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Evolutionary gain of red blood cells in a commensal bivalve (Galeommatoidea) as an adaptation to a hypoxic shrimp burrow

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Evolutionary transitions from free-living to symbiotic lifestyles often lead to dramatic changes in morphological, ecological and physiological characteristics. Galeommatoidea represents a highly diverse superfamily of Bivalvia, with > 620 described species in multiple free-living and symbiotic clades and, thereby, provides a unique opportunity to investigate the character evolution associated with lifestyle transitions. *Barrimysia cumingii* is a commensal galeommatoidean that lives in the deep burrow of the strahlaxiid shrimp *Neaxius acanthus*. The burrow is a sulphide-rich reducing environment owing to poor water circulation and decay of seagrass leaves accumulated by the shrimp. In this study, we found that abundant spherical red blood cells (RBCs; ~10 μm in diameter) with one prominent nucleus occur in the haemocoel of *B. cumingii*. This is the first report of RBCs for the superfamily. Our phylogenetic reconstruction based on four-gene DNA sequences indicates a relatively recent divergence of *B. cumingii* from free-living ancestors without RBCs and the origin of RBCs in association with the colonization of the shrimp burrow. Other bivalve lineages with RBCs tend to occur in hypoxic habitats, including mangrove swamps and deep-sea hydrothermal vents and cold seeps, suggesting that *B. cumingii* has also obtained RBCs as a physiological adaptation to within-host-burrow hypoxia.

ADDITIONAL KEYWORDS: adaptation – *Barrimysia cumingii* – commensalism – erythrocyte – Mollusca – *Neaxius* – phylogeny – Strahlaxiidae – symbiosis.

INTRODUCTION

Symbiosis, the living together of different species of organisms (de Bary, 1879), is ubiquitous and diverse in the sea and plays an important role in the maintenance of marine biodiversity (Morton, 1988; Nybakken & Bertness, 2004). Animals involved in such interactions often show remarkable morphological, behavioural, physiological and ecological adaptations to the symbiotic life accompanied by gain and/or loss of various traits (e.g. MacGinitie, 1939; Morton, 1988; Pillay, 2010). Comparison of symbiotic species with their non-symbiotic (free-living) relatives is one of the most effective approaches to understand the types of adaptations that are required for a symbiotic lifestyle (Leung, 2014). However, we still know little about the evolutionary origins of symbiotic lifestyles in marine environments.

The bivalve superfamily Galeommatoidea (with the monotypic family Galeommatidae *sensu* Ponder, 1998) exhibits tremendous species diversity in tropical shallow-water environments (Bouchet *et al.*, 2002; Paulay, 2003). This group includes a large number of free-living and symbiotic species and thus provides an invaluable

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opportunity to investigate the adaptations associated with lifestyle transitions (Goto *et al.*, 2012; Li *et al.*, 2012, 2016). A large majority of free-living galeommatoideans live attached to the undersurface of rocks or rock crevices in the intertidal zone (Lützen & Nielsen, 2005), whereas most symbiotic species live on the body surface or inside the burrow of other marine invertebrates (Boss, 1965; Morton & Scott, 1989; Goto *et al.*, 2018). Recent molecular phylogenetic analyses have suggested multiple evolutionary transitions between free-living and symbiotic lifestyles in this group (Goto *et al.*, 2012; Li *et al.*, 2016).

Barrimysia cumingii (A. Adams, 1856), a rare galeommatoidean in the tropical western Pacific, is unique in having a crimson body (Figs 1, 2) that contrasts with the whitish tissue of all other species in the superfamily. Kano & Haga (2011) have suggested the presence of red blood cells (RBCs or erythrocytes) in its haemocoel, but this remains unconfirmed. *Barrimysia cumingii* is a commensal that lives exclusively in the burrow of the strahlaxiid shrimp *Neaxius acanthus* (Kneer *et al.*, 2008; Seike & Goto, 2017). The burrow of this species comprises a vertical, cylindrical tube and a large basal chamber (Kneer *et al.*, 2008; Seike & Goto, 2017). Inside the burrow is a nutrient-rich, reducing environment, because the shrimp actively gathers fragments of seagrass leaves in the vicinity of the burrow opening and accumulates them in the basal chamber (Vonk *et al.*, 2008).

