



## Phylogenetic placement of the enigmatic worm-like Rhodopemorpha slugs as basal Heterobranchia

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### ABSTRACT

Rhodopemorphs are small, interstitial or psammobiotic heterobranch slugs, which have been troubling to place phylogenetically. Their small size and habit of living in or among sediment have led to a correlated reduction and simplification of morphology, and consequently to contradictory phylogenetic signal from anatomical features. When morphological data have previously been used to generate phylogenetic hypotheses, these were vulnerable to the effects of homoplasy. We collected multiple species of *Rhodope*, along with another rhodopemorph genus *Helminthope*, to produce DNA sequence data for the first time to test their monophyly. We sequenced mitochondrial and nuclear genes, and analysed the data under maximum likelihood and Bayesian inference. By analysing these data in a broader heterobranch dataset, we also examined the placement of Rhodopemorpha within Heterobranchia. Despite rhodopemorphs showing aspects of a euthyneurous (and pentaganglionate) condition, their placement in a molecular phylogeny occurs outside the taxon defined by this term (i.e. Euthyneura = Pentaganglionata). Instead, model-based inferences placed rhodopemorphs among basal heterobranch taxa, usually as a clade sister to a shelled group (Murchisonellidae) that was recently removed from the Pyramidelloidea. The Rhodopemorpha + Murchisonellidae clade is herein termed Allomorpha. Three-dimensional reconstruction methods have elucidated potential morphological homologies for Rhodopemorpha and comparisons with Murchisonellidae have also uncovered morphological support for this placement. Thus, we consider the phylogenetic placement of Rhodopemorpha solved, although relationships among lower Heterobranchia lineages remain challenging.

### INTRODUCTION

The tiny worm-like animals in Rhodopemorpha have caused problems for systematic biologists for over a century. As stated by Lang (1896: 282): “There are, no doubt, serious obstacles in the way of those who seek to establish the relationship of these animals within the Mollusca. The chief of these is the want of a heart and the entire absence of a shell, and a foot, even in the embryo.” Highly-adapted for living in interstitial environments, rhodopemorphs have lost many morphological attributes that could be used to establish their evolutionary origins. In addition, taxa living in these environments are also expected to have high levels of morphological homoplasy, due to the selective constraints of living interstitially (Hanken & Wake, 1993). This has been demonstrated for Rhodopemorpha, whereby cladistic morphological analyses (artificially) placed them as sister to Acochlidia (Salvini-Plawen & Steiner, 1996; Wägele & Klussmann-Kolb, 2005; Schrödl & Neusser, 2010). These types of taxonomic problems are in need of solution by molecular approaches.

*Rhodope veranii* was first described as a gastropod (Koelliker, 1847), but some subsequent authors emphasized its turbellarian-like affinities (Bergh, 1882; Trinchese, 1887). In fact, Schultze (1854) had independently described Koelliker’s organism as a turbellarian, unaware of its description as a gastropod. Later, Graff (1883) made the connection between the two, but *Rhodope* still moved back and forth between Mollusca and Turbellaria (Salvini-Plawen, 1991; see also Marcus & Marcus, 1952). Bergh (1882) did not support a molluscan connection, but still correctly predicted its developmental strategy, which Trinchese (1887) confirmed. The contradictions are perhaps best illustrated by Ihering’s (1876) treatment of Rhodopidae, which he erected as a family-level name. Although he included the Rhodopidae within what we know as ‘Opisthobranchia’, he also proposed that Nudibranchia were primitive and a link to Turbellaria (Ihering, 1880). The unique set of morphological attributes in Rhodopemorpha have generated phylogenetically disparate alternative sister-group hypotheses, and show all the hallmarks of classic ‘Problematica’ (Haszprunar, Rieger & Schuchert, 1991; Jenner & Littlewood, 2008).

Despite a growing consensus for placement in the gastropod group Euthyneura by the mid-twentieth century (e.g. Riedl, 1959), rhodopemorphs' exact phylogenetic affinities remained uncertain (Haszprunar & Heß, 2005). Affinities with Gymnomorpha (Salvini-Plawen, 1970) were later rejected since rhodopemorphs were thought to lack the diagnostic procererebrum or cerebral glands (Haszprunar & Huber, 1990; Salvini-Plawen, 1991) and the nervous system was more similar to opisthobranchs than pulmonates (Haszprunar & Huber, 1990). Rhodopemorphs also appeared to be excluded from the Nudibranchia by lacking the synapomorphic special vacuolar cells in the integument/epidermis (see Salvini-Plawen, 1991). However, a suggested affinity with dorid nudibranchs has been a long-standing hypothesis (Graff, 1883; Böhmig, 1893; Thiele, 1931; Böttger, 1955; Odhner, 1968; Haszprunar & Künz, 1996; Bouchet *et al.*, 2005). Notably, Lang & Hescheler (1900) declared that if *Rhodope* were a mollusc, it could only be a highly derived form. Salvini-Plawen (1991) erected the taxon Rhodopemorpha to include both *Rhodope* and *Helminthope*, the latter genus introduced for a truly meiofaunal organism. At that time, he considered rhodopemorphs as a highly specialized offshoot of lower opisthobranchs, of uncertain rank.

Throughout the systematic history of the Rhodopemorpha, independent sources of data have prompted contradictory conclusions. No data derived from DNA have yet been applied to the debate, because all attempts to extract DNA have failed (Schrödl & Wägele in Haszprunar & Heß, 2005). We have returned to this long-standing question with renewed efforts to generate and apply molecular data to the problem. As a result we now have novel data from species representing both described genera in the Rhodopidae, *Rhodope* and the monotypic *Helminthope*. We also

include data representing the morphotypes of all valid Rhodopidae (Fig. 1).

