



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- μ 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

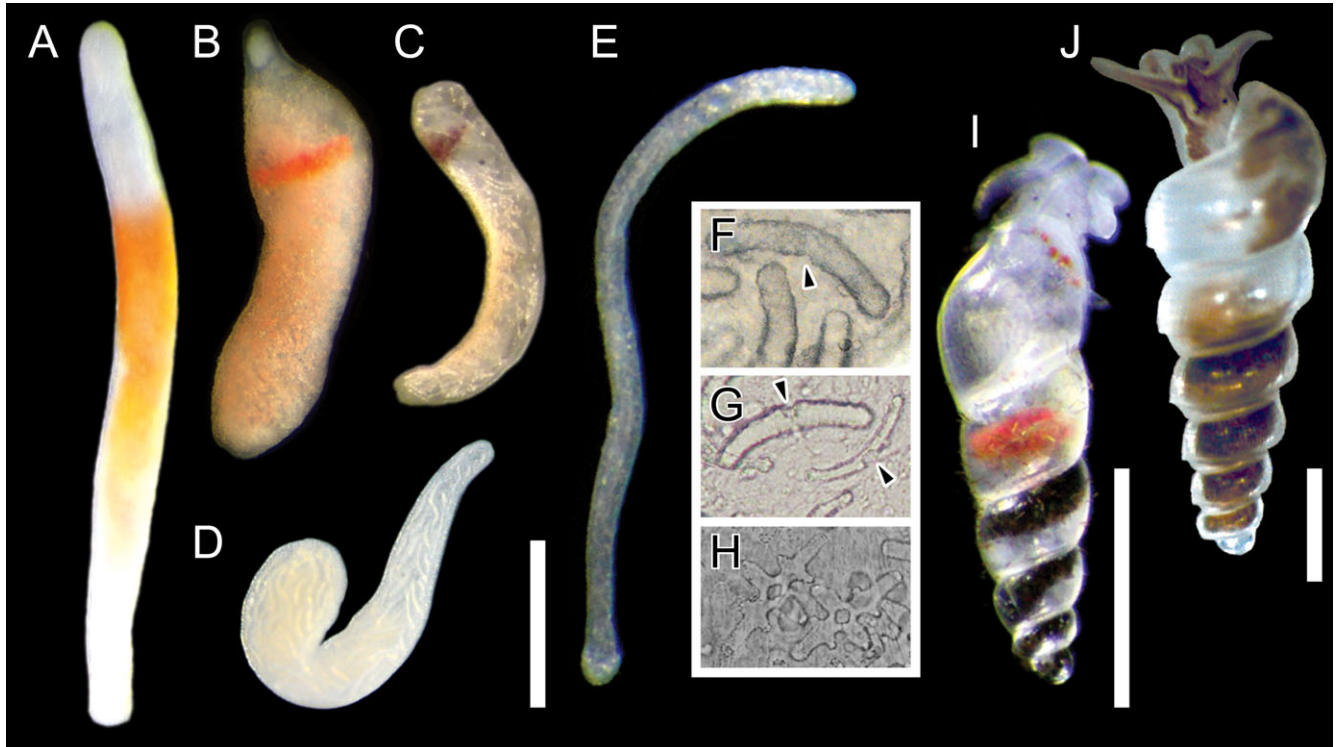


Figure 1. Morphotypes of Rhodopidae and Murchisonellidae, and a selection of spicule types in Rhodopidae. Microphotographs of live specimens: all dorsal views, head directed dorsally, taken with incident light. Microphotographs of spicules in transmitted light. **A.** *Rhodope* cf. *veranii*. Specimen 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 *Koloanella 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 μ m. Spicules are between 80 and 150 μ 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 |
|--|-------------------|-----------------|-----------------|-----------------|-----------------|
| Rhodopemorpha | | | | | |
| <i>Helminthope psammobionta</i> | WAM S99395 | KY806801 | – | KY806791 | KY806819 |
| ' <i>Rhodope</i> ' cf. <i>crucispiculata</i> | ZSM Mol 20160226 | KY806802 | KY806810 | KY806792 | KY806820 |
| <i>Rhodope roskei</i> | ZSM Mol 20050860 | KY806803 | KY806812 | KY806794 | – |
| <i>Rhodope rousei</i> | AMS C.469553 | KY806804 | KY806813 | KY806795 | KY806822 |
| <i>Rhodope</i> sp. | ZSM Mol 20160228 | KY806805 | KY806814 | KY806796 | KY806823 |
| <i>Rhodope</i> cf. <i>veranii</i> | ZSM Mol 20100300 | KY806807 | KY806816 | KY806798 | KY806825 |
| <i>Rhodope</i> cf. <i>transtrosa</i> | AMS C.469762 | KY806806 | KY806815 | KY806797 | KY806824 |
| <i>Rhodope marcusii</i> | ZSM Mol 20160227 | – | KY806811 | KY806793 | KY806821 |
| Lower Heterobranchia | | | | | |
| <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 |
| Ringipleura | | | | | |
| <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 | KY806800 | KY806809 | KY806790 | KY806818 |
| <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 | KY806808 | KY806817 | KY806799 | – |
| Panpulmonata | | | | | |
| <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 beauii</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 GGAGCTCCGGTTTGAACCTCAGATC | 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 CATCTAGGGCATCACAGACC 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 CTTGGCAAATGCTTTCCG | 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