This study is the first to confirm that *B. cumingii* has abundant RBCs in the haemocoel based on our observation using optical and scanning electron microscopy. We also investigated the phylogenetic position of this reddish commensal clam within the Galeommatoidea based on partial sequences of two nuclear RNA genes (18S and 28S), one nuclear protein-coding gene (histone *H3*) and one mitochondrial protein-coding gene (*COI*) from 47 ingroup and seven outgroup species. The obtained phylogeny enabled us to infer the evolutionary origin of the symbiotic lifestyle and RBCs in the lineage leading to *B. cumingii*.

MATERIAL AND METHODS

Sampling and observation of red blood cells

We collected three specimens of *B. cumingii* from the burrows of *N. acanthus* on the intertidal gravel/sand flats of Edateku Island and Itton, Amami-Oshima Island, the Ryukyu Archipelago, southern Japan, during 2010–2012. Two specimens collected from Edateku (#1 and #2) were stored in pure ethanol for molecular analyses. One of these (#1) was observed for RBCs

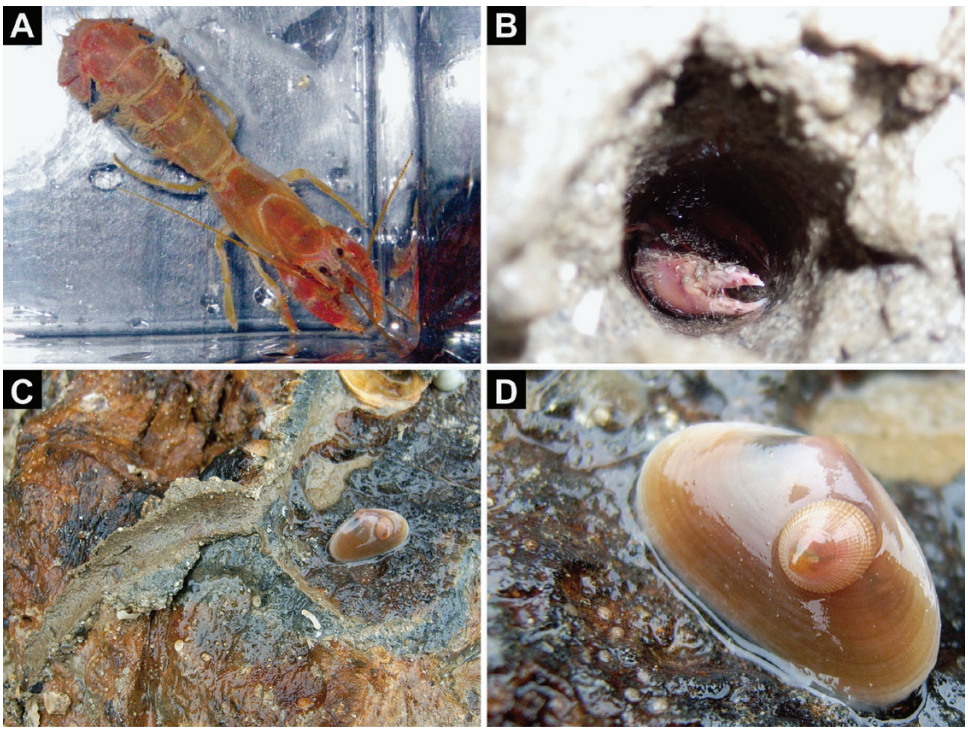

Figure 1. Red-blooded clam and limpet, *Barrimysia cumingii* (Bivalvia: Galeommatoidea) and *Zacalantica* sp. (Gastropoda: Phenacolepadidae) attached to *B. cumingii*, and their host shrimp *Neaxius acanthus* (Crustacea: Strahlaxiidae). A, *N. acanthus*. B, *N. acanthus* at opening of its burrow. C, *B. cumingii* attaching to burrow wall. D, *B. cumingii* with attached *Zacalantica* sp.

under light and dissecting microscopes before preservation. The shell morphology, hinge structure and internal anatomy of specimen #1 are shown in the Supporting Information (Fig. S1). The single specimen from Itton (#3) was stored in 70% ethanol after being fixed in 10% formaldehyde. The mantle tissue of this specimen was prepared using standard techniques (cleaned, freeze-dried, mounted on a stub and coated with platinum) for observation with scanning electron microscopy (Hitachi S-4800).