The Rhodopemorpha–Murchisonellidae relationship recovered with molecular data was first highlighted in a conference abstract (Wilson, Jörger & Schrödl, 2010). This reignited morphological attention using modern 3D-reconstruction techniques (Brenzinger, Wilson & Schrödl, 2011; Brenzinger, Haszprunar & Schrödl, 2013; Brenzinger, Wilson & Schrödl, 2014). Here we formally publish the molecular results and summarize the current understanding of the evolution of the group.

## MATERIAL AND METHODS

### *Specimen collection and DNA extraction*

Rhodopemorphs were collected from both subtidal and intertidal coarse sand and coralline-algal turf samples (Table 1). Two main methods of collection were used. In the first, a sample was elutriated in a large bucket of approximately three times the volume of the sand sample, so that agitation and centrifugal forces caused by swirling the liquid separated the animals from the sediment. The elutriate was then concentrated in a 250- $\mu$ m sieve and sorted under a dissecting microscope. In the second method, a decantation technique was used (see Jörger *et al.*, 2014), after one to two days of enrichment of the samples. *Helminthope psammobionta* was collected by Katrine Worsaae using traditional meiofaunal techniques (described by Worsaae & Rouse, 2008).

For most specimens, DNA was extracted from an individual using a DNeasy Blood and Tissue kit (Qiagen) or the NucleoSpin



**Figure 1.** Morphotypes of Rhodopidae and Murchisonellidae, and a selection of spicule types in Rhodopidae. Microphotographs of live specimens: all dorsal views, head directed dorsally, taken with incident light. Microphotographs of spicules in transmitted light. **A.** *Rhodope* cf. *veranii*. Specimen from near Pula, Croatia. **B.** *Rhodope* cf. *transtrosa* from Lord Howe Island; note everted buccal bulb. **C.** *Rhodope* sp. from Bocas del Toro, Panama, with purple band. **D.** *Rhodope marcusii* from São Sebastião, Brazil; posterior end contracted. **E.** *Helminthope* sp. from Bocas del Toro, Panama (photo by J.L. Norenburg). **F.** Curved spicule of *R. marcusii*; note notch in concave side (arrowhead). **G.** Curved spicule of *Rhodope* sp. shown in **C**, note notch on convex side (arrowheads). **H.** Cross-shaped spicules of *Rhodope* cf. *crucispiculata* from Bocas del Toro, Panama; note central hole. **I.** Murchisonellid *Koloanelia minutissima* from Nelson Bay, Port Stephens, New South Wales, Australia. **J.** *Murchisonella anabathron* from Moreton Bay, Queensland, Australia (photo by A. Dinapoli). Scale bars (one bar for all *Rhodope*, J, K): all c. 500  $\mu$ m. Spicules are between 80 and 150  $\mu$ m.

PHYLOGENETIC POSITION OF RHODOPEMORPHA

**Table 1.** Voucher and GenBank accession numbers of sequences generated in this study (in bold) and additional sequences retrieved from GenBank for phylogenetic analyses.

Taxon	Voucher	18 S rRNA	28 S rRNA	16 S rRNA	COI
<b>Rhodopemorpha</b>					
<i>Helminthope psammobionta</i>	WAM S99395	<b>KY806801</b>	–	<b>KY806791</b>	<b>KY806819</b>
' <i>Rhodope</i> ' cf. <i>crucispiculata</i>	ZSM Mol 20160226	<b>KY806802</b>	<b>KY806810</b>	<b>KY806792</b>	<b>KY806820</b>
<i>Rhodope roskei</i>	ZSM Mol 20050860	<b>KY806803</b>	<b>KY806812</b>	<b>KY806794</b>	–
<i>Rhodope rousei</i>	AMS C.469553	<b>KY806804</b>	<b>KY806813</b>	<b>KY806795</b>	<b>KY806822</b>
<i>Rhodope</i> sp.	ZSM Mol 20160228	<b>KY806805</b>	<b>KY806814</b>	<b>KY806796</b>	<b>KY806823</b>
<i>Rhodope</i> cf. <i>veranii</i>	ZSM Mol 20100300	<b>KY806807</b>	<b>KY806816</b>	<b>KY806798</b>	<b>KY806825</b>
<i>Rhodope</i> cf. <i>transtrosa</i>	AMS C.469762	<b>KY806806</b>	<b>KY806815</b>	<b>KY806797</b>	<b>KY806824</b>
<i>Rhodope marcusii</i>	ZSM Mol 20160227	–	<b>KY806811</b>	<b>KY806793</b>	<b>KY806821</b>
<b>Lower Heterobranchia</b>					
<i>Acteon tornatilis</i>		GQ845182/3	GQ845177	GQ845190	GQ845172
<i>Architectonica perspectiva</i>		FJ917220/1	FJ917231	FJ917251	FJ917269
<i>Cima</i> sp.		FJ917206	FJ917228	FJ917260	*
<i>Cornirostra pellucida</i>		FJ917215	FJ917225	FJ917249	FJ917282
<i>Kolonella minutissima</i>		*	FJ917237	FJ917258	FJ917277
<i>Graphis</i> sp.		FJ917209	FJ917230	FJ917262	FJ917281
<i>Hydatina physis</i>		AY427515	AY427480	DQ986637	DQ974651
<i>Larochella alta</i>		*	FJ917242	FJ917261	FJ917280
<i>Micromelo undatus</i>		DQ923446	DQ927214	DQ986638	DQ974653
<i>Murchisonella anabathron</i>		*	FJ917238	FJ917259	FJ917278
<i>Omalogyra fusca</i>		FJ917217	FJ917233	FJ917253	FJ917272
<i>Omalogyra</i> sp.		FJ917204	FJ917234	FJ917254	FJ917273
<i>Orbitestella</i> sp.		EF489352	EF489377	EF489333	EF489397
<i>Orbitestella parva</i>		FJ917207	FJ917239	FJ917250	FJ917268
<i>Pupa solidula</i>		AY427516	AY427481	EF489319	DQ238006
<i>Rictaxis punctocaelatus</i>		EF489346	EF489370	GQ845193	EF489393
<i>Rissoella elongatospira</i>		FJ917203	FJ917232	–	FJ917270
<i>Rissoella rissoaformis</i>		FJ917214	FJ917226	FJ917252	FJ917271
<i>Valvata piscinalis</i>		FJ917222	FJ917224	FJ917248	FJ917267
<b>Ringipleura</b>					
<i>Armina loveni</i>		AF249196	–	AJ223394	AF249781
<i>Doris kerguelenensis</i>		AJ224771	–	AF249233	AF249780
<i>Bathydoris clavigera</i>		AY165754	AY427444	AF249222	AF249808
<i>Dendronotus dalli</i>		AY165757	AY427450	AF249252	AF249800
<i>Gymnodoris ceylonica</i>	UQ, Lizard Island	<b>KY806800</b>	<b>KY806809</b>	<b>KY806790</b>	<b>KY806818</b>
<i>Microglyphis</i> sp.		LC150579	–	LC150585	LC150585
<i>Pleurobranchus peroni</i>		AY427494	AY427455	EF489333	DQ237993
<i>Ringicula doliaris</i>		LC150577	LC150580	LC150582	LC150582
<i>Ringiculopsis foveolata</i>		LC150578	LC150581	LC150584	LC150584
<i>Tomthompsonia antarctica</i>		AY427492	AY427452	EF489330	DQ237992
<i>Vayssierea</i> sp.	ZSM Mol 20071333	<b>KY806808</b>	<b>KY806817</b>	<b>KY806799</b>	–
<b>Panpulmonata</b>					
<i>Acroloxus lacustris</i>		AY282592	EF489364	EF489311	AY282581
<i>Albinaria caerulea</i>		AY546382	–	AY546342	X83390
<i>Amphibola crenata</i>		EF489337	EF489356	EF489304	JF439216
<i>Ancylus fluviatilis</i>		AY282593	EF489365	EF489312	DQ328270
<i>Arianta arbustorum</i>		AY546383	AY014136	AY546343	AY546263
<i>Arion silvaticus</i>		AY145365	AY145392	AY947380	AY987918
<i>Boonea seminuda</i>		AY145367	AY145395	AF355163	–
<i>Bosellia mimetica</i>		AY427498	AY427460	DQ480203	HM187642
<i>Carychium minimum</i>		EF489341	EF489361	EF489308	HQ171535
<i>Chilina</i> sp.		EF489338	EF489357	EF489305	EF489382
<i>Cyerce nigricans</i>		AY427500	AY427463	EU140843	DQ237995
<i>Cylindrobulla beaultii</i>		*	EF489371	EF489321	GQ996665
<i>Eulimella ventricosa</i>		FJ917213	FJ917235	FJ917255	*
<i>Glacidorbis rusticus</i>		FJ917211	FJ917227	FJ917264	FJ917284

