal spicules are distributed across the body but are numerous inside the tentacles, in the foot, and in the area of the pharynx (Figure 2B–D).

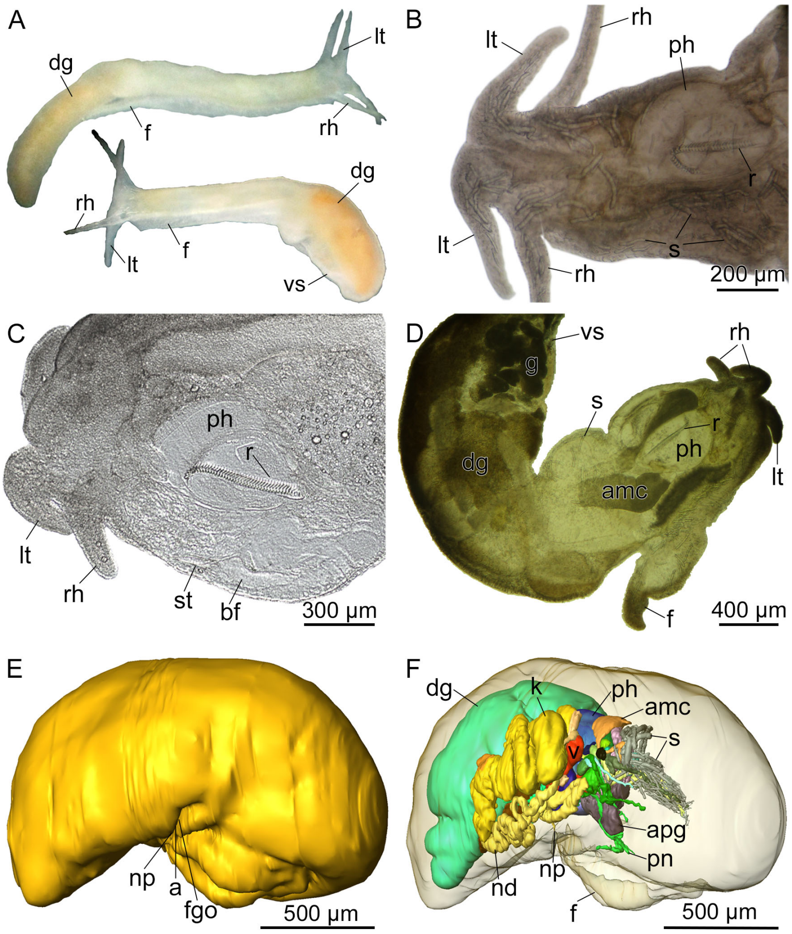

Figure 2. Living specimen (**A**), squash preparations (**B**–**D**) and 3D reconstructions (**E**,**F**) of *Potamohedyle espinosai* n. gen. n. sp. (**A**) External morphology, approx. 5 mm body length. (**B**) Head with pharynx and radula (dorsal view). (**C**) Head (ventral view). (**D**) Right view;.(**E**) Positions of the female gonopore, nephropore and anus in the reconstructed, contracted body (right view). (**F**) General anatomy (right view). Abbreviations: amc, anterior male copulatory organs; a, anus; apg, anterior pedal gland; bf, basal finger; dg, digestive gland; f, foot; fgo, female gonopore; g, gonad; k, kidney; lt, labial tentacle; nd, nephroduct; np, nephropore; ph, pharynx; pn, pedal nerve; r, radula; rh, rhinophore; s, spicule; st, stylet; v, ventricle; vs, visceral sac.

The male genital opening is located at the base of the right rhinophore. The female gonopore, anus and nephropore are positioned dextroventrally (from anterior to posterior, respectively) at the junction of the head–foot complex with the visceral sac (Figure 2E), with the anus and the nephropore being in close proximity.

3.3. *Internal Microanatomy*

The anterior part of the body is filled with the central nervous system, the anterior part of the digestive system (oral tube, pharynx, radula, salivary glands, and oesophagus), and the anterior male copulatory organs. The visceral sac contains the posterior part of the digestive system (digestive gland and intestine), the female reproductive system and the ovotestis, and the circulatory and excretory systems.

3.3.1. Central Nervous System (CNS)

The CNS of *Potamohedyle espinosai* n. gen. n. sp. is euthyneurous, following the general acochlidimorph bauplan (Figures 3 and 4). It consists of the paired cerebral, pedal, pleural, rhinophoral, optic, and buccal ganglia; three distinct ganglia of the visceral nerve cord; and the unpaired presumed osphradial ganglion. All ganglia except the buccal ganglia are located pre-pharyngeally. All ganglia show the same histology, i.e., an outer cortex with dark-stained nuclei and an inner neuropil with light-stained nerves (Figure 5).

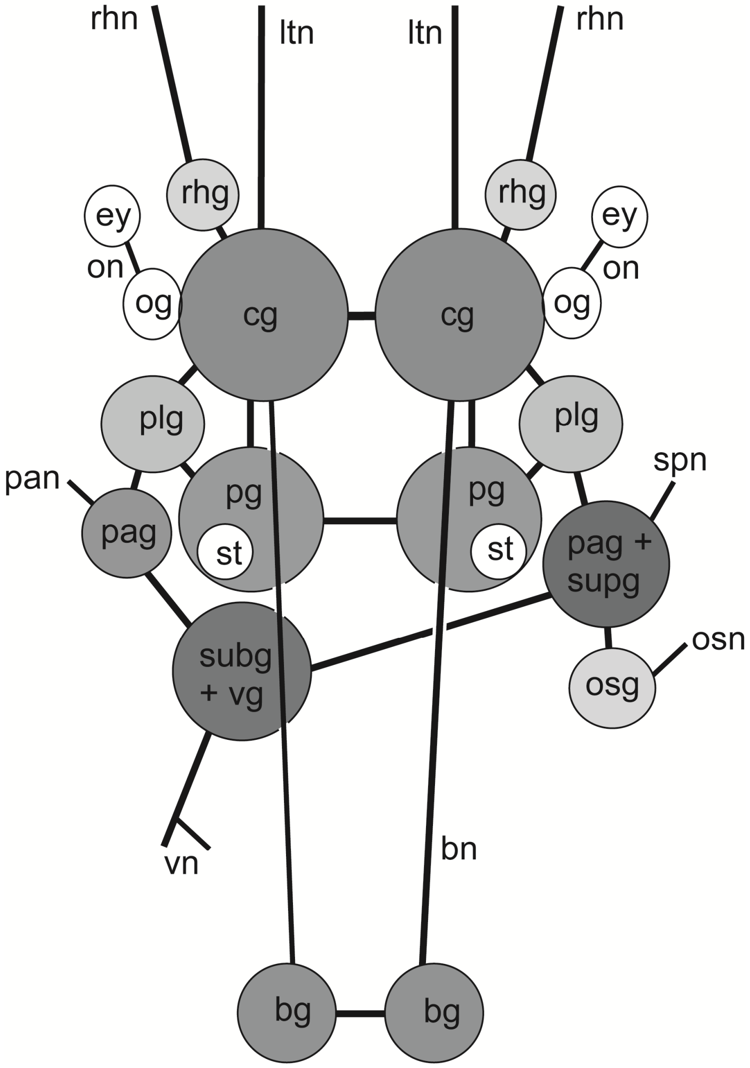

Figure 3. Schematic overview of the central nervous system (CNS) of *Potamohedyle espinosai* n. gen. n. sp. Not to scale. Abbreviations: bg, buccal ganglion; bn, cerebro–buccal connective; cg, cerebral ganglion; ey, eye; ltn, labial tentacle nerve; og, optic ganglion; on, optic nerve; osg, osphradial ganglion; osn, osphradial nerve; pag, parietal ganglion; pan, parietal nerve; pg, pedal ganglion; plg, pleural ganglion; rhg, rhinophoral ganglion; rhn, rhinophoral nerve; spn, supraintestinal + parietal nerve; st, statocyst; subg, subintestinal ganglion; supg, supraintestinal ganglion; vg, visceral ganglion; vn, visceral nerve.

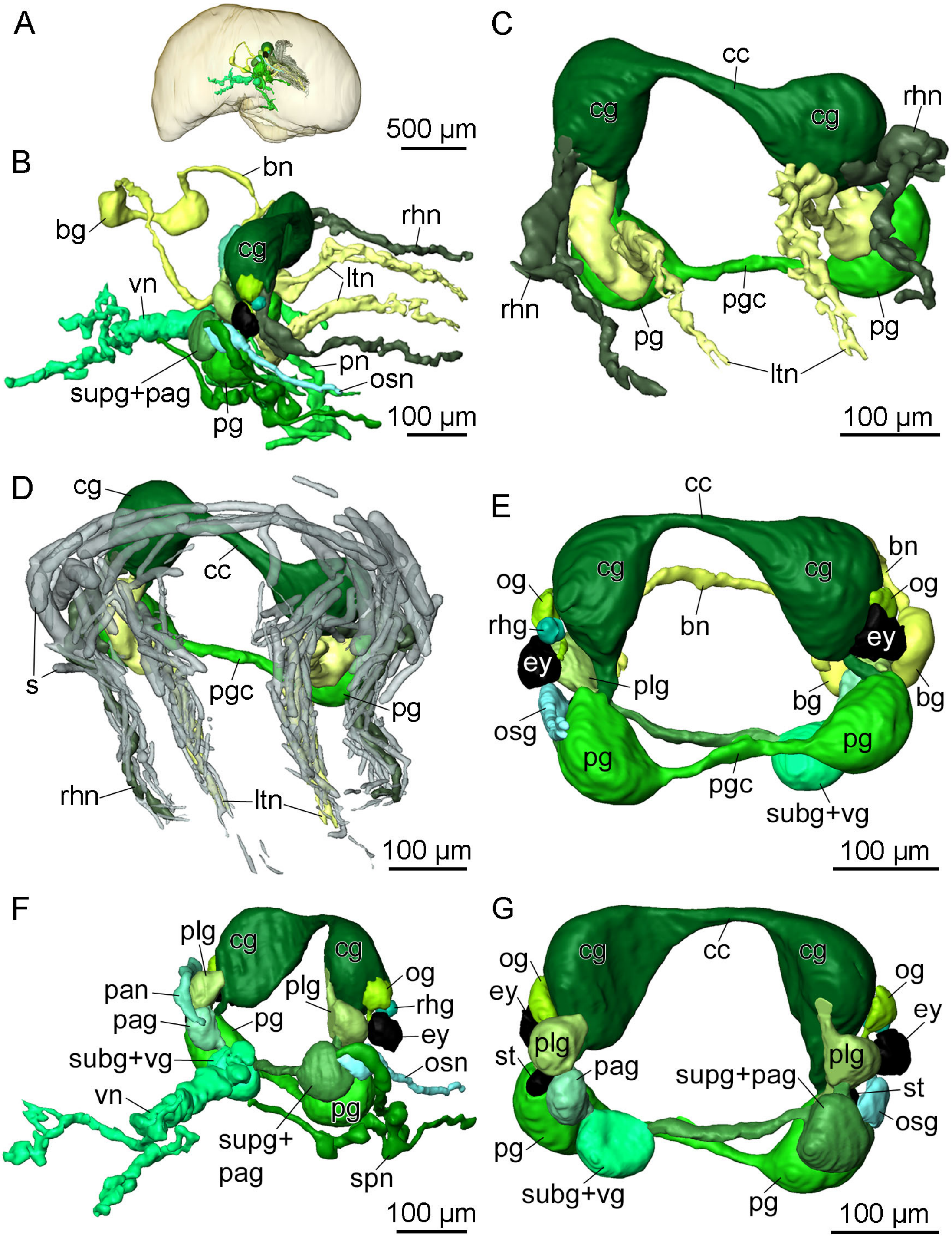

Figure 4. Three-dimensional reconstructions of the central nervous system (CNS) of *Potamohedyle espinosai* n. gen. n. sp. (**A**) Relative position of the CNS (right view). (**B**) Complete CNS (right view). (**C**) Cerebral and pedal ganglia with cerebral nerves (anterior view). (**D**) CNS protected by spicules (anterior view). (**E**) All major ganglia without nerves (anterior view). (**F**) Visceral nerve cord ganglia and visceral nerve (posterolateral right view). (**G**) Ganglia without buccal ganglia (posterior view). Abbreviations: bg, buccal ganglion; bn, cerebro–buccal connective; cc, cerebral commissure; cg, cerebral ganglion; ey, eye; ltn, labial tentacle nerve; og, optic ganglion; osg, osphradial ganglion; osn, osphradial nerve; pag, parietal ganglion; pan, parietal nerve; pg, pedal ganglion; pgc, pedal commissure; plg, pleural ganglion; pn, pedal nerve; rhg, rhinophoral ganglion; rhn, rhinophoral nerve; s, spicule; spn, supraintestinal + parietal nerve; st, statocyst; subg, subintestinal ganglion; supg, supraintestinal ganglion; vg, visceral ganglion; vn, visceral nerve.