Extraction and sequencing of DNA

DNA sequences were determined for one of the Edateku specimens (#2) and were used for phylogenetic analyses along with the published sequences of Galeommatoidea (Goto *et al.*, 2012; Valentich-Scott *et al.*, 2013). Sequences were also determined and analysed for an undetermined species of the subfamily Galeommatinae (*sensu* Huber, 2015) from the underside of an intertidal rock in Uwajima, Ehime, Japan. This free-living bivalve has a translucent, elongated trigonal shell that is partly covered by the colourless mantle with many papillae. We tentatively call the bivalve '*Melliteryx*' sp. 1 for its closest resemblance to *Melliteryx puncticulata*, although it also resembles *B. cumingii* in shell morphology.

Total genomic DNA was isolated from the tissue following previously described methods (Goto *et al.*, 2012). Polymerase chain reactions (PCRs) were used to amplify ~1700 bp of 18S, ~1000 bp of 28S and ~350 bp of *H3*. Amplifications were performed in 20 μL mixtures consisting of 0.4 μL of forward and reverse primers (20 μM each; Supporting Information, Table S1), 2.0 μL ExTaq buffer, 1.6 μL dNTPs (2.5 μM each), 0.1 μL ExTaq polymerase (TaKaRa, Otsu, Japan), 14.5 μL distilled water and 1.0 μL genomic DNA. Thermal cycling was performed with an initial denaturation for 3 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 55 °C and 2 min at 72 °C, with a final 3 min extension at 72 °C. The sequencing reaction was performed using PCR primers and internal primers (Supporting Information, Table S1) and a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and electrophoresed on an ABI 3130 sequencer (Applied Biosystems). The obtained sequences were deposited in the DDBJ/EMBL/GenBank databases with accession numbers LC386847, LC386848, LC387592 and LC387593 (Supporting Information Table S2).

Phylogenetic analyses

Sequences of the 18S, 28S, *H3* and *COI* genes were aligned using the program Muscle (Edgar, 2004) as implemented in the software Seaview (Galtier *et al.*, 1996; Gouy *et al.*, 2010) with default settings; poorly aligned regions were corrected by eye. The alignments of *H3* and *COI* sequences (328 and 658 bp, respectively) had no indels and were therefore unambiguous. We used Gblocks v. 0.91b (Castresana, 2000; Talavera & Castresana, 2007) to eliminate ambiguously aligned regions for 18S and 28S datasets. Lengths of sequences before and after the Gblocks treatment were 1847 and 1755 bp (18S) and 1212 and 953 bp (28S), respectively.

Phylogenetic trees were constructed based on the combined dataset (18S + 28S + *H3* + *COI*) using Bayesian inference and maximum likelihood (ML) methods. The Bayesian analysis was performed using MrBayes v. 3.2.1 (Ronquist & Huelsenbeck, 2003) with substitution models chosen by Kakusan 4 (Tanabe, 2011) for each gene and codon position (SYM+γ for 18S, GTR+γ for 28S, *H3* first, *H3* second, *COI* second and *COI* third, JC69+Homogeneous for *H3* third, and HKY85+γ for *COI* first). Two independent runs of Metropolis-coupled Markov chain Monte Carlo were carried out simultaneously for 8 000 000 generations, sampling trees every 100 generations. A consensus tree and posterior probabilities (PPs) for branches were computed from 120 000 trees; the first 20 000 trees for each run were discarded to make sure the four chains reached stationarity by referring to the average standard deviation of split frequencies (Ronquist & Huelsenbeck, 2003). Maximum likelihood analysis was performed using RAxML (Stamatakis, 2006) as implemented in raxmlGUI v. 1.31 (Silvestro & Michalak, 2012). Datasets were partitioned by gene and codon position, and the GTR+γ model was implemented for all partitions. Robustness of tree topology was evaluated by bootstrap support (BS) values from 1000 replications. The resulting Bayesian tree was used to assess the evolutionary origin of the symbiotic lifestyle in *B. cumingii*. The transitions between free-living and symbiotic lifestyles in the Galeommatoidea were mapped using the Trace Character History function of Mesquite v. 3.03 (Madison & Madison, 2015) under the maximum likelihood reconstruction algorithm (Mk1 model). The lifestyle data for terminal taxa were obtained in this study and from previous literature (Kneer *et al.*, 2008; Goto *et al.*, 2012, 2014; Valentich-Scott *et al.*, 2013).