Continued

**Table 1.** *Continued*

Taxon	Voucher	18 S rRNA	28 S rRNA	16 S rRNA	COI
<i>Hedylopsis ballantinei</i>		HQ168429	HQ168442	HQ168416	HQ168454
<i>Latia neritoides</i>		EF489339	EF489359	EF489307	EF489384
<i>Lymnaea stagnalis</i>		EF489345	EF489367	AY577461	EF489390
<i>Odostomia plicata</i>		GU331938	GU331928	GU331948	GU331957
<i>Onchidella floridana</i>		AY427521	AY427486	EF489317	EF489392
<i>Ophicardelus ornatus</i>		DQ093442	DQ279994	DQ093486	DQ093530
<i>Otina ovata</i>		EF489344	EF489363	EF489310	EF489389
<i>Phallomedusa solida</i>		DQ093440	DQ279991	DQ093484	DQ093528
<i>Planorbis planorbis</i>		EF012192	EF489369	EF489315	EF012175
<i>Pseudunela marteli</i>		HQ168431	HQ168444	HQ168418	HQ168456
<i>Siphonaria alternata</i>		AY427523	AY427488	HQ386678	HQ386679
<i>Trimusculus</i> sp.		KM281008	KM281088	KM281035	KM281117
<i>Turbonilla lactea</i>		GU331941	GU331931	GU331951	GU331960
Euopisthobranchia					
<i>Agalaja tricolorata</i>		DQ923447	DQ927215	AM421854	AM421902
<i>Akera bullata</i>		AY427502	AY427466	AF156127	AF156143
<i>Aplysia californica</i>		AY039804	AY026366	AY569552	AF077759
<i>Aliculastrum cylindricum</i>		DQ923458	DQ927228	–	DQ974671
<i>Bulla vermicosa</i>		DQ923451	DQ927219	DQ986636	DQ974661
<i>Cavolinia uncinata</i>		DQ237964	DQ237983	–	DQ237997
<i>Cylichna gelida</i>		EF489349	EF489374	EF489326	–
<i>Diaphana</i> sp.		DQ923456	EF489373	EF489325	EF489394
<i>Gastropterion rubrum</i>		–	DQ237990	AM422902	AM421865
<i>Haminoea hydatis</i>		AY427504	AY427468	EF489323	DQ238004
<i>Philine aperta</i>		DQ093438	DQ279988	AY345016	AY345016
<i>Phillinopsis depicta</i>		–	AM421954	AM421831	AM421892
<i>Pyrrunculus</i> sp.		DQ923465	DQ927237	–	DQ974678
<i>Runcina africana</i>		DQ923466	DQ927240	KJ022780	DQ974680
<i>Scaphander lignarius</i>		EF489348	EF489372	EF489324	DQ974663
<i>Spongiobranchaea australis</i>		DQ237969	DQ237988	–	DQ238002
<i>Tyrodina perversa</i>		AY427496	AY427458	AY345024	AY345024
<i>Umbraculum umbraculum</i>		AY165753	AY427457	EF489322	DQ256200
Outgroups					
<i>Campanile symbolicum</i>		AF055648	HM003649	AY010507	AY296828
<i>Littorina littorea</i>		X91970	AJ488672	DQ093481	AJ622946
<i>Aperostoma palmeri</i>		DQ093435	DQ279983	DQ093479	DQ093523
<i>Melanella eburnea</i>		AF120519	AF120576	DQ280051	AF120636

Voucher deposition: Australian Museum, Sydney (AMS), Bavarian State Collection of Zoology (ZSM), University of Queensland, Cheney Lab (UQ), Western Australian Museum, Perth (WAM).