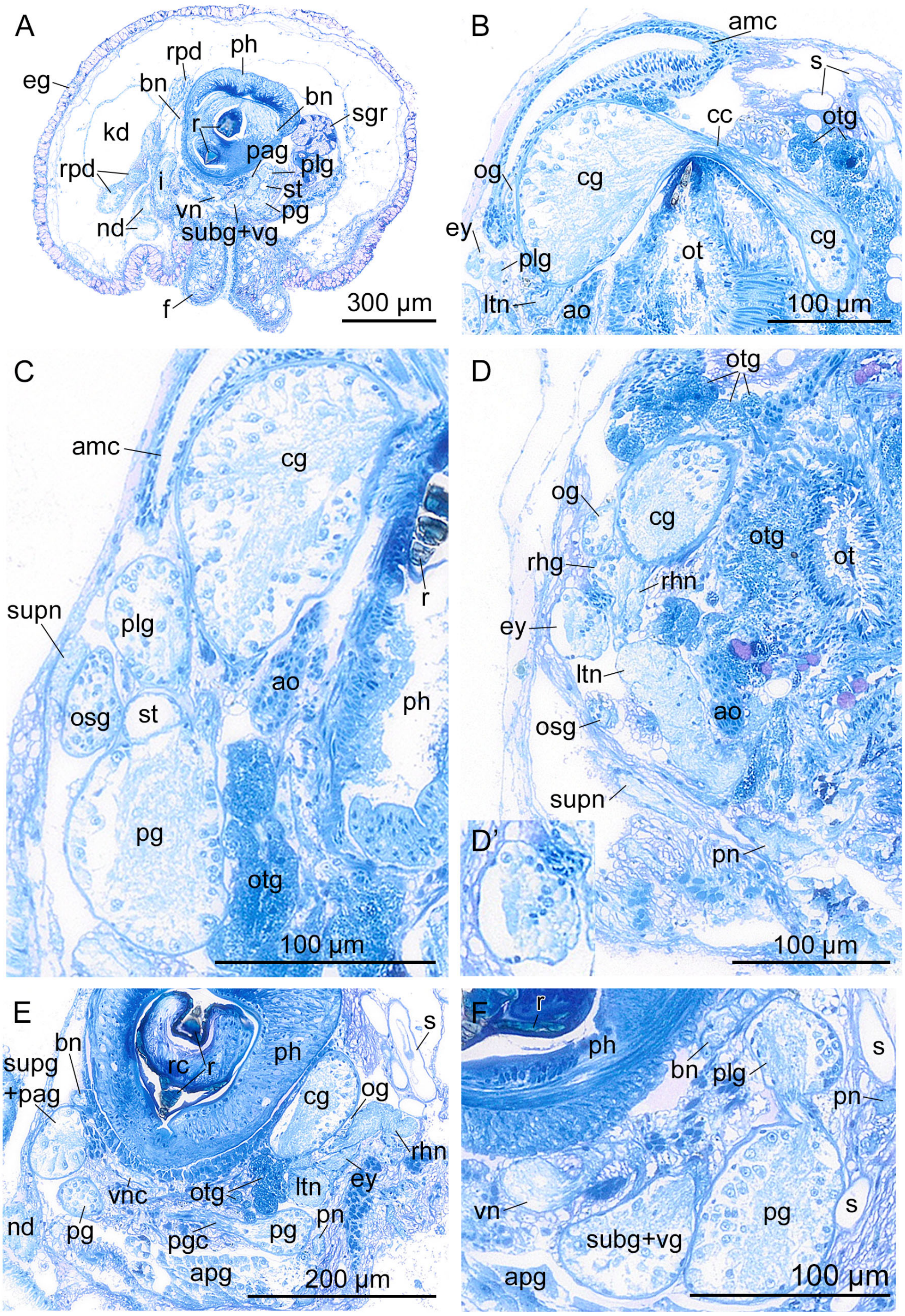

Figure 5. Histological cross-sections of the CNS of *Potamohedyle espinosai* n. gen. n. sp. (**A**) Overview. (**B**) Cerebral ganglia and commissure. (**C**) Nerve ring ganglia and statocyst. (**D**) Optic ganglion and eye. (**E**) Pedal commissure and cerebral nerves. (**F**) Pleural, pedal, and the fused subintestinal + visceral ganglia. Abbreviations: amc, anterior male copulatory organs; ao, aorta; apg, anterior pedal gland; bn, cerebro–buccal connective; cc, cerebral commissure; cg, cerebral ganglion; eg, epidermal gland; ey, eye; f, foot; i, intestine; kd, wide lumen of kidney; ltn, labial tentacle nerve; nd, nephroduct; og, optic ganglion; osg, osphradial ganglion; ot, oral tube; otg, oral tube gland; pag, parietal ganglion; pg, pedal ganglion; pgc, pedal commissure; ph, pharynx; plg, pleural ganglion; pn, pedal nerve; r, radula; rc, radula cushion; rhg, rhinophoral ganglion; rhn, rhinophoral nerve; rpd, renopericardioduct; s, spicule cavity; sgr, right salivary gland; st, statocyst; subg, subintestinal ganglion; supg, supraintestinal ganglion; supn, supraintestinal + parietal nerve; vg, visceral ganglion; vn, visceral nerve; vnc, connective of supg + pag and subg + vg.

The two large cerebral ganglia (~130 µm in diameter each) are connected by a short cerebral commissure (Figures 3, 4C and 5B). The labial tentacle nerve emerges anteroventrally of each cerebral ganglion (Figures 4B,C and 5B,D,E). A rhinophoral nerve emerges more centrally of the anterior side of each cerebral ganglion (Figures 3, 4B,C and 5D,E). The cerebral ganglia, the labial tentacle nerves, and rhinophoral nerves are densely surrounded by spicules (Figure 4D). Only on the right side was a small rhinophoral ganglion (~30 µm in diameter) (Figures 4E and 5D) detected laterally near the base of the rhinophoral nerve. Accessory ganglia are absent. A small optic ganglion (~50 µm in diameter) (Figures 4E–G and 5B,D,E) is situated laterally to each cerebral ganglion. Small, unpigmented eyes (~60 µm in diameter) are slightly beneath the optic ganglia (Figures 4E,F and 5B,D,E). Only on the right side was a thin optic nerve, which connects the optical ganglion and the eye, detected. The paired pedal ganglia (~110 µm in diameter each) (Figures 3, 4E and 5C,F) lie ventrally to the cerebral ganglia and are connected with the latter via short cerebro–pedal connectives. The pedal commissure (Figures 4E and 5E) is slightly longer and thinner than the cerebral commissure. Four pedal nerves (Figures 4B and 5D–F) emerge from each pedal ganglion: two anterodorsally, one anteroventrally, and one posteroventrally. A statocyst (Figures 4G and 5A,C,F) is attached posterodorsally to each pedal ganglion. The static nerve could not be detected. The pleural ganglion (~70 µm in diameter) (Figures 4F,G and 5A,C,F) is located posteriorly to the cerebro–pedal connective and is connected via a short connective to both cerebral and pedal ganglia forming the pre-pharyngeal nerve ring. The visceral nerve cord is connected to each pleural ganglion and lies posteriorly to the pedal commissure. Three distinct ganglia are situated on the visceral nerve cord: the left parietal ganglion (~70 µm in diameter) (Figures 3 and 4F,G), the fused subintestinal/visceral ganglion (~80 µm in diameter) (Figures 4F,G and 5A,F), and the fused supraintestinal/parietal ganglion (~85 µm in diameter) (Figures 4F,G and 5E). The connectives from the left pleural to the parietal ganglion, the parietal to the subintestinal/visceral ganglion, and the supraintestinal/parietal to the right pleural ganglion are short. In contrast, the connective from the subintestinal/visceral to the supraintestinal/parietal ganglion (Figures 4G and 5E) is much longer. A nerve emerges dorsally from the supraintestinal/parietal ganglion (Figures 4F and 5C) and posterodorsally from the left parietal ganglion (Figure 5F). A thick visceral nerve (Figures 4B,F and 5F) emerges from the subintestinal/visceral ganglion and extends into the visceral sac, where it divides into two branches. A presumed osphradial ganglion (~60 µm in diameter) (Figures 4E,G and 5C) is attached to the supraintestinal/parietal ganglion. An osphradial nerve emerges anteriorly (Figure 4F). No osphradium was detected. The paired buccal ganglia (~70 µm in diameter) (Figure 4B,C) are located post-pharyngeally. There is a short buccal commissure (Figure 4B) ventrally to the transition of the pharynx with the oesophagus. The cerebro–buccal connectives are long and thin. Gastro-oesophageal ganglia are absent.

3.3.2. Digestive System

The digestive system of *Potamohedyle espinosai* n. gen. n. sp. consists of the oral tube, oral tube glands, pharynx, radula, salivary glands, oesophagus, digestive gland, and intestine (Figure 6A). The mouth opening lies ventrally between the labial tentacles. The oral tube (Figures 5B and 6A–C) is relatively short and not ciliated. The oral tube glands (Figure 6B,C) are stained uniformly grainy dark blue (Figure 7A). Openings of the oral tube glands were not detected. The voluminous pharynx (Figures 5A,C, 6C–F, and 7B) is composed of complex muscle tissue, with staining varying from dark to bright blue. Posteroventrally, the pharynx tapers into the radula sac (Figures 6F and 8A). The radula lies on the radula cushion (Figure 5E), is hook-shaped (~500 µm) (Figures 5E and 6F), and comprises a longer upper (~300 µm) and shorter lower (~200 µm) ramus (Figure 8A). The radula formula is 40 × 1.1.1, with 25–27 rows in the upper ramus and 13–15 rows in the lower ramus. Each row includes a central rhachidian tooth and one lateral tooth on each side.