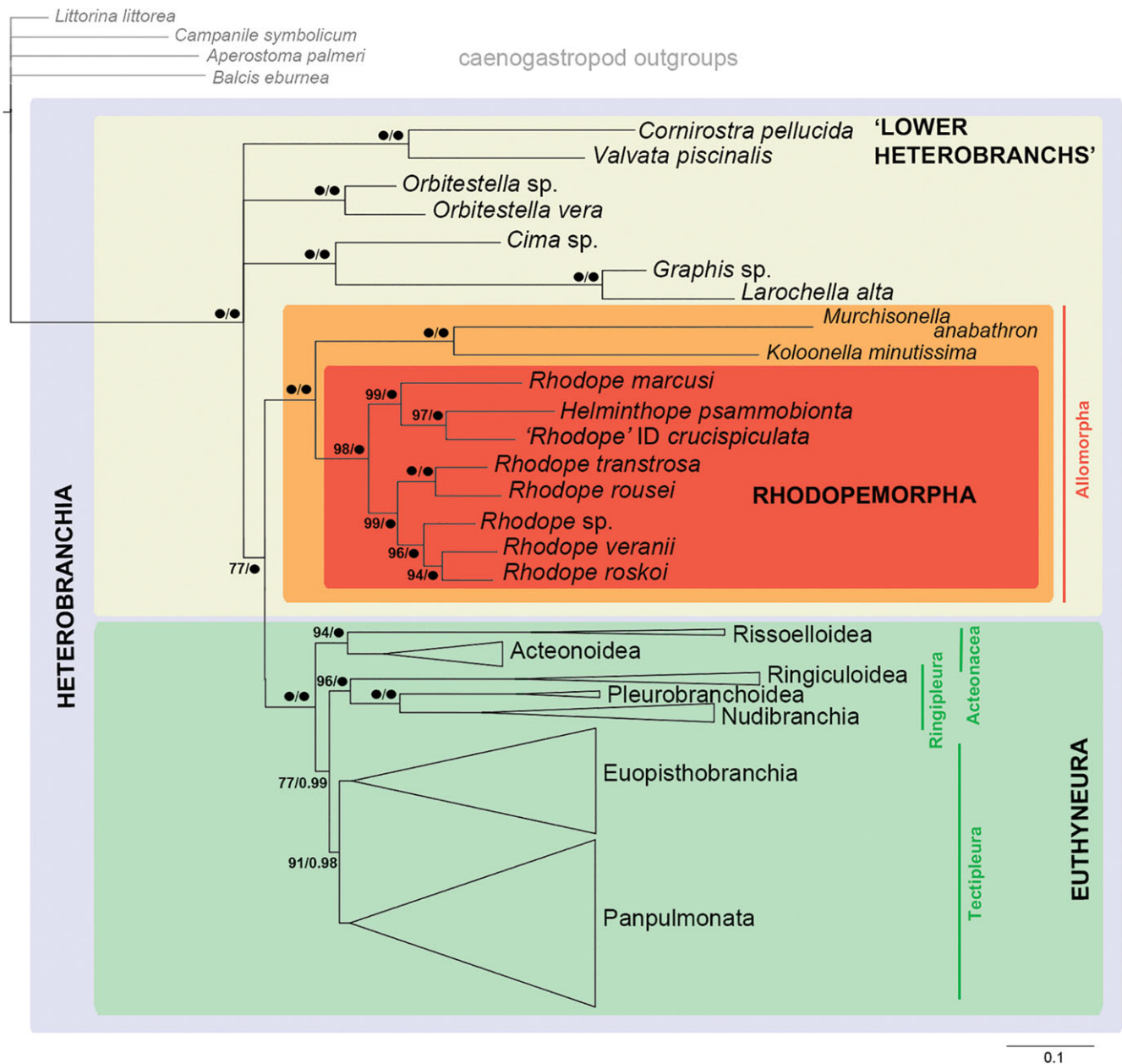


Figure 2. Maximum-likelihood topology of the reduced dataset (i.e. long-branch taxa removed) showing position of Rhodopemorpha in relation to other heterobranch groups. Within-Euthyneura clades have been collapsed and higher-taxon names applied. Support values given as ML/PP (PP converted into percentages);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