\*Available sequences in GenBank were not included, as they are potentially contaminated.

Tissue kit (Macherey and Nagel), according to manufacturer's instructions, but instead eluting the DNA twice in 30–50 µl. For low-yield samples from which it was difficult to obtain PCR products, the combined elution was then concentrated using a Microcon-30 kDa Centrifugal Filter Unit with Ultracel-30 membrane (Merck Millepore). One microliter of this concentrated genomic DNA (approximately 10 ng/µl) was then used in a Whole Genome Amplification technique (Genomiphi, GE Healthcare), and a dilution series from these used as a template in subsequent PCR reactions.

#### PCR and data preparation

To take advantage of the broadest existing data for Heterobranchia, we sequenced a commonly used set of markers: mitochondrial cytochrome *c* oxidase subunit I (COI) and 16 S rDNA, and nuclear 18 S rDNA and 28 S rDNA. M13 tailed primers were sometimes used for COI samples that were difficult to amplify. The primers used are presented in Table 2. Amplifications were carried out using standard

PCR programs (Table 2). Bi-directional sequences were assembled and edited in Sequencher v. 5 (Gene Codes Corporation, Ann Arbor, MI) or Geneious v. 7-8 (Kearse *et al.*, 2012).

All sequences were checked via BLAST searches in GenBank (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) against potential contaminations. Although the Rhodopemorpha COI sequences are highly derived and show no close similarities with gastropod sequences deposited in GenBank (last accessed December 2016), they can be translated and were generated in independent laboratories with multiple samples. These sequences can be unambiguously aligned to each other and so were determined to be authentic rhodopemorph sequences and thus included in the present study.

#### Ingroup context and outgroup selection

There is yet no phylogenetic consensus regarding euthyneuran relationships and many major shifts in thinking have occurred in the past decade or so (summarized by Schrödl *et al.*, 2011; Wägele *et al.*, 2014; Kano *et al.*, 2016). To account for some phylogenetic

**Table 2.** Primers and amplification programs used in this study.

Primer name	Primer sequence	Program	Reference
COI: LCO/ HCO	GGTCAACAAATCATAAAGATATTGG TAAACTTCAGGGTGACCAAAAAATCA	95–98 °C 30 s (95–98 °C 5 s, 48–52 °C 5 s, 72 °C 20 s) × 35–37, 72 °C 60 s	Folmer <i>et al.</i> (1994)
COI: LCO1490- M13/ HCO2198-M13	CGCCAGGGTTTTCCAGTCACGACGGTCAACAAATCATAAAGATATTGG TCACACAGGAAACAGCTATGACTAACTTCAGGGTGACCAAAAAATCA	95 °C 30 s (95 °C 5 s, 42 °C 5 s, 72 °C 20 s) × 35–37, 72 °C 60 s	Folmer <i>et al.</i> (1994), Messing (1983)
16 S: 16Sf/ 16 Sr	CGGCCGCCTGTTTATCAAAAACAT GGAGCTCCGGTTGAACCTCAGATC	95–98 °C 30 s (95–98 °C 5 s, 48–55 °C 5 s, 72 °C 20 s) × 35–40, 72 °C 60 s	Simon <i>et al.</i> (1994), Schwenk <i>et al.</i> (1998)
18 S: 18A1/ 700 R/ 470 F/ 1500 R/ 1155 F/ 1800 R	CCTACTTCTGGTTGATCCTGCCAGT CGCGGCTGCTGGCACCAGAC CAGCAGGCACGCAAAATTACCC CATCTAGGCATCACAGACC CTGAAACTTAAAGGAATTGACGG TAATGATCCTCCGCAGGTT	98 °C 30 s (98 °C 5 s, 52–65 °C 5 s, 72 °C 20–25 s) × 28–40, 72 °C 60 s	Vonnemann <i>et al.</i> (2005), Wollscheid & Wägele (1999)
18 S: 18S1F/5 R	TACCTGGTTGATCCTGCCAGTAG CTTGCCAAATGCTTTCCG	95 °C 30 s (95 °C 5 s, 52–65 °C 5 s, 72 °C 20–25 s) × 28–40, 72 °C 60 s	Giribet <i>et al.</i> (1996), Whiting <i>et al.</i> (1997)
28 S: 28SC1/ 28SD3	ACCCGCTGAATTTAAGCAT GACGATCGATTGCACGTCA	95–98 °C 90 s (95–98 °C 15 s, 52–65 °C 5 s, 72 °C 25 s) × 28–40, 72 °C 60 s	Dayrat <i>et al.</i> (2001), Vonnemann <i>et al.</i> (2005)
28 S: 28Sa/ 28Srd5b	GACCCGTCTTGAAACACGGA CCACAGCGCCAGTTCTGCTTAC	95 °C 90 s (95 °C 15 s, 58 °C 5 s, 72 °C 25 s) × 28–40, 72 °C 60 s	Mallatt & Sullivan (1998) & references therein

uncertainty, we chose to test our newly generated data in a broadly-based heterobranched framework. Caenogastropod outgroups were chosen because of continuing robust support for Apogastropoda (Zapata *et al.*, 2014) and trees were rooted with the caenogastropod stem. Each taxon was represented by at least three of the four selected genes (Table 1).