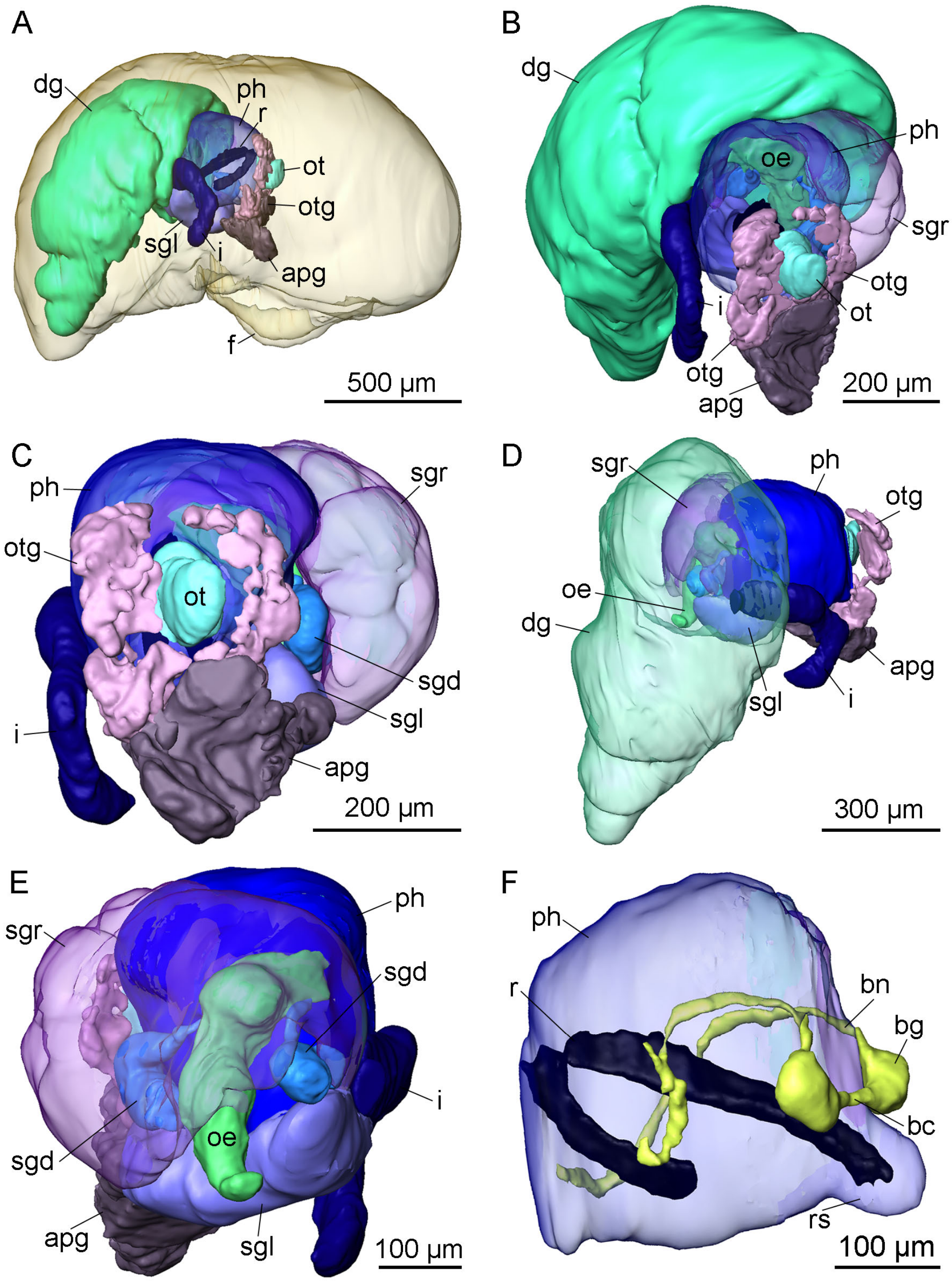

Figure 6. Three-dimensional reconstructions of the digestive system of *Potamohedyle espinosai* n. gen. n. sp. (**A**) Relative position of the digestive system inside the body (right view). (**B**) Complete digestive system (anterolateral view). (**C**) Oral tube glands and anterior pedal gland (anterior view). (**D**) Complete digestive system (posterolateral view). (**E**) Salivary glands and ducts (posterior view, digestive gland omitted). (**F**) Pharynx with radula and buccal ganglia. Abbreviations: apg, anterior pedal gland; bc, buccal commissure; bg, buccal ganglion; bn, cerebro–buccal connective; dg, digestive gland; f, foot; i, intestine; oe, oesophagus; ot, oral tube; otg, oral tube gland; ph, pharynx; r, radula; rs, radula sac; sgd, salivary gland duct; sgl, left salivary gland; sgr, right salivary gland.

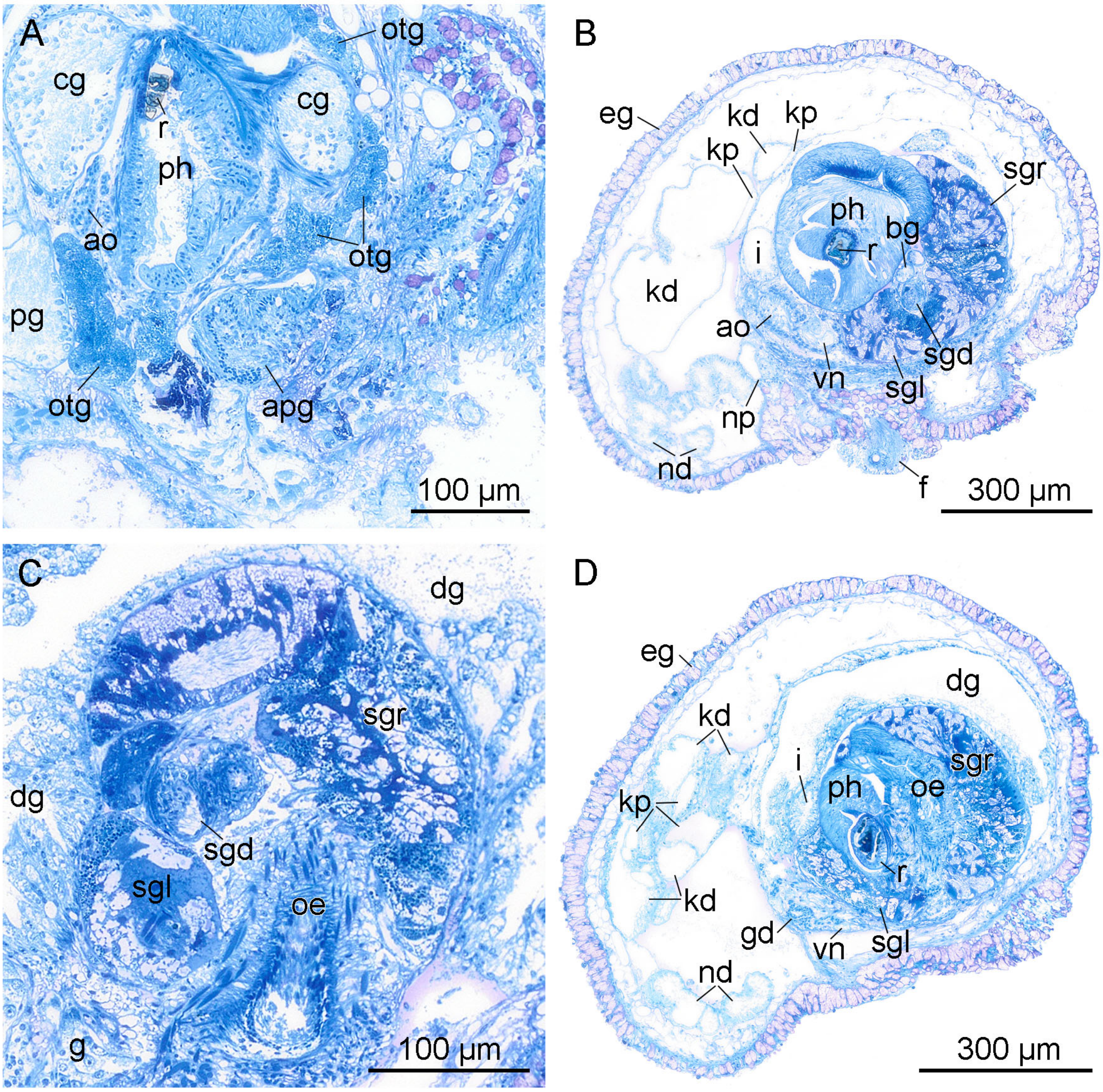

Figure 7. Histological cross-sections of the digestive system of *Potamohedyle espinosai* n. gen. n. sp. (**A**) Oral tube glands and anterior pedal gland. (**B**) Salivary glands and pharynx with radula. (**C**) Salivary glands and duct. (**D**) Digestive gland and intestine. Abbreviations: ao, aorta; apg, anterior pedal gland; bg, buccal ganglion; cg, cerebral ganglion; dg, digestive gland; eg, epidermal gland; f, foot; g, gonad; gd, gonoduct; i, intestine; kd, distal part of kidney; kp, proximal part of kidney; nd, nephroduct; np, nephropore; oe, oesophagus; otg, oral tube gland; pg, pedal ganglion; ph, pharynx; r, radula; sgd, salivary gland duct; sgl, left salivary gland; sgr, right salivary gland; vn, visceral nerve.

The rhachidian tooth (Figure 8C,D) is triangular, with a prominent central cusp and eight to nine lateral denticles (Figure 8D′) on each side. The overlapping, plate-shaped lateral teeth (Figure 8B–D) are elongated with one pointed denticle and a notch (Figure 8D) into which the denticle of the respective posterior tooth fits perfectly. The paired salivary glands (Figures 6C,E and 7B–D) are situated at the posterior end of the pharynx. They have a narrow lumen, and their glandular tissue shows a variety of staining properties, ranging from dark purple/blue to bright purple to grainy blue (Figure 7B,C). The salivary gland ducts form muscular salivary pumps (Figures 6E and 7B,C) and discharge into the pharynx at the transition to the oesophagus. The latter (Figures 6E and 7C,D) extends posterodorsally from the pharynx and connects to the anterior part of the digestive gland (Figure 6D). The sac-like digestive gland (Figures 6A,B,D and 7C,D) tapers posteriorly and occupies large portions of the visceral sac. A distinct histological stomach could not be detected. The short, ciliated intestine (Figures 6A–D and 7B,D) emerges from the digestive gland anterolaterally on the right side. The anus opens dextroventrally slightly anterior to the nephropore to the exterior (Figure 2E).