RESULTS

Habitat

In the burrows of *N. acanthus*, *B. cumingii* were attached to the burrow wall with byssal threads (Fig. 1). These bivalves were found only in the lower part of the burrow. The wall in this region was blackish because of reducing conditions. The red-blooded limpets of the family Phenacolepadidae also occurred

in the burrows (up to seven individuals per burrow), at both Edateku and Itton. Some of the limpets were attached to the shell surface of *B. cumingii* (Fig. 1D). No other commensals were found within *N. acanthus* burrows.

Red blood cells

The soft body of *B. cumingii* was crimson in live specimens (Fig. 2A). The mantle was reflected along the shell margin and displayed numerous, fine reddish tentacles (Fig 2A). The ctenidia include outer and inner demibranches, whose anterior extremities are connected to leaf-shaped labial palps (Fig. 2B; Supporting Information, Fig. S1). The dark brown digestive gland and white gonad occupy anterior and posterior parts of the visceral mass, respectively (Fig. 2B; Supporting Information, Fig. S1). Under light microscopy, numerous reddish grains that are assumed to represent RBCs were observed in the haemocoel of the mantle and tentacles (Supporting Information, Movie 1). Each RBC contained a single nucleus (Fig. 2C). Under scanning electron microscopy of the mantle haemocoel, RBCs of *B. cumingii* were spherical in shape and ~10 μm in diameter (Fig. 2D).

Phylogeny and ancestral state reconstruction

Within the monophyletic Galeommatoidea (PP, 1; BS, 100%), *B. cumingii* was recovered in a robust clade (PP, 1; BS, 99%; clade I in Fig. 3) that approximately corresponds to the Galeommatinae *sensu* Huber (2015). The free-living '*Melliteryx*' sp. 1 was identified as the sister taxon to *B. cumingii* (PP, 1; BS, 100%; Fig. 3).

Ancestral state reconstruction suggested a symbiotic lifestyle for the common ancestor of the Galeommatoidea (Fig. 4). Reverse transitions from symbiotic to free-living lifestyles seemed to have occurred at least four times in the evolutionary history of the superfamily (Fig. 4). Further reversal from free-living to symbiotic was strongly suggested for two terminal lineages in clade I, one of which corresponds to the origin of the symbiotic habit of *B. cumingii* (Fig. 4).

DISCUSSION

We identified abundant RBCs in the haemocoel of *B. cumingii* (Fig. 2), this being the first example recorded for the Galeommatoidea (superorder Imparidentia). Red blood cells have previously been confirmed in six