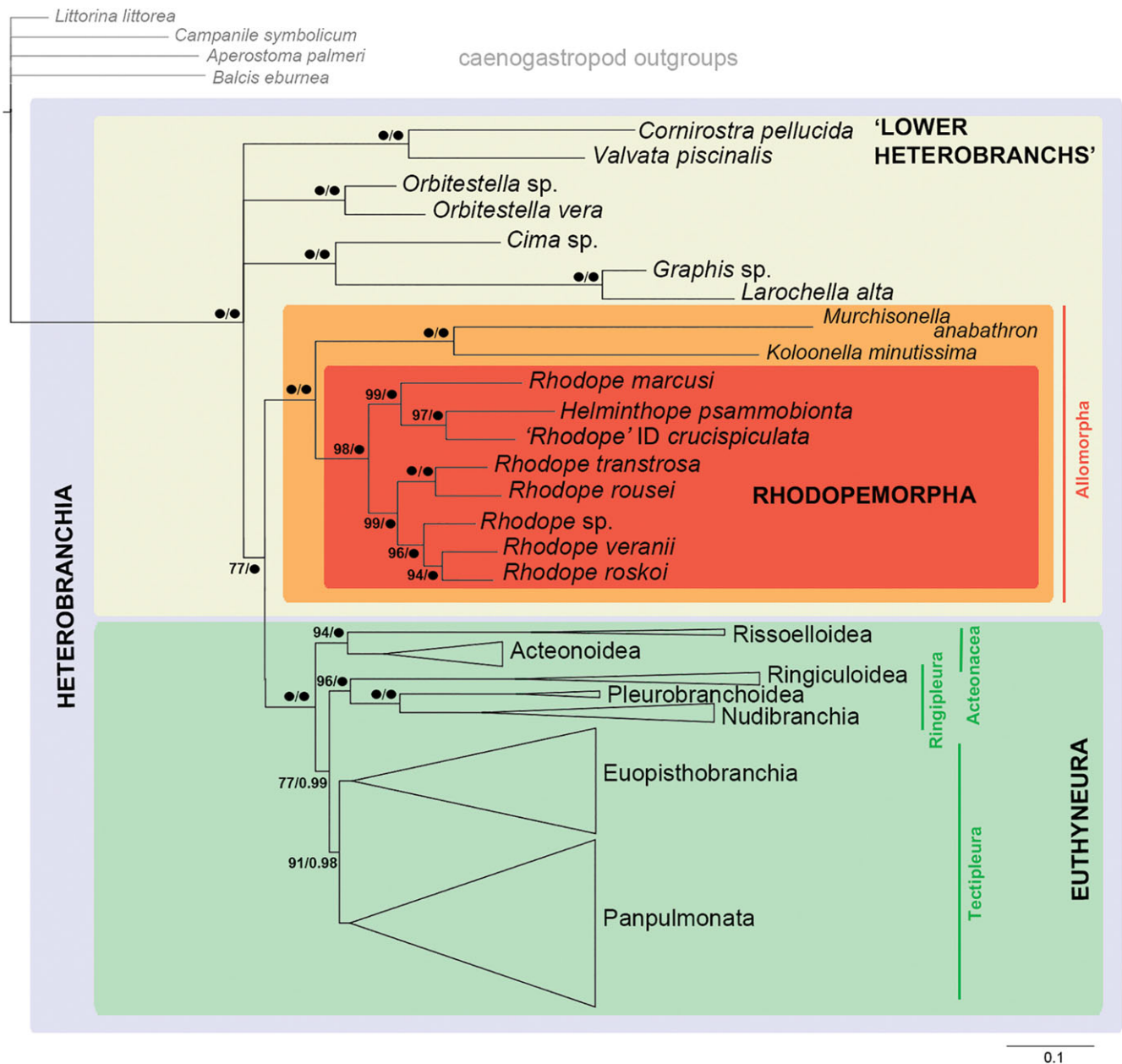
Rhodopemorph sampling included morphotypes (Fig. 1A–E) that closely match all of the described species and included: *H. psammobionta* Salvini-Plawen, 1991 from its type locality, Bermuda (an undescribed congener is shown in Fig. 1E); a morphotype similar to *Rhodope* (?) cf. *crucispiculata* Salvini-Plawen, 1991 from Panama (the first record since its description from Tunisia; spicules shown in Fig. 1H); *R. marcusii* Salvini-Plawen, 1991, sampled from São Sebastião, very close to the type locality near São Paulo; a paratype of *R. roskei* Haszprunar & Heß, 2005 from the type locality, Roscoff; *R. rousei* Brenzinger *et al.* 2011 from its type locality in South Australia; *R. sp.* from Belize; *R. cf. transtrosa* Salvini-Plawen, 1991 from Lord Howe Island (type locality unknown); and *R. cf. veranii* Koelliker, 1847 from Croatia (type locality Sicily). Where possible, morphology of living specimens was documented while they were crawling and extended, and photographs were taken with macro lenses or through stereomicroscopes (Fig. 1A–E, I, J); squeezed specimens were also observed under compound microscopes to document details of inner anatomy such as spicule morphology (Fig. 1F–H). Sample information is summarized in Table 1; where species were not collected from the type locality their identification was qualified as ‘cf.’, conveying some level of uncertainty. The two Murchisonellidae previously sequenced by Dinapoli & Klussmann-Kolb (2010) as *Ebala* sp. and *Murchisonella* sp. are herein identified as *Kolonella minutissima* (Laseron, 1951) and *Murchisonella anabathron* (Hedley, 1906), following Warén (2013) (Fig. 1I, J; Table 1). Ideally, these identifications should be re-examined at a later date, as the collection sites of the Dinapoli & Klussmann-Kolb specimens were not from the type localities of these species.