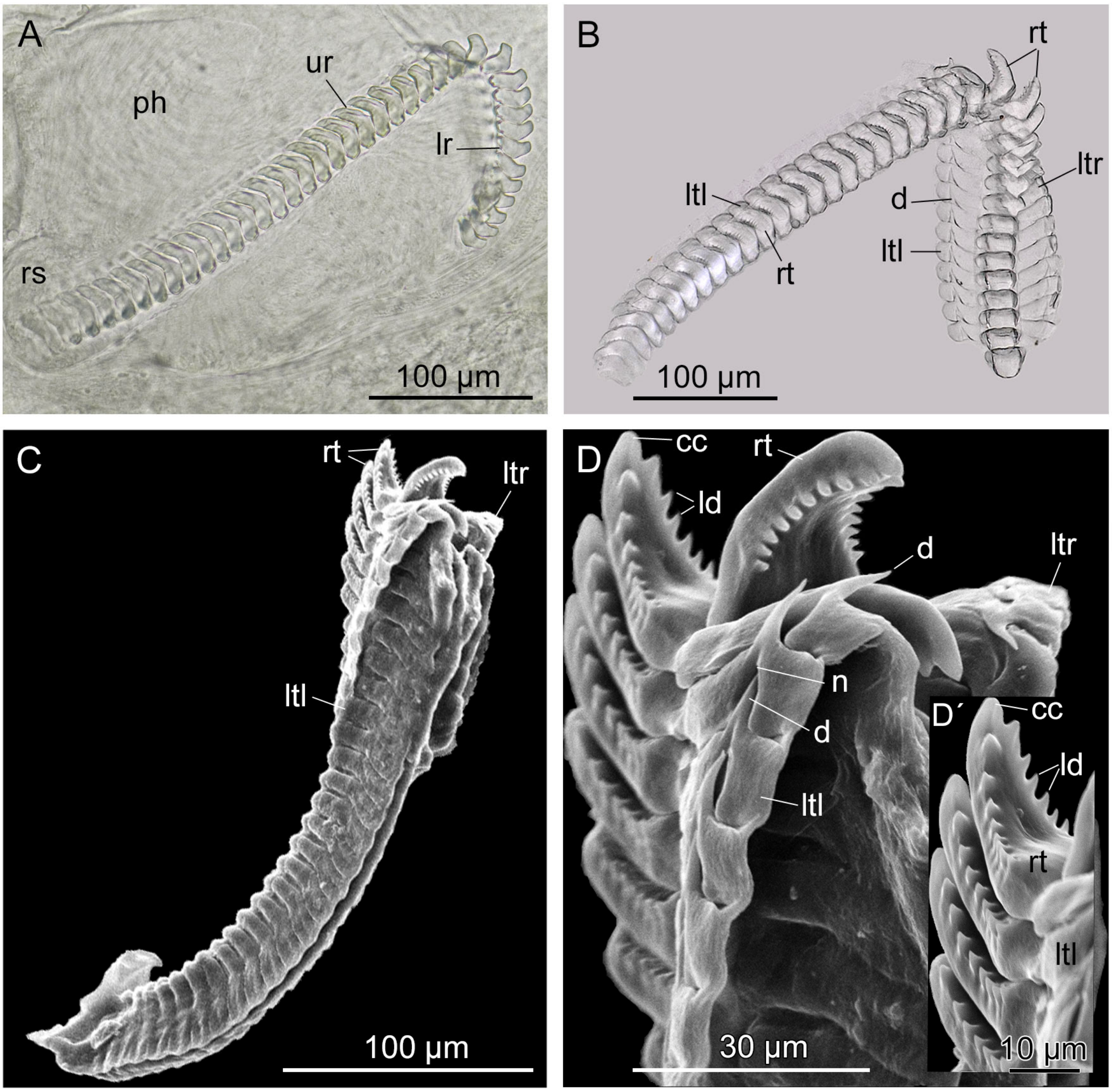

Figure 8. Light microscopic images (**A**,**B**) and SEM micrographs (**C**,**D**) of the radula of *Potamohedyle espinosai* n. gen. n. sp. (**A**) Squash preparation of the pharynx (right view). (**B**) Dissected radula (right view). (**C**) Complete radula (dorsal view). (**D**) Rhachidian teeth and left lateral teeth (dorsal view). (**D′**) Rhachidian teeth. Abbreviations: cc, central cusp; d, denticle; ld, lateral denticle; lr, lower ramus; ltl, left lateral tooth; ltr, right lateral tooth; n, notch; ph, pharynx; rs, radula sac; rt, rhachidian tooth; ur, upper ramus.

3.3.3. Circulatory and Excretory Systems

The circulatory and excretory systems (Figure 9) are situated on the right side (Figure 10A) of the visceral sac and consist of a two-chambered heart surrounded by a pericardium, one to three separated renopericardioducts, a complex kidney, and a long, looped nephroduct.

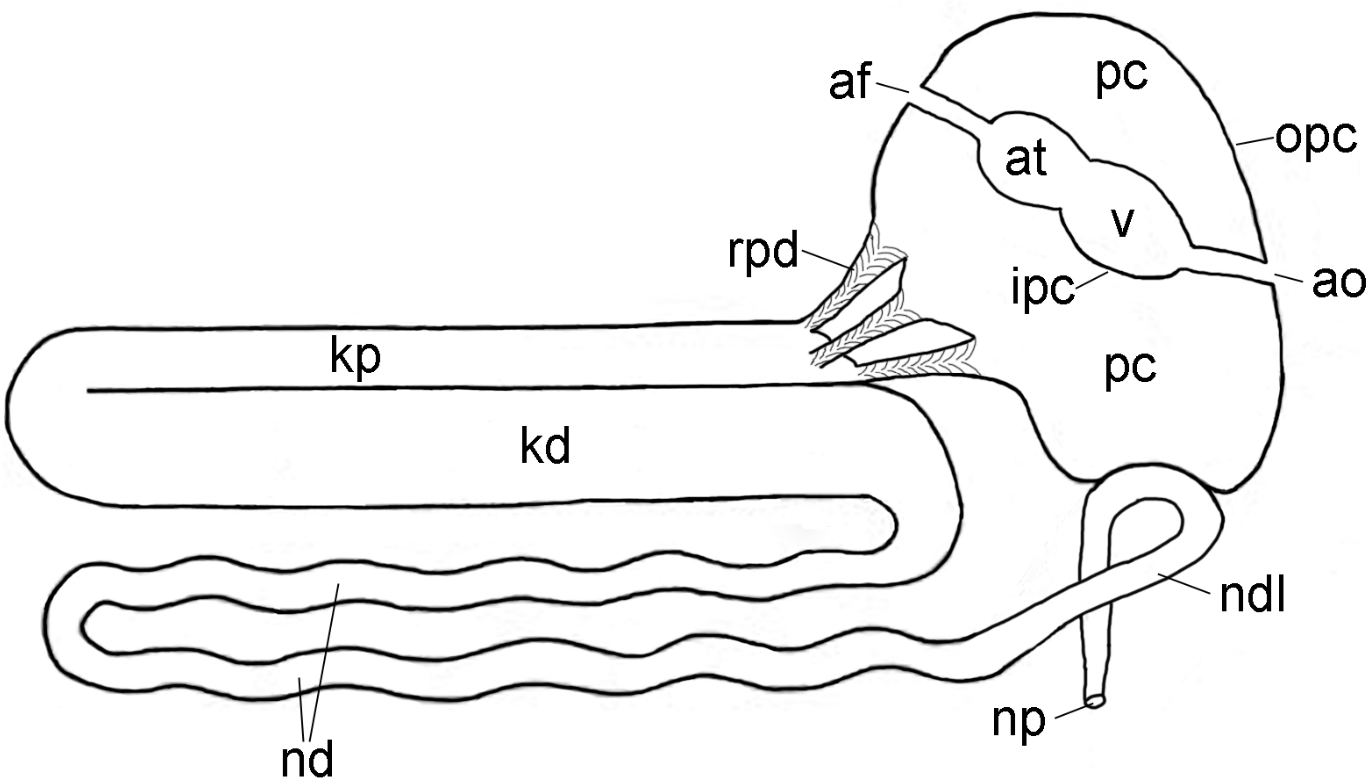

Figure 9. Schematic overview of the circulatory and excretory systems of *Potamohedyle espinosai* n. gen. n. sp. Not to scale. Abbreviations: af, afferent haemolymph flow; ao, aorta; at, atrium; ipc, inner epithelium of the pericardial cavity; kd, distal part of the kidney; kp, proximal part of the kidney; nd, nephroduct; ndl, distal loop of the nephroduct; np, nephropore; opc, outer epithelium of the pericardial cavity; pc, pericardium; rpd, renopericardioduct; v, ventricle.

The two-chambered heart lies anteriorly to the kidney and is divided into an anterior muscular ventricle and a posterior thin-walled atrium (Figures 9 and 10B,D,E). The aorta emerges anteriorly to the heart (Figure 10C,D), extending towards the head, where it bifurcates into two branches approximately in the region of the pedal ganglia. These branches proceed toward the tentacles and terminate in blood sinuses. The aorta, together with the visceral nerve, are surrounded by muscle tissue (Figure 10G,H). The heart is surrounded by a spacious, thin-walled pericardium (Figures 9 and 10D,E). Dorsal vessels are absent. The complex kidney corresponds to the typical acochlidimorph type II kidney, which is internally divided into a proximal part with a narrow lumen and a distal part with a wide lumen (Figure 10C,G,H). Both lumina converge in the posterior part of the kidney. One to three densely ciliated renopericardioducts (Figure 10D,F,H) open separately from the pericardium and discharge into the proximal part of the kidney. One of the three renopericardioducts opens into the narrow lumen of a small, anterodorsal, and isolated part of the kidney, the connection of which to the rest of the kidney was not detected. A long, looped nephroduct (type IIb based on Neusser, Jörger and Schrödl [34]) (Figure 10B,C,G) emerges anteroventrally from the distal kidney. It forms a distal loop (Figures 9 and 10B) lying near the pericardium before opening to the exterior via the nephropore (Figures 2E and 10G), which is positioned dextroventrally and slightly posterior to the anus.

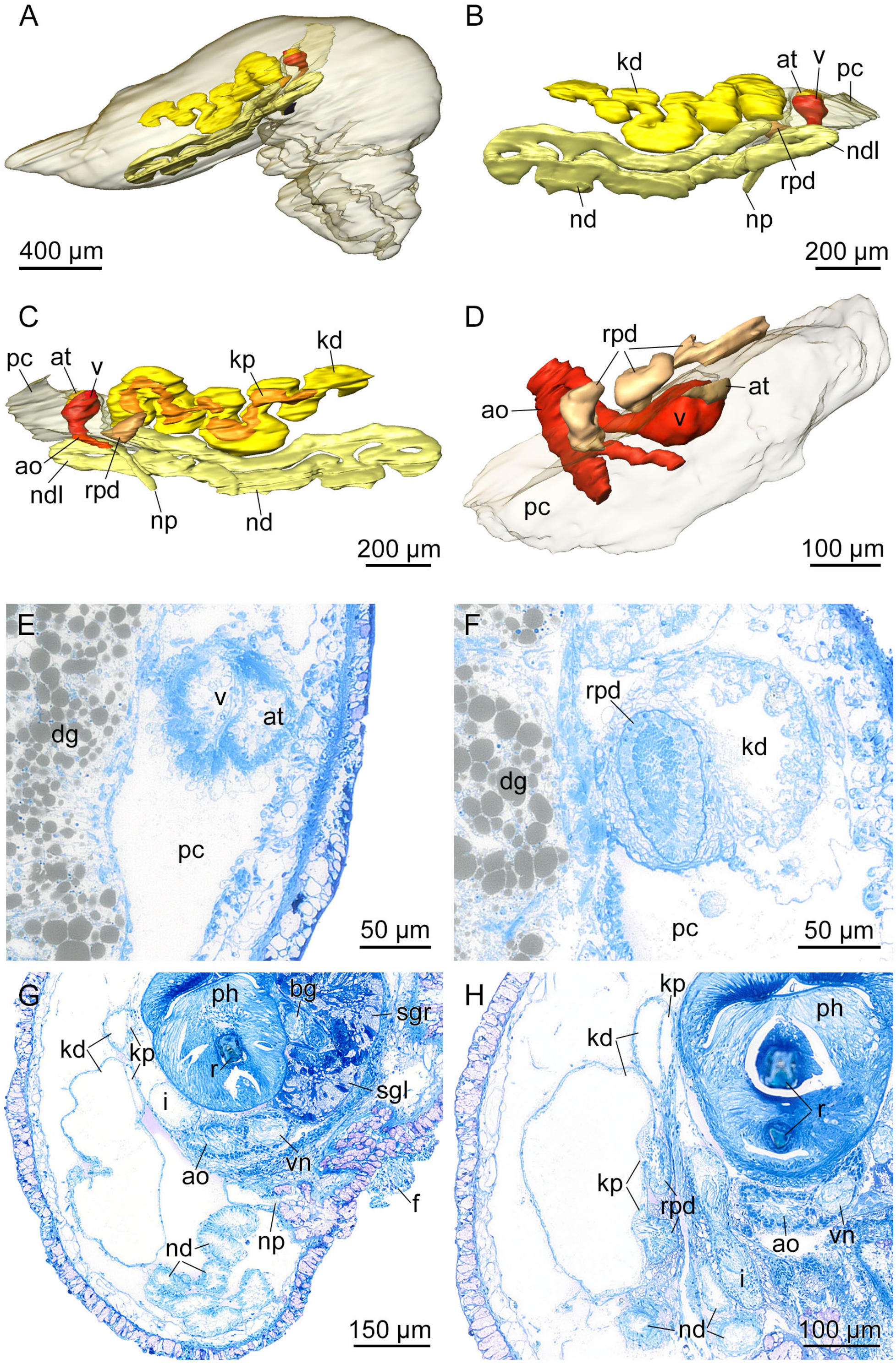

Figure 10. Three-dimensional reconstructions (**A**–**D**) and histological cross-sections (**E**–**H**) of the circulatory and excretory systems of *Potamohedyle espinosai* n. gen. n. sp. (**A**) Relative position of the organ systems in the body (right view). (**B**,**C**) Complete organ system (right/left views). (**D**) Renopericardioducts and pericardium surrounding the heart (right view, tilted forward). (**E**) Ventricle and atrium. (**F**) Renopericardioduct. (**G**) Looped nephroduct and nephropore. (**H**) Kidney. Abbreviations: ao, aorta; at, atrium; bg, buccal ganglion; dg, digestive gland; f, foot; i, intestine; kd, distal part of the kidney; kp, proximal part of the kidney; nd, nephroduct; ndl, distal loop of the nephroduct; np, nephropore; pc, pericardium; ph, pharynx; r, radula; rpd, renopericardioduct; sgl, left salivary gland; sgr, right salivary gland; v, ventricle; vn, visceral nerve.