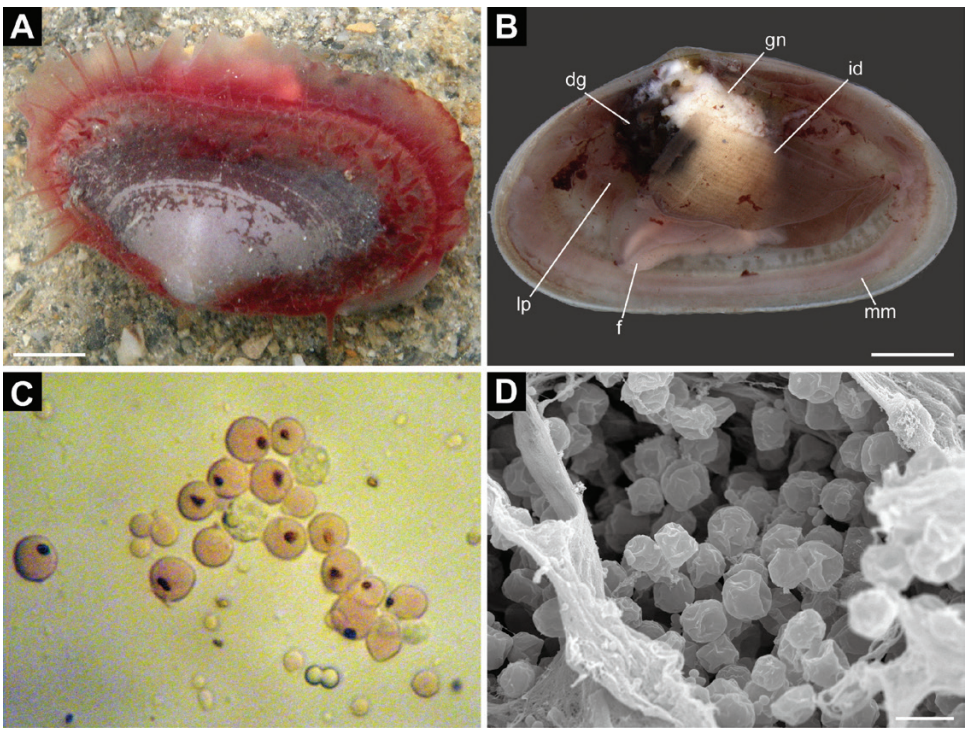

Figure 2. *Barrimysia cumingii* and its red blood cells (RBCs). A, specimen #3 with extended tentacles. B, specimen #1, showing internal organs; outer demibranch removed. C, light microscopy of RBCs, specimen #1. D, scanning electron microscopy image of RBCs, specimen #3. Abbreviations: dg, digestive gland; f, foot; gn, gonad; id, inner demibranch; lp, labial palp; mm, mantle margin. Scale bars: 10 mm (A), 3 mm (B), 10 μm (D).