### Analyses

Alignments were generated for each marker using the MAFFT add-on in Geneious (Katoh & Standley, 2013). Ambiguous parts of the 16 S, 28 S and 18 S rRNA alignments were masked using GBlocks (Talavera & Castresana, 2007) by applying the settings for a less stringent selection. The COI alignment was checked manually via translation into amino acids and single base indels resulting in shifts of the reading frame were removed manually. Models of evolution were applied to the alignment partitioned according to PartitionFinder v. 1.1.1 (Lanfear *et al.*, 2012) using the Bayesian information criterion. The selected partitions were 16 S (1–343 bp, GTR + I + G), 18 S (344–1999 bp, SYM + I + G), 28 S (2000–2802 bp, GTR + I + G) and COI (2803–3475 bp, GTR + I + G). Maximum-likelihood (ML) analyses were conducted with RAxML-HPC (Stamatakis, 2006) through the Cipres Science Gateway (<https://www.phylo.org>) with the GTR + I + G model applied to all four partitions. Branch support was assessed using 1000 bootstrap (BS) pseudoreplicates under the rapid bootstrap algorithm. Bayesian inference (BI) analyses were carried out in MrBayes (Ronquist *et al.*, 2012) running default priors employing six iterations of 10 million generations with eight chains sampling every 1000 generations (temperature 0.02). A consensus was then built from the trees remaining after the default burn-in was removed and support was estimated as posterior probabilities (PP). To understand the effect of long branches on the topology, we also repeated analyses removing *Architectonica*, *Omalogyra*, *Armina* and *Dendronotus* from the dataset. This reduced dataset was analysed as above by ML and BI.

### RESULTS

With long-branched taxa removed (Fig. 2; Supplementary Material Figs S1, S2), there was strong support for a monophyletic



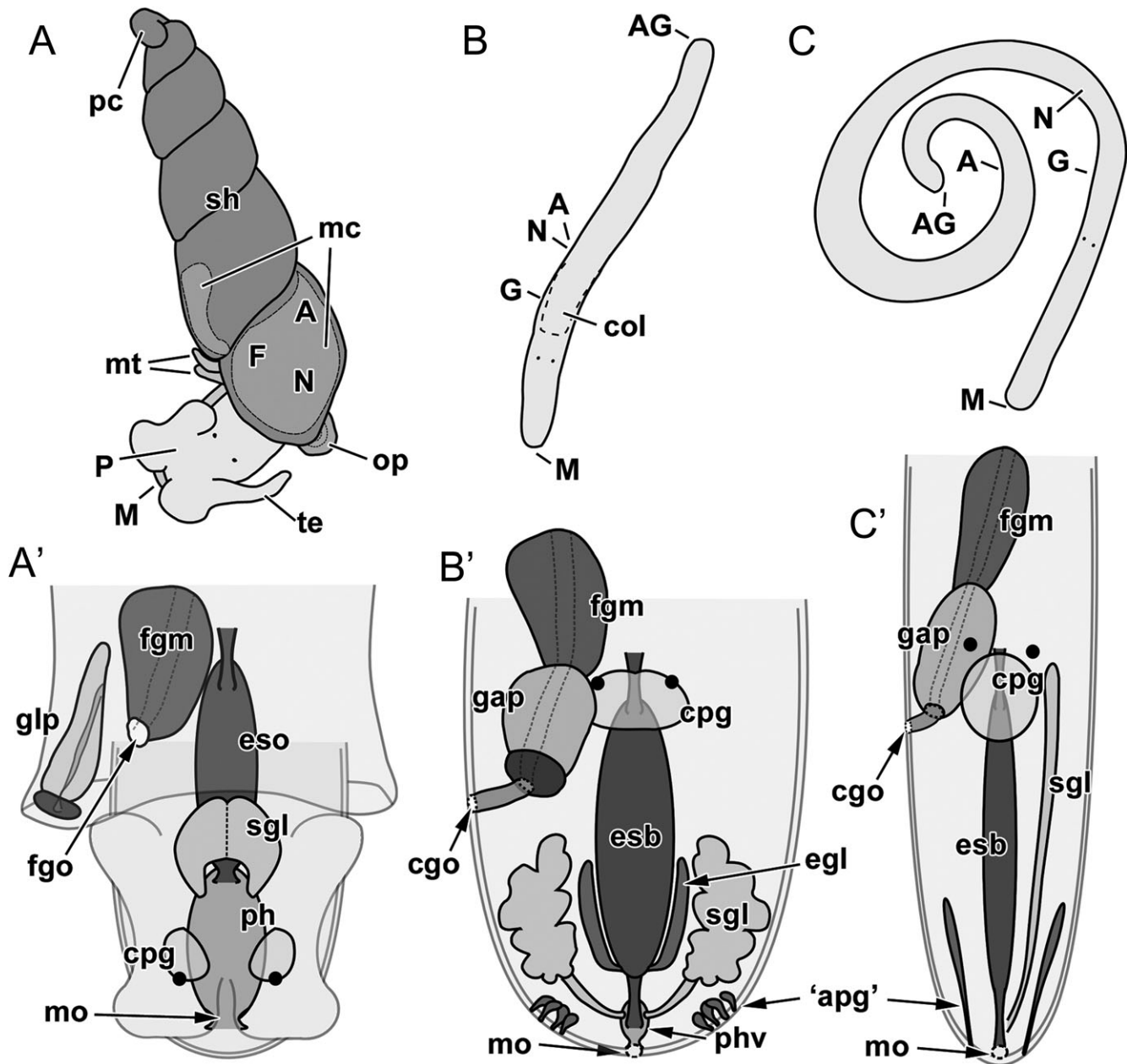
**Figure 2.** Maximum-likelihood topology of the reduced dataset (i.e. long-branch taxa removed) showing position of Rhodopemorpha in relation to other heterobranch groups. Within-Euthyneura clades have been collapsed and higher-taxon names applied. Support values given as ML/PP (PP converted into percentages); black dots indicate maximal support.

Heterobranchia (BS 100, PP 1.00) and topologically consistent Euthyneura (BS 100, PP 1.00), Tectipleura (BS 91, PP 0.98), Euopisthobranchia (BS 77, PP 0.99) and Panpulmonata (BS 4, PP 0.99) in concatenated analyses. Recent hypotheses of heterobranch relationships such as Ringipleura (Kano et al., 2016) were strongly supported. ML analyses placed Pyramidelloidea in a typical position within Panpulmonata (Supplementary Material Fig. S1); however, in BI analyses of this dataset, pyramidellids were sister to Tectipleura (Supplementary Material Fig. S2; PP 1.00).