3.3.4. Reproductive System

Potamohedyle espinosai n. gen. n. sp. is a sequential hermaphrodite. The reconstructed specimen herein shows fully developed male reproductive organs with the female organs in early stages of development.

The reproductive system (Figure 11) is divided into the anterior male copulatory organs (Figures 2D, 12A and 13A,B), situated in the head on the right side of the body, and a posterior part consisting of the female reproductive organs, sperm-storing receptacles, and the gonad. The anterior and posterior reproductive organs are connected by a subepidermal vas deferens (Figure 12B).

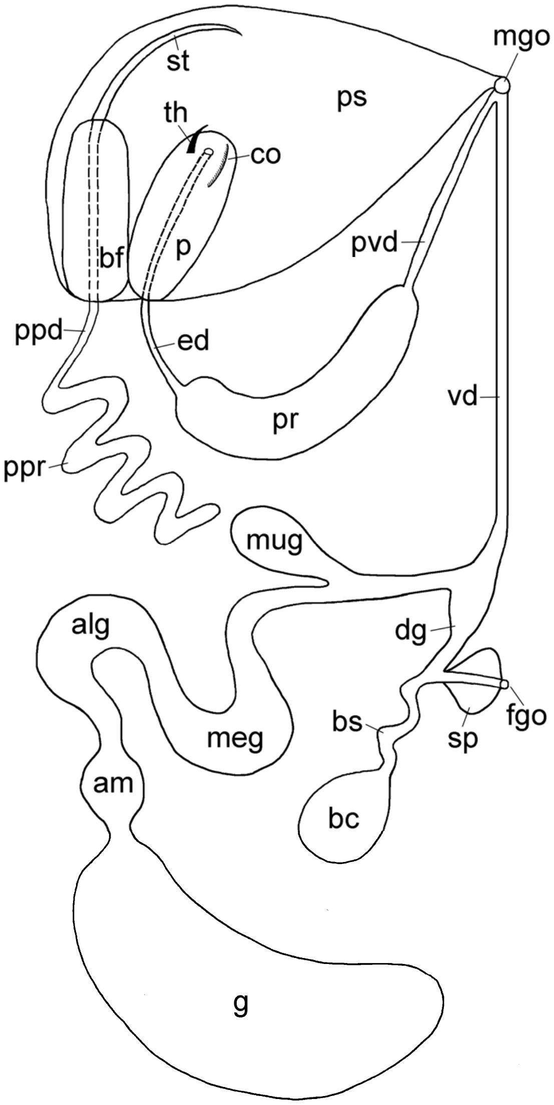

Figure 11. Schematic overview of the reproductive system of *Potamohedyle espinosai* n. gen. n. sp. Not to scale. Abbreviations: alg, albumen gland; am, ampulla; bc, bursa copulatrix; bf, basal finger; bs, bursa stalk; co, comb-like structure; dg, distal gonoduct; ed, ejaculatory duct; fgo, female gonopore; g, gonad; meg, membrane gland; mgo, male gonopore; mug, mucous gland; p, penis; pr, prostate; ppd, paraprostatic duct; ppr, paraprostate; ps, penial sheath; pvd, posterior-leading vas deferens; sp, sphincter; st, stylet on basal finger; th, thorn on penis; vd, vas deferens.

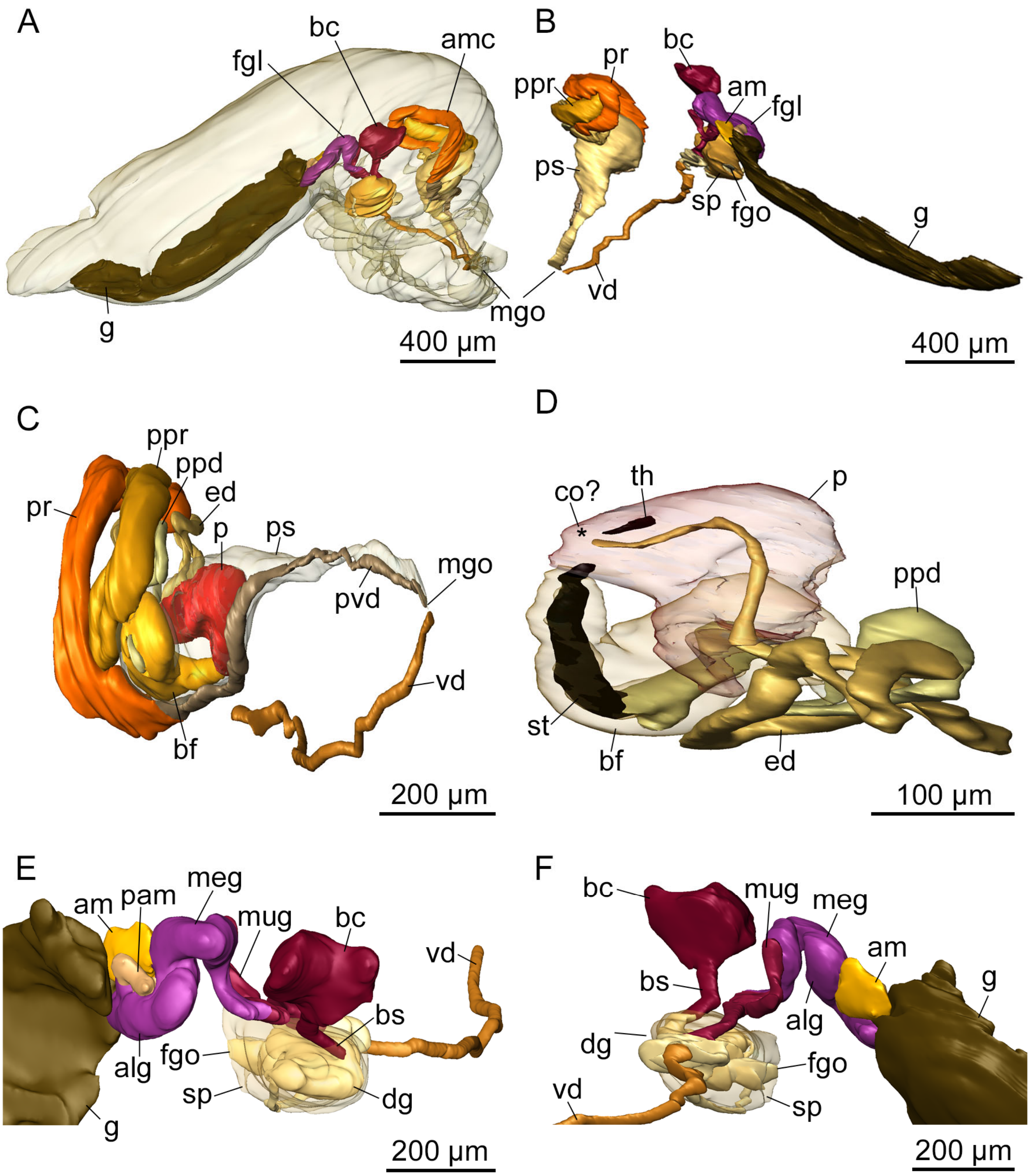

Figure 12. Three-dimensional reconstructions of the reproductive system of *Potamohedyle espinosai* n. gen. n. sp. (**A**) Position of the reproductive system in the body (right view). (**B**) Complete organ system (left view). (**C**) Anterior male copulatory organs (right view). (**D**) Penis and basal finger with stylet. (**E**,**F**) Posterior reproductive system (right/left views). Abbreviations: alg, albumen gland; am, ampulla; amc, anterior male copulatory organs; bc, bursa copulatrix; bf, basal finger; bs, bursa stalk; co?, putative position of comb-like structure; dg, distal gonoduct; ed, ejaculatory duct; fgl, female gland; fgo, female gonopore; g, gonad; meg, membrane gland; mgo, male gonopore; mug, mucous gland; p, penis; pam, post-ampullary gonoduct; pr, prostate; ppd, paraprostatic duct; ppr, paraprostate; ps, penial sheath; pvd, posterior-leading vas deferens; sp, sphincter; st, stylet on basal finger; th, thorn on penis; vd, vas deferens.