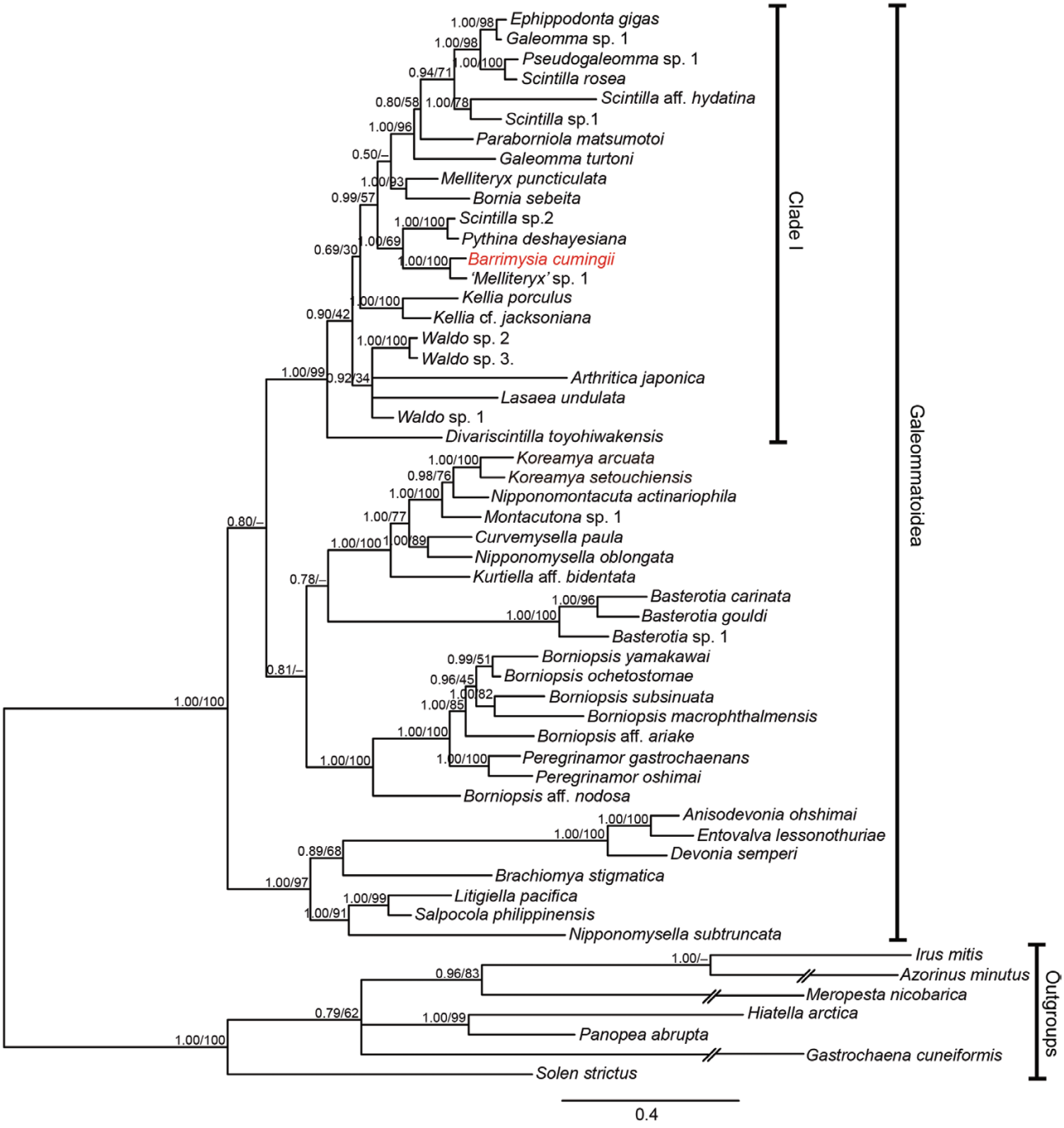

Figure 3. Molecular phylogenetic tree based on combined dataset (18S + 28S + *COI* + *H3*). Numbers above branches indicate Bayesian posterior probabilities followed by maximum-likelihood bootstrap support values (as percentages).

bivalve families: Vesicomyidae, Xylophagaidae and Solenidae of Imparidentia (Lankester, 1872; Ansell & Nair, 1968; Terwilliger *et al.*, 1983), and Arcidae, Glycymerididae and Crassatellidae of Archiheterodonta (Booth & Mangum, 1978; Cohen & Nemhauser, 1980; Taylor *et al.*, 2005). Griesbach (1891) observed alleged RBCs from several other bivalve families, including the Tellinidae (Imparidentia); those observations are, however, now known to be unreliable and need to be confirmed by further examination (Kluytmans *et al.*, 1983; Mangum, 1992). Likewise, Florkin (1960) reported 'erythrocytes' from various bivalve families, but he apparently referred to extracellular haemoglobins instead of RBCs (Mangum, 1992).