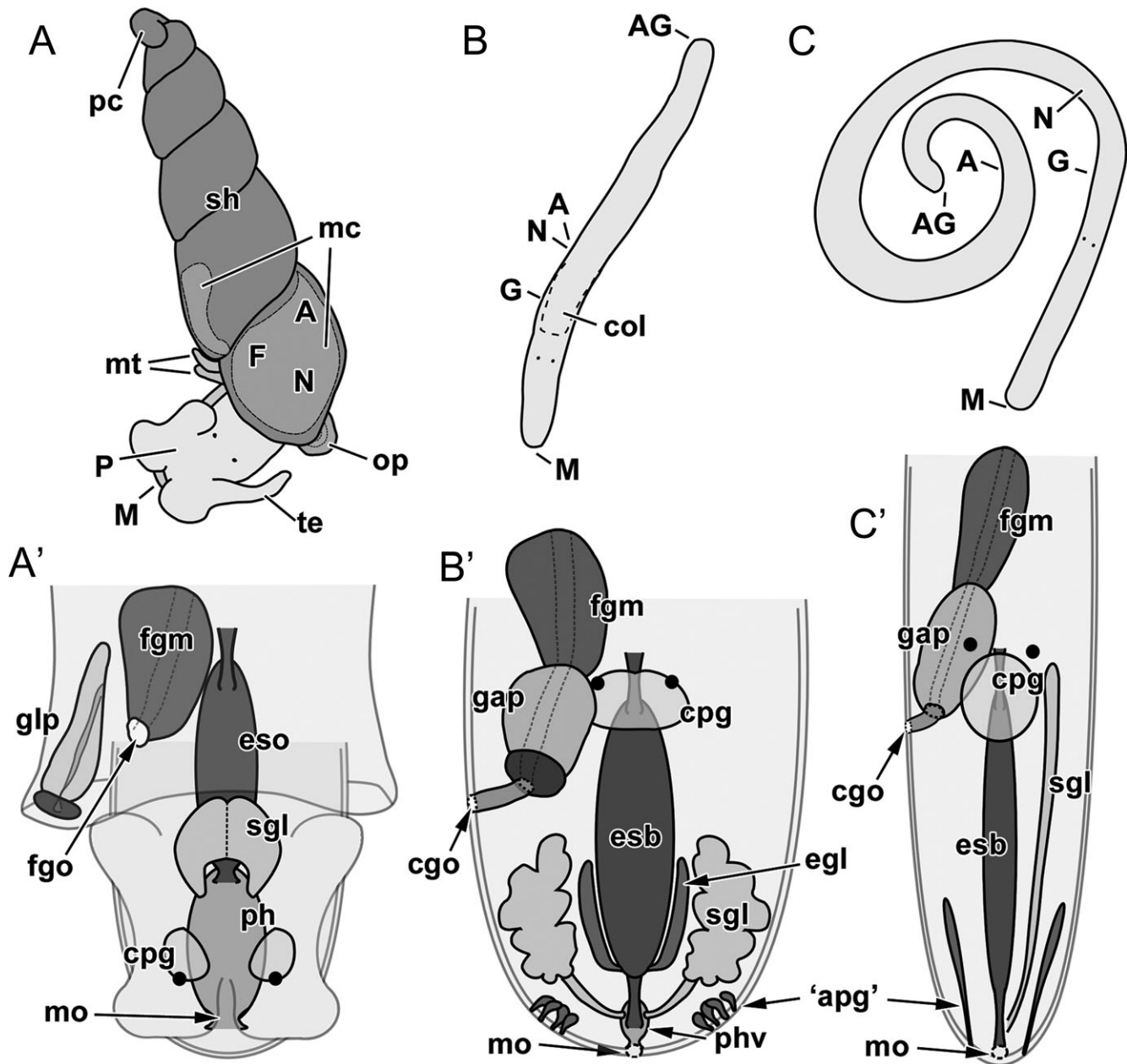


Figure 3. Comparative morphology of a murchisonellid (*Koloanella*), *Rhodope* and *Helminthope*. Upper row: general organization of body with morphological landmarks, dorsal views. **A.** *Koloanella minutissima* (after Brenzinger *et al.*, 2014; fig. 1A’). **B.** *Rhodope* cf. *veranii* (after Fig. 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; Odnher, 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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