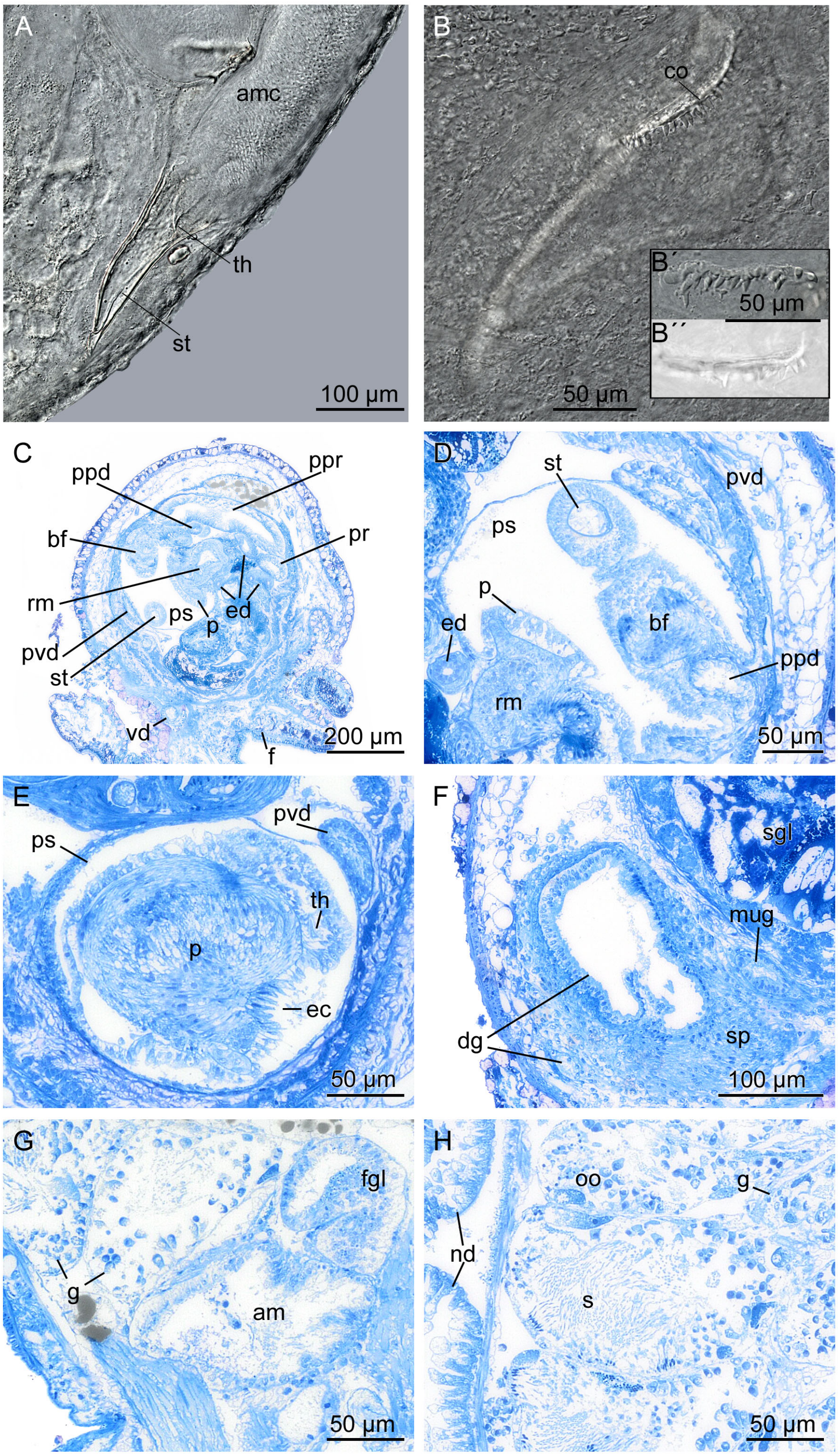

Figure 13. Squash preparations (**A**,**B**) and histological cross-sections (**C**–**H**) of the reproductive system of *Potamohedyle espinosai* n. gen. n. sp. (**A**) Anterior male copulatory organs. (**B**,**B′**,**B″**) Comb-like structure. (**C**) Anterior male copulatory organs. (**D**) Basal finger and stylet. (**E**) Penis with thorn. (**F**) Distal gonoduct with sphincter. (**G**) Ampulla. (**H**) Gonad follicles. Abbreviations: am, ampulla; amc, anterior male copulatory organs; bf, basal finger; co, comb-like structure; dg, distal gonoduct; ec, elongated cells; ed, ejaculatory duct; f, foot; fgl, female gland; g, gonad; mug, mucous gland; nd, nephroduct; p, penis; pr, prostate; ppd, paraprostatic duct; ppr, paraprostate; ps, penial sheath; pvd, posterior-leading vas deferens; rm, retractor muscle of the penis; sgl, left salivary gland; sp, sphincter; st, stylet on basal finger; th, thorn on penis; vd, vas deferens.

The ovotestis is sac-like and composed of follicles where both sperm cells and oocytes are produced (Figures 12B and 13G). A pre-ampullary gonoduct connects to the tube-like ampulla (Figure 12E,F). The epithelium of the ampulla is thin and non-glandular (Figure 13G). At the time of examination, the ampulla lacked sperm cells and there was no developed receptaculum seminis. A post-ampullary gonoduct leads to three female nidamental glands: the albumen gland, membrane gland, and mucus gland, from proximal to distal, respectively (Figure 12E,F). The albumen gland and membrane gland are tube-like, while the mucus gland is sac-like (Figure 12E,F). These glands are not fully developed yet; therefore, they all are of a glandular tissue, though no histological differences were detected (Figure 13F,G).

The mucus gland then leads to the distal gonoduct (Figure 12E,F). The bursa copulatrix is connected to the distal gonoduct by the bursa stalk (Figure 12F). The distal gonoduct passes through a muscular sphincter (Figure 12E,F) and opens to the exterior via the female gonopore (Figure 2E) located dextroventrally anterior to the anus and nephropore. The subepidermal vas deferens emerges from the distal gonoduct (Figure 12E,F) and extends to the head region, connecting the posterior reproductive system to the anterior male copulatory organs (Figure 12C,D). Slightly posterior to the male genital opening, positioned at the base of the right rhinophore, the vas deferens connects to the posterior leading vas deferens (Figures 12C and 13D,E), leading to the prostate (Figure 12C). The latter consists of glandular tissue (Figure 13C) through which the ejaculatory duct continues. The tubular ejaculatory duct leads into the muscular penis and opens at its tip (Figure 12D). The retractor muscle (Figure 13C) is located on the left side of the penis. A hollow penial stylet is absent, but near the tip of the penis, there is a small solid thorn approximately 40 µm in length (Figures 12D and 13E). Attached to the base of the penis there is a muscular basal finger (Figures 12C and 13C,D) with a retractable hollow stylet measuring 170 to 210 µm. The sac-like paraprostate (Figure 12C) is connected to the basal finger via the paraprostatic duct (Figures 12D and 13C,D). In addition to the hollow stylet of the basal finger and the solid thorn of the penis, there is a third cuticular structure on the anterior male copulatory organs that is visible in the squash preparations (Figure 13A,B): a comb-like structure (~85 µm) with several small thorns (Figure 13B,B′,B″). However, this structure was not detected in the histological section series and therefore was not reconstructed. Near the thorn of the penis, there are elongated cells forming a semicircle containing large dark blue nuclei (Figure 13E), which are possibly remnants of the comb-like structure. The penis and the basal finger are enclosed within the thin-walled penial sheath (Figure 12C,D and Figure 13C–E), which opens to the exterior via the male gonopore at the base of the right rhinophore.

3.4. Phylogenetic Placement

Potamohedyle espinosai n. gen. n. sp. clusters within a clade containing all known species of acochlidimorph freshwater slugs (*Tantulum* not included), as well as interstitial marine and brackish Pseudunelidae. In rooted RAxML analyses defining the three Microhedylacea as outgroups, *Strubellia* is sister to a clade including *Potamohedyle* and all representatives of Acochlidiidae and Pseudunelidae. In this phylogenetic hypothesis, *Potamohedyle* is sister to the clade *Pseudunela*+ (*Wallacellia*+(*Palliohedyle* + *Acochlidium*) (see Figure 14). All known genera are monophyletic with high bootstrap support (BS). However, relationships among genera are largely not well-supported, and the inclusion of *Potamohedyle* in the phylogenetic analyses of acochlidimorph Hedylopsacea renders Acochlidiidae (currently combining *Strubellia*, *Wallacellia*, *Palliohedyle* and *Acochlidium*) paraphyletic due to the exclusion of *Strubellia*. In unrooted IQtree analyses, the relationships among the genera are slightly different: *Potamohedyle espinosai* n. gen. n. sp. is sister to *Strubellia* (however, with no significant BS) and (*Potamohedyle* + *Strubellia*) are sister to a clade composed of *Pseudunela* + (*Wallacellia* + (*Palliohedyle* + *Acochlidium*). In single-gene trees of the two fast-evolving mitochondrial markers, *Potamohedyle* is sister to marine/brackish

Pseudunelidae (based on 16S rRNA, pairwise identity 83.97–87.91%) or to *Strubellia* (based on COI, pairwise identity 78.26–82.46%).

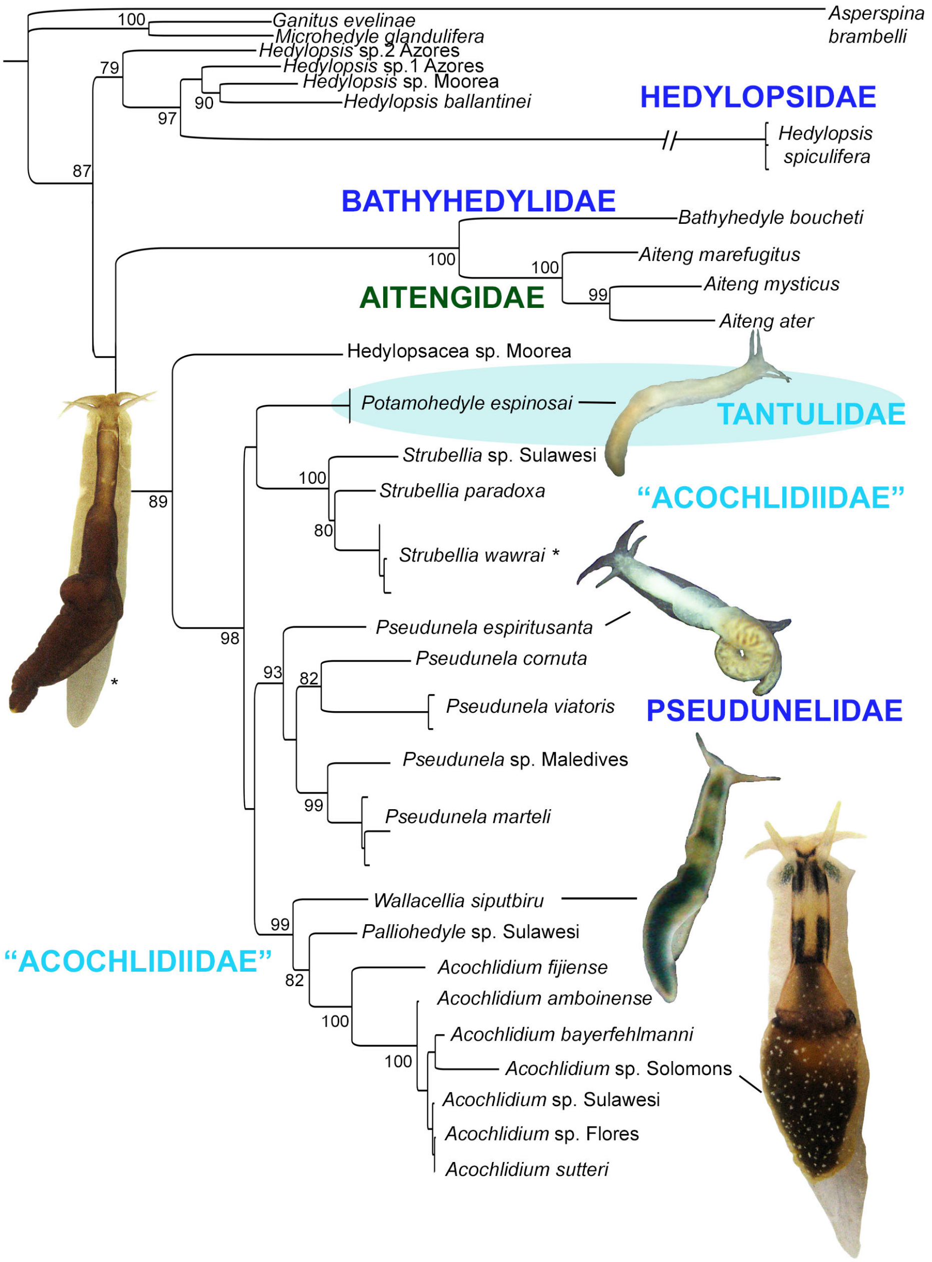

Figure 14. Maximum likelihood tree of Hedylopsacea based on the concatenated four marker data set (18S rRNA, 28 rRNA, 16S rRNA and COI). Bootstrap values (BS) above 75 are given.

4. Discussion

4.1. Anatomical Resemblance and Peculiarities of *Potamohedyle espinosai* n. gen. n. sp.

Potamohedyle espinosai n. gen. n. sp. externally most closely resembles minute and interstitial *Tantulum* and representatives of the genus *Pseudunela* (see Figure 14), but differs to those in its larger body size, which refers to its benthic opposed to interstitial lifestyle. In contrast to the other benthic slugs in limnic systems, it lacks body coloration, which is otherwise used to camouflage the slugs in their environment. Furthermore, *Potamohedyle* only has rudimentary, i.e., pigment-lacking and externally not visible, eyes, which is otherwise only known from interstitial species among Acochlidimorpha. With this external morphology, it seems well adapted to a new microhabitat for freshwater slugs, a life on the underside of stones and among the surrounding mud, and is unlikely to venture along the light-exposed sides of the volcanic gravel in the river.