In this reduced taxon set, the lower Heterobranchia formed a basal grade of Euthyneura, consisting of five well-supported lineages. These were *Valvata* + *Cornirostra* (BS 100, PP 1.00), *Orbitestella* (BS 100, PP 1.00), *Cima* + *Graphis* + *Larochella* (see Warén, 2013 for identification and discussion of these individuals) (BS 100, PP 1.00) and Murchisonellidae + Rhodopemorpha (BS 100, PP 1.00). In the all-taxa dataset (Supplementary Material

Figs S3, S4), which included *Architectonica* + *Omalogyra*, support values for that basal heterobranch lineage were high (BS 100, PP 1.00).

Using our datasets, BI analyses appeared to be affected by the very long branch leading to *Architectonica* + *Omalogyra*, resulting in the position of Murchisonellidae varying among analyses. In the ML analyses of the all-taxa dataset, Murchisonellidae were always the sister group to Rhodopemorpha (BS 82), but BI results showed Murchisonellidae switching to become sister of *Architectonica* + *Omalogyra* (although with no significant support). In the reduced dataset, in which long-branched taxa had been removed, Murchisonellidae were the sister to Rhodopemorpha in both ML (BS 100) and BI analyses (PP 1.00). The average standard deviation of split frequencies for the full dataset was 0.038361 and, for the reduced dataset, 0.035036, indicating somewhat better convergence of likelihood values in the reduced dataset. In all analyses,



**Figure 3.** Comparative morphology of a murchisonellid (*Koloanella*), *Rhodope* and *Helminthope*. Upper row: general organization of body with morphological landmarks, dorsal views. **A.** *Koloanella minutissima* (after Brenzinger *et al.*, 2014; fig. 1A"). **B.** *Rhodope cf. veranii* (after Fig. 1A). **C.** *Helminthope psammobionta* (after Brenzinger *et al.*, 2013; fig. 2). Lower row: schematic dorsal views of anterior body showing potential synapomorphies (see text) in anterior digestive tract and distal gonoduct with its associated glands. **A'.** *Koloanella* (after Brenzinger *et al.*, 2014). **B'.** *Rhodope* (after Brenzinger *et al.*, 2011 and unpublished data). **C'.** *Helminthope* (after Brenzinger *et al.*, 2013). Abbreviations (upper row): A, position of anus; AG, position of adhesive gland; col, position of coloured transverse band; F, position of female genital opening; G, position of common genital opening; M, position of mouth; mc, extent of mantle cavity; mt, tentacles at mantle margin; N, position of nephropore; op, operculum; P, position of penis; pc, protoconch (sinistral); sh, shell (teleoconch, dextral); te, tentacle. Abbreviations (lower row): 'apg', anterior pedal glands (*sensu* Brenzinger *et al.*, 2013); cgo, common genital opening (in *Rhodope* and *Helminthope*); cpg, cerebropneural ganglion (black circle are eyes); egl, oesophageal glands; esb, oesophageal bulb; eso, oesophagus (vacuolated); fgm, female glandular mass; fgo, female genital opening (in *Koloanella*); gap, glandular apparatus (with central duct as extension of gonoduct); glp, glandular pocket (with open groove opposite to female genital opening); mo, mouth opening; ph, pharynx; phv, vestigial pharynx (in *Rhodope*); sgl, salivary gland (unpaired in *Helminthope*, medially fused (?) in *Koloanella*).

Rhodopemorpha were placed with the lower heterobranchs and not within Euthyneura.

Relationships within a monophyletic Rhodopemorpha (BS 98, PP 1.00) showed two well-supported lineages (Fig. 2). One consisted of *Rhodope marcusii*, *Helminthope psammobionta* and '*R.*' cf. *crucispiculata* (BS 99, PP 1.00). The other consisted of *R. rousei*, *R. cf. transtrosa*, *R. sp.*, *R. rosikoi* and *R. cf. veranii* (BS 99, PP 1.00).

## DISCUSSION

The sister group to the Rhodopemorpha slugs was the Murchisonellidae snails and this relationship was robustly supported across most analyses. This negates the long-standing idea that Rhodopemorpha are allied with Nudibranchia (Graff, 1883; Böhmig, 1893; Thiele, 1931; Böttger, 1955; Odhner, 1968; Haszprunar & Künz, 1996; Bouchet *et al.*, 2005) or with any other

'opisthobranch' taxon. The minute, shelled Murchisonellidae were long considered to belong to Pyramidelloidea and were only relatively recently repositioned as basal heterobranchs on the basis of molecular data (Dinapoli & Klussmann-Kolb, 2010; in contrast to a revised panpulmonate placement of Pyramidelloidea). The apparent morphological disparity between the shelled Murchisonellidae and the interstitial worm-like Rhodopemorpha makes a close relationship counterintuitive at first glance (Figs 1, 3A–C). Following initial recovery of this new clade, 3D-reconstruction techniques have clarified the anatomy of *Rhodope* (Brenzinger *et al.*, 2011), *Helminthope* (Brenzinger *et al.*, 2013) and the murchisonellid *Kolonella* (Brenzinger *et al.*, 2014). These studies have identified potential synapomorphies within several organs that appear unaffected by the strong modification of rhodopids: (1) a modified anterior digestive tract with a reduced (Murchisonellidae) or almost fully lost pharynx (Rhodopidae) and an oesophagus that is at the same time enlarged and vacuolated (murchisonellids) or forms a large, bulb-like organ (rhodopids) and (2) a pair of histologically distinct glands that form a pocket adjacent to the female genital opening in murchisonellids and a tubular apparatus as an extension of the distal gonoduct in rhodopids (shown in Fig. 3A'–C'). Further similarities that require additional examination of (lower heterobranch) outgroups are the potentially derived infaunal habitat of both families and the presence of subepidermal calcium concretions (calcium cells in the neck and foot in murchisonellids, ubiquitous spicules in rhodopids) and glandular cells at the posterior edge of the foot or tail ('opercular' glands in murchisonellids, adhesive glands in rhodopids) (Brenzinger *et al.*, 2013, 2014). Details of the nervous system may turn out to be valuable characters for future comparisons, such as configuration of the cerebral nerves (one with a double root in rhodopids) and the visceral loop, but for now both remain largely unknown in murchisonellids (Brenzinger *et al.*, 2014, unpublished data).