Concerning its sensory and nervous features, *Potamohedyle espinosai* n. gen. n. sp. shows some peculiarities in comparison to its closed relatives, which also might be related to the differences in lifestyle. In contrast to the other tantulid *Tantulum elegans,* it bears an osphradial ganglion and lacks accessory ganglia. An osphradial ganglion is typically found in aquatic hedylopsacean Acochlidimorpha [34,35,47], but is absent in (semi-)terrestrial Aitengidae [48], in interstitial and limnic *Tantulum* (but see the discussion below), as well as in all marine interstitial microhedylacean Acochlidimorpha. The corresponding chemosensory organ—the osphradium—is so far only described for limnic *Strubellia wawrai* as an inconspicuous pit at the right side of the head–foot complex [35], but is also likely present in *Acochlidium* and simply might have been overlooked in smaller-sized Hedylopsacea. The absence of an osphradium and osphradial ganglion in (semi-)terrestrial Aitengidae is unsurprising, as in other gastropod clades, the fate of this typical molluscan sensory organ, which is probably plesiomorph at least for Gastropoda [49], is related to the habitat choice. Within gastropods, its occurrence is limited to aquatic taxa, in which it is originally found in the mantle cavity anterior to the gills (originally as a paired organ (e.g., in Patellogastropoda), while in the evolutionary context of torsion, it is reduced to a single chemosensory organ) and has been lost various times independently in the transitions of different groups of gastropods to land [50]. No osphradial ganglion was detected in limnic *Tantulum*; however, it possesses an additional ganglion attached to the supraoesophageal ganglion that was interpreted as penial ganglion innervating the male copulatory organ [28]. This interpretation was based on the emerging nerve that leads towards the copulatory organ, but its destination might have been misinterpreted [35], leading to a potentially erroneous conclusion on the absence of an osphradial ganglion. Based on current knowledge, the presence of an osphradial ganglion is likely diagnostic for the family Tantulidae. The previous misinterpretation of the tantulid osphradial ganglion was also driven by the hypothesis of Edlinger [51] that the gastropod osphradium was functionally replaced by another olfactory structure—the Hancock's organ—in opisthobranch gastropods. Therefore Neusser and Schrödl [28] hypothesized that in *T. elegans*, the osphradium was replaced by the Hancock's organ. Studies of *Strubellia* [35], as well as *Pseudunela* [34,52], have revealed, however, that both sensory organs—the Hancock's organ and the osphradium—can co-occur, which shows that they are not homologous and likely have different origins.

The function of the osphradium was long debated, and a variety of putative functions were proposed (for a review, see [49]). Originally, the osphradium was considered an organ for testing the water [53]. Later, Hulbert and Yonge [54] assumed that the organ was involved in the detection of the amount of sediment swept into the mantle cavity. Further studies suggested functions related to, e.g., the location of food sources [55], the detection of O_2 and CO_2 levels in lymnaeid snails [56], or the synchronization of spawning and reproductive behaviour [57]. The morphology and ultrastructure of the osphradium was thoroughly investigated [57–59], which revealed, according to Taylor and Miller [59], that the morphology of the osphradium in caenogastropods is influenced by the diet and life habits, e.g., simple osphradia are prevalent in herbivores, while carnivorous/predatory species have large and complex osphradia. At first glance, this interpretation seems unlikely in the acochlidimorph *Strubellia wawrai* in which the osphradium is considered as simple because it is a small pit with a dense microvillous border, but the species feed on protein-rich egg capsules [35]. However, a similar "simple" osphradium as in herbivores might be sufficient for food detection in an oophagous slug feeding on stationary and immobile egg capsules. More studies on Acochlidimorpha are needed for a better understanding of the presence and sensory function of the mostly still undetected or at least inconspicuous osphradium.

There is another difference in cerebral features between both species of Tantulidae. While precerebral accessory ganglia are absent in *Potamohedyle espinosai* n. gen. n. sp. (as in all hedylopsacean acochlidimorphs, regardless their body size and habitat), they are present in small, interstitial *Tantulum elegans*. These nervous aggregations were reported for numerous minute, marine mesopsammic slugs, such as microhedylacean acochlidimorphs [26], cephalaspideans [60], rhodopemorphs [61], sacoglossan *Platyhedyle* [62], and pseudovermid nudibranchs [63]. Accessory ganglia thus evolved multiple times independently and were discussed as special adaptations to the interstitial habitat, providing an additional nervous capacity in slugs with a minute body size [61]. Accordingly, precerebral accessory ganglia might be an apomorphy for marine interstitial microhedylacean Acochlidimorpha and evolved a second time independently in small, interstitial limnic *Tantulum*.

While the digestive, circulatory, and excretory systems of *Potamohedyle* are best discussed in an ecological context and provide little diagnostic characters for taxonomy and systematics, the anatomical characters of the reproductive system are most promising for hypotheses on the systematic relationships of *Potamohedyle*. The reproductive system of *Potamohedyle espinosai* n. gen. n. sp. resembles that of limnic *Tantulum, Strubellia* and *Acochlidium* in being a sequential (protandric) hermaphrodite [28,35,64], whereas *Pseudunela* and *Wallacellia* are simultaneous hermaphrodites [23,52].

Within Acochlidimorpha, there is a clear differentiation of reproductive organs and therefore related reproductive strategies. While all marine interstitial Microhedylacea are aphallic and sperm transfer occur by spermatophores [65], an internal vas deferens and cephalic male copulatory organs evolved at the base of Hedylopsacea and reproductive organs became more complex along the hedylopsacean stemline. Deep-sea Bathyhedylidae and (semi)terrestrial Aitengidae possess a simple penis and sperm transfer happens by copulation [36,48]. While marine interstitial *Helicohedyle* retain the simple penis, in Hedylopsidae, a paraprostate evolved as a second gland in addition to the prostate, and both discharge into a hollow penial stylet, suggesting hypodermic injection as mode of sperm transfer [66]. Freshwater acochlidimorphs developed a basal finger injection system, and penises are equipped with more or less elaborated apical cuticular structures. *Strubellia* shares the open external sperm groove with *Hedylopsis,* and the penis is equipped with a basal thorn, probably functioning as coupling device during copulation [35]. Copulation is the mode of sperm transfer in Tantulidae as well. Based on our phylogenetic hypothesis (see Figure 14), interstitial *Tantulum* has secondarily lost both the basal finger injection system and the penial apical cuticle. In contrast, *Potamohedyle* has a penial thorn and evolved a cuticular comb-like structure, similar to one described in *Wallacellia* [23]. After the split of Tantulidae, a hollow penial stylet is present in all remaining hedylopsaceans, i.e., *Pseudunela*, *Wallacellia*, *Palliohedyle,* and *Acochlidium*, and based on current knowledge, hypodermic injection is the exclusive mode of sperm transfer. Limnic *Wallacellia*, *Palliohedyle,* and *Acochlidium* evolved complex, elaborated male copulatory organs with the presence of a large "rapto-penis" with semicircles of spines and thorns functioning as a grappling organ in *Acochlidium* [64]. In summary, *Potamohedyle* stands at the beginning of a 'copulatory arms race' of benthic living limnic acochlidimorphs. Table 3 summarizes the main taxonomic anatomical characters of *Potamohedyle espinosai* n. gen. n. sp., comparing it to its closed relatives among freshwater and brackish Hedylopsacea. *Potamohedyle* n. gen. n. sp. shows a unique combination of characters, which justifies the erection of the new genus. Based on the geographic vicinity and our morphological and microanatomical data, *Potamohedyle* best fits within the family Tantulidae. Unfortunately, our taxonomic placement can currently not be backed-up by molecular data, since *Tantulum elegans* is unavailable for molecular studies. Discrepancies to *Tantulum elegans*, i.e., the larger body size, the multiplication of renopericardioducts, and the presence of a cuticular comb associated with the copulatory organ, can be explained as individual adaptations to different habitats and therefore lifestyles and reproductive strategies. The *Tantulum elegans* anatomy shows several modifications and simplifications [27,28], which can be interpreted as secondary adaptations to its interstitial lifestyle and miniaturization. In consequence, the diagnosis of the family Tantulidae, originally established by Rankin in 1979, is herein modified and specified (see Section 3.1).

Table 3. Comparison of the internal anatomy of different freshwater and brackish acochlidimorph species.

	***Potamohedyle espinosai* n. gen. n. sp.**	***Tantulum elegans***	***Pseudunela cornuta***	***Pseudunela espiritusanta***	***Strubellia paradoxa***	***Strubellia wawrai***	***Wallacellia siputbiru***	***Acochlidium amboinense***	***Acochlidium bayer-fehlmanni***	***Acochlidium fijiense***
Data source	present study	[27,28]	[67,68]	[52]	[69,70]	[35]	[23]	[71]; §	[72–74]; §	[64,75]; §
Accessory ganglia	-	+	-	-	-	-	-	?	?	?
Eyes	unpigmented	unpigmented	unpigmented	pigmented	pigmented	pigmented	pigmented	pigmented	pigmented	pigmented
Osphradial ganglion	+	+	+	+	+	+	+	?	?	?
Gastro-oesophageal ganglia	-	+	+	+	+	+	+	?	?	?
Salivary pumps	+	+	-	-	-	+	+	?	?	?
Radula formula	40 × 1.1.1	1.1.2	50 × 1.1.1.	67 × 1.1.2	38 × 1.1.2	40–60 × 1.1.2	40 × 1.1.2	52 × 2.1.2	56 × 1.1.2	50 × 1.1.2
Digestive gland	sac-like	sac-like	sac-like	sac-like	sac-like	sac-like	sac-like	diverticular	diverticular	diverticular
N° renopericardioducts	1–3	1	1	1	1	1	1	up to 25	up to 42	up to 8
Dorsal vessels	-	-	-	-	-	-	-	+	+	+
Hermaphrodite genital system	sequential, protandric	sequential, protandric	simultaneous	simultaneous	sequential, protandric	sequential, protandric	simultaneous	?	?	sequential, protandric
Internal vas deferens	+	+	+	+	-	-	+	+	+	+
External sperm groove	-	-	-	-	+	+	-	-	-	-
Penial stylet [µm]	-	-	~600	~80	-	-	~1600	?	+	+
Penial apical cuticle	thorn (40 µm), comb	-	-	-	thorn (50 µm)	thorn (150 µm)	comb	+	thorn	thorn
Bulb underlying penis	-	-	-	-	-	-	+	-	-	-
Basal finger stylet [µm]	170–210	-	~110	~340	~600	~750	~200	?	+	+
Grappling organ	-	-	-	-	-	-	+	+	+	+

Abbreviations: + present; - absent; § own unpublished data; ? no data available.

4.2. Ecology of Freshwater Slugs

Potamohedyle espinosai n. gen. n. sp. lives on the underside of volcanic stones near the river mouth and up to 300 m upstream. Based on the habitat and the co-occurring malacofauna, it can be assumed that it can tolerate a temporary saltwater impact (possibly during spring tides and storms), but it usually inhabits freshwater. It shares this benthic freshwater habitat with limnic Acochlidiidae in the Indo-Pacific, which are found on the underside of stones in coastal rivers [23,35,76]. For future ecological surveys, a genus level identification key to freshwater slugs is provided in Supplementary File S1.

Adaptations in the circulatory and excretory systems play crucial roles in the transition from marine to limnic systems and are thus of major evolutionary importance. Like other freshwater Acochlidimorpha, *Potamohedyle espinosai* n. gen. n. sp. shows complex, highly efficient circulatory and excretory systems adapted to life in freshwater. Typically, for marine euthyneurans, the exclusively marine microhedylacean Acochlidimorpha have a simple, small, sac-like kidney with a short nephroduct (termed type I in [34]). In contrast, *Potamohedyle espinosai* n. gen. n. sp. possesses the type of excretory system typical of all limnic and (at least temporary) brackish acochlidimorph taxa described in detail (*T. elegans*, *P. cornuta*, *P. espiritusanta*, *S. paradoxa*, and *S. wawrai*) (termed type IIb in [34]) that is characterised by a complex, internally divided kidney and a long, looped nephroduct. A similar complex kidney together with a short nephroduct is found in close marine relatives, such as *Hedylopsis*, *Pseudunela marteli*, and *Pseudunela viatoris* (termed type IIa in [34]). A long, looped nephroduct is generally interpreted to enhance salt reabsorption from the primary urine before excretion [77]. Thus, the complex kidney and the long, looped nephroduct in freshwater and brackish acochlidimorphs can be seen as unique adaptations to their habitat or at least as pre-requisites for a habitat transition [34,52]. Due to osmotic pressure, slugs in freshwater are exposed to constant water inflow and must secrete high amounts of diluted urine. Representatives of freshwater Acochlidimorpha manage this via their long nephroduct.

Surprisingly, *Potamohedyle espinosai* n. gen. n. sp. has one to three ciliated renopericardioducts, which open from the pericardium into the proximal part of the kidney. Usually, gastropods have one pair of renopericardioducts [78]. Most acochlidimorph species possess only one renopericardioduct, e.g., all Microhedylacea and hedylopsacean *Tantulum elegans*, *Pseudunela*, *Strubellia*, and *Wallacellia* [23,28,34,35,52]. In contrast, representatives of the genus *Acochlidium* show multiplications of renopericardioducts, culminating in 42 renopericardioducts in *A. bayerfehlmanni* [74]. An increased number of renopericardioducts was recently discussed as an adaptation to the limnic habitat, as more ducts generally provide a faster flow rate of the primary urine [74]. However, not enough data are available comparing the number of renopericardioducts in juveniles and adults. Thus, it is unclear if the number of renopericardioducts increases in older specimens, if it is a species-specific feature, or a combination of both. In one subadult specimen of *Potamohedyle espinosai* n. gen. n. sp., a separated small part of the kidney with one renopericardioduct is present without a connection to the rest of the kidney. This might hint at the successive development of renopericardioducts during ontogeny. However, the ratio of body surface and volume is more inconvenient in smaller slugs or juveniles than in larger specimens, leading to greater osmotic stress. Comparative analyses of different ontogenetic stages are needed to reveal strategies for overcoming osmotic stress in small slugs and juveniles.

Snails co-occurring with *Potamohedyle espinosai* n. gen. n. sp. in the same habitat belong to the hygrophilan Planorbidae (1 sp.), caenogastropod Thiaridae (1 sp.), neritimorph Neritidae (2 ssp.), and Neritiliidae (2 ssp.). While Planorbidae and Thiaridae only inhabit freshwater systems, Neritimorpha occur in marine, freshwater, and terrestrial environments. The kidney in Neritimorpha is a similarly complex, tubular organ subdivided into a proximal glandular portion in which the renopericardioduct discharges and a non-glandular distal region, the so-called bladder, which empties into a short ureter [79]. Little [80] emphasized that the circulatory and excretory systems of freshwater neritids are remarkable similar to those of marine representatives in morphology but greatly differ in function, as salts are removed from the urine by the glandular part of the kidney, producing hyposmotic urine. Thirty-two gastropod snail families have invaded freshwater systems [24], whereas Acochlidimorpha comprise the only freshwater slugs with just a few species. Apparently, snails are more successful in invading freshwater habitats than slugs. This might be due to the presence of a more or less robust shell and, additionally in some snails, the operculum, which protects the animal from high osmotic stress. Slugs lacking these protective structures are more susceptible to water inflow and consequently must evolve specialized morphological structures, as discussed above. Furthermore, slugs without a protective shell and operculum are more prone to the risk of predation. Large benthic Acochlidiidae probably avoid predation by camouflaging, while smaller and colourless *Potamohedyle* hide under stones and in the sediment.

Most freshwater snails are herbivorous, feeding on macrophytes via grazing or rasping off parts of the substrate with their radulae or additionally using their jaws to rip off parts of leaves or filaments [81]. Many of these snails seem to be rather unspecific grazers and can, next to their favourite plants and algae, also feed on fungal hyphae, detritus, or bacteria. Among panpulmonate Viviparidae and Bithyniidae, facultative filter-feeding of the breathing water has evolved in addition to grazing on epiphytic diatoms using their radula [81]. Furthermore, among prosobranch Nassariidae, carnivorous predators, such as *Anentome helena*—a complex of species—can occur, which can present a serious threat to the native gastropod fauna as introduced species [82]. No direct feeding observations are available for *Potamohedyle espinosai* n. gen. n. sp., but this new freshwater slug shows a digestive system typical of carnivorous gastropods, having large salivary glands and a short, straight intestine [83]. Additionally, it is characterized by the presence of muscular pumps that are located distally at the salivary gland ducts just before discharging the pharynx. Few records of muscular pumps are known from other gastropod lineages. For example, Morse [84] reported muscular salivary gland duct bulbs in the marine carnivorous heterobranch sea slug *Pleurobranchaea californica*, acting as reservoirs for saliva and pumping the secretion into the pharynx. Within Acochlidimorpha, these pumps are present in the marine mesopsammic *Helicohedyle dikiki* and the limnic *Tantulum elegans*, *Strubellia wawrai*, and *Wallacellia siputbiru* [23,28,35,47]; however, the exact function is unknown.

Strubellia and *Acochlidium* are known to feed on hard egg capsules [35] of co-occurring neritids in Indo-Pacific streams, and the same feeding mode was assumed for *Wallacellia* [23]. Although neritid egg capsules are calcareous and well protected [85], they pierce the calcareous egg capsules with their slender and serrated rhachidian tooth and then suck out the nutritious fluids. (Semi)terrestrial Aitengidae were observed to feed on gelatinous egg masses [86] and are assumed to be active predators on other co-occurring molluscs (own unpubl. data). Thus, ovivory, i.e., egg predation, is a known feeding strategy in hedylopsacean Acochlidimorpha, with highly specialised egg feeders such as *Strubellia* and facultative egg feeders like *Aiteng*.

The rhachidian tooth in *Potamohedyle espinosai* n. gen. n. sp. is triangular and denticulated, pointing to a rasping rather than piercing function. It resembles the radula of Aitengidae [48], and *Potamohedyle espinosai* n. gen. n. sp. might be a facultative egg feeder as well. Observations of living specimens of *Potamohedyle espinosai* in the field and feeding experiments in the laboratory with a variety of potential prey are necessary to confirm or reject our hypothesis of facultative ovivory and reveal the feeding strategy in *Potamohedyle*.

4.3. Evolutionary Scenarios of Freshwater Colonisation

Molecular data cluster the Caribbean *Potamohedyle* within a clade of Indo-Pacific freshwater and marine interstitial slugs (Figure 14). Its phylogenetic relationships to the established genera of limnic Acochlidiidae (*Strubellia*, *Palliohedyle*, *Acochlidium*, and *Wallacellia*) and marine/brackish Pseudunelidae are poorly resolved based on the current taxon sample (including all known genera except *Tantulum*) and the set of genetic standard markers used. Our phylogenetic hypothesis indicates an independent evolutionary origin of the limnic slugs *Strubellia* from the remaining genera of Acochlidiidae (*Palliohedyle*, *Acochlidium*, and *Wallacellia*), thereby rendering this family paraphyletic. Reestablishing the family Strubellidae Rankin, 1979, is required by our phylogenetic hypothesis, but this is beyond the scope of the present study on Caribbean *Potamohedyle* and will be postponed until the current hypothesis on the evolutionary relationships of riverine slugs is confirmed by independent genetic markers, which will hopefully lead to a better-resolved phylogenetic hypothesis in future research.

Our phylogenetic hypotheses unambiguously indicate a single evolutionary transition from the marine environment to limnic systems in the common ancestor of Caribbean Tantulidae and Indo-Pacific Acochlidiidae. Under this novel evolutionary scenario, the ancestor of Indo-Pacific Pseudunelidae secondarily returned to the marine, interstitial environment via brackish and potentially benthic forms, such as the recent *Pseudunela espiritusanta*, which is sister to its marine and interstitial congeners. This corrects previous hypotheses based on cladistic analyses using morphological and ecological characters, which proposed two independent events of freshwater colonization [26]. In the absence of the possibility to include a tantulid in their molecular clock estimates on the evolution of Acochlidimorpha, Jörger et al. [25] speculated that Caribbean *Tantulum* might present a relic of a former pan-Tethys (early (approx. 120 mya)–late Cretaceous (approx. 90 mya +)) distribution of limnic slugs. While the major radiation of limnic slugs in the Indo-Pacific is comparably young and was estimated to have occurred in the Paleogene (approx. 66 mya–23 mya), the transition from marine to limnic systems in the common ancestor of Tantulidae + Acochlidiidae under the current scenario is likely much older and might date back to the early Cretaceous, according to the chronogram of Acochlidimorpha in Jörger et al. [25].

The currently known distribution of freshwater slugs nevertheless remains mysterious. Even under the hypothesis of Caribbean Tantulidae as a relic of a former pan-Tethys distribution, the question of the dispersal abilities of freshwater acochlidimorphs remains unresolved. Based on current knowledge, limnic Acochlidimorpha exclusively inhabit coastal streams and rivers (but see the discussion on *Tantulum* below) and have never been encountered in freshwater without direct access to the sea, such as lakes.

Indo-Pacific freshwater Acochlidiidae are suspected to be amphidromous, i.e., pelagic veliger larvae hatch in freshwater, are swept down the river to the sea, spend a certain time as marine pelagic veliger larvae, then morphologize into 'adhesive'-type larvae, recolonize freshwater systems, crawl upstream as juveniles, and reproduce in freshwater [35]. An amphidromous lifestyle is present both in vertebrates (e.g., fishes) and in invertebrates (e.g., crustaceans and gastropod molluscs) [87] and allows for high dispersal abilities, i.e., long-distance, pelagic dispersal across the open ocean [88]. Amphidromy is also known from Indo-Pacific neritids [89] with which Acochlidimorpha might co-evolve. As *Potamohedyle* inhabits a coastal river, an amphidromous lifestyle is possible. In contrast, amphidromy in *Tantulum*, which lives in a spring marsh 411.5 m high up in the mountains, seems questionable, even though not impossible, as the marsh is connected to a river system discharging into the sea. More likely, however, the dispersal of *Tantulum* occurs passively via extra-aquatic dispersal by birds [90], as in the co-occurring minute bivalve *Pisidium punctiferum*, which is also known to be distributed by birds. The question remains of whether direct development evolved in *Tantulum* or if hatched larvae are really swept down the river system and follow the amphidromous cycle.

The discovery of another riverine slug in the Caribbean might represent a relic of a more successful radiation of freshwater slugs in this region in former times. At the same time, it shows how novel discoveries of missing links can change evolutionary hypotheses and uncover novel survival strategies or microhabitats in known groups. More primary taxonomic work is needed, especially in environmentally healthy freshwater habitats, before such putative relic species are lost to science.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/hydrobiology3040018/s1, Supplementary file S1: Key to freshwater slug species.

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