The monophyly of Rhodopemorpha was well-supported here, but the monophyly of its constituent genera was not. *Rhodope* was recovered as paraphyletic since the white-bodied *R. marcusii* was recovered as more closely related to the equally colourless *Helminthope psammobionta* and '*R.*' cf. *crucispiculata* than to the remaining *Rhodope* that have a pigmented band. There is some justification to reconsider the generic placement of '*R.*' cf. *crucispiculata*, which shows a true meiofaunal habit and associated morphology like *Helminthope*, and was described with some generic uncertainty. Haszprunar & Heß (2005) thought that '*R.*' *crucispiculata* warranted a new genus and did not belong to either *Rhodope* or *Helminthope*. Salvini-Plawen (1991) described this species very briefly and indicated some doubt about its generic designation; type material apparently does not exist (W. Sterrer, personal communication). In the same paper, he described the genus *Helminthope* as being defined by a wide nervous system with free ganglia, by the differentiation of precerebral ganglia, and the axial connection of the foregut and midgut without an anterior caecum. Brenzinger *et al.* (2013) reinterpreted some of the original views and also identified the presence of only a single salivary gland, a posteriorly shifted anus and a derived kidney with a superficial resemblance to an unpaired protonephidium as potential synapomorphies of *Helminthope* (Fig. 3C, C'). Clearly, further work is required to compare the morphology of '*R.*' *crucispiculata* with that of *H. psammobionta*. It must be noted that our specimen of '*R.*' cf. *crucispiculata* was sampled far from the type locality and differs in the morphology of its spicules ('snowflake-like' see Fig. 1H, *vs* straight, cross-shaped in Rieger & Sterrer, 1975: fig. 36); wider geographic sampling and phylogeographic approaches are necessary to understand taxonomic boundaries. It is very likely that cryptic species will be uncovered in the future, as is common with other interstitial and simplified organisms (e.g. Jörger *et al.*, 2012; Leasi & Norenburg, 2014). The grouping of *R. marcusii* (Fig. 1D) with '*R.*' cf. *crucispiculata* and *H. psammobionta* is less easy to understand and is currently only supported by the lack of pigmentation in this clade as compared with the remaining clade

of orange-, red- or purple-banded *Rhodope* (Fig. 1A–C); the position of this species needs to be retested when missing data are available and intra- and interspecific variation in spicule morphology should be examined (compare Fig. 1F, G).

The relationships among the remaining *Rhodope* species show some correlation with geography. The two Australian species (*R. rousei* + *R.* cf. *transtrosa*; Fig. 1B) were recovered as sister taxa and the Caribbean species (*R.* sp.; Fig. 1C) was sister to a pair of European species (*R. roskei* + *R.* cf. *veranii*; Fig. 1A). However, these patterns must be considered as very preliminary, since we know that many undescribed rhodopemorphs exist. There are records from Madeira (Graff, 1883), Norway (Karling, 1966), Lord Howe Island, temperate Australia, Belize, Moorea (NGW, personal observation), Guadeloupe and Papua New Guinea (P. Bouchet, BB personal observation), Brazil, Thailand and the Azores (KJ, MS personal observation), Guam (Carlson & Hoff, 1981) and the Galapagos Islands (Arnaud, Poizat & Salvini-Plawen, 1986). Further sampling will undoubtedly reveal more species and allow more robust testing of evolutionary and biogeographic hypotheses.

Among our sampled taxa, two species still have an unresolved taxonomy. The identity of our specimen of *R.* cf. *veranii* is uncertain; the type from Messina (Sicily, western Italy) has only a transverse bar of dark red colour (Koelliker, 1847), whereas all subsequent studies on the species (including Graff, 1883; Böhmig, 1893; Riedl, 1959 and our present study) attribute the name to brick-red to orange-coloured animals from the northern Adriatic that show an additional longitudinal marking (see Fig. 1A; discussed by Brenzinger *et al.*, 2011). Resampling from the type locality is the only way to resolve this. The same approach is not possible for *R. transtrosa*. This species lacks a precise type locality, having been described from specimens found in an aquarium in Vienna (Salvini-Plawen, 1991), derived from live aquarium rock from Sri Lanka. This creates a challenge to nomenclatural stability. Here we report a species that matches the external colouration reported for *R. transtrosa* (see Fig. 1B), collected from Lord Howe Island, a volcanic remnant island emerging from the Lord Howe Rise, about 600 km east of the Australian mainland. Although we do not suggest that this specimen truly represents the unknowable *R. transtrosa* and do not imply that the aquarium rocks were collected from Lord Howe Island, we have identified our specimen as *R.* cf. *transtrosa* because of its colouration.

In all analyses, the clade Rhodopemorpha + Murchisonellidae is placed outside the node that defines the taxon Euthyneura and should be considered as a basal heterobranch lineage. We introduce the clade name Allomorpha (Greek: allo- indicating divergent or different; morph indicating shape or form, referring to the divergent body forms of the two clades) to describe this lineage. Their nervous system clearly shows elements of the euthyneurous/pentagangliate condition (Riedl, 1960; Salvini-Plawen, 1991; Brenzinger *et al.*, 2013), which however, must not be used as a justification for their artificial inclusion in Euthyneura (Schrodl *et al.*, 2011). Although this work has placed Rhodopemorpha into a phylogenetic context, there is much still to do in order to embed the group into a wider lower heterobranch framework and to uncover the full range of rhodopemorph diversity worldwide.

## SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Molluscan Studies* online.

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