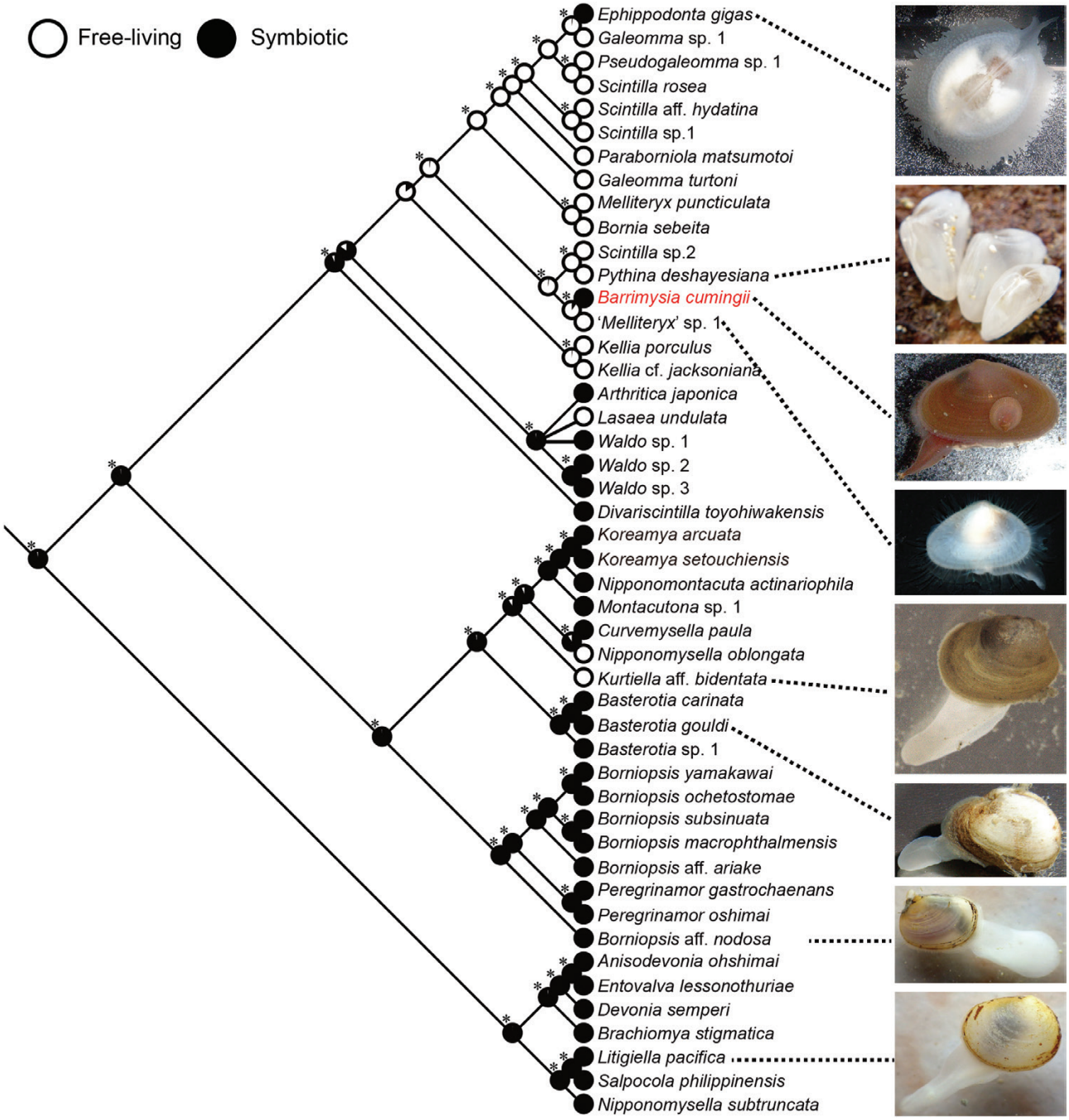

Figure 4. Likelihood reconstruction of evolutionary transitions between free-living and symbiotic lifestyles, mapped onto Bayesian four-gene tree. Pie charts illustrate relative likelihoods of states at each node; asterisks indicate significant support (ancestral states with raw likelihood scores > 2.0). Photographs show shell shapes and body colours of representative galeommatoidean species.

The remote phylogenetic relationships between Vesicomyidae, Xylophagaidae, Solenidae and Galeommatoidea (Bieler *et al.*, 2014) suggest at least four independent origins of RBCs in the Imparidentia. Bivalve RBCs are usually nucleated, flattened ellipsoidal in shape and ~10 µm in diameter (Ansell & Nair, 1968; Cohen & Nemhauser, 1980; Cohen & Tamburri, 1998; Taylor *et al.*, 2005). The spherical appearance in *B. cumingii* (Fig. 2C, D), along with the terminal position of the species in the present

phylogeny (Fig. 3), further corroborates the independent (and relatively recent) origin of RBCs in the evolutionary history of Galeommatoidea.

Many bivalves with RBCs live in sulphide-rich and oxygen-poor environments (Terwilliger, 1998; Kano & Haga, 2011). For example, vesicomyids inhabit deep-sea chemosynthetic ecosystems, including hydrothermal vents and cold seeps (Terwilliger *et al.*, 1983). Extracellular haemoglobins are also common in bivalve groups that live in reducing sediments (e.g. Solemyidae and Lucinidae; Terwilliger, 1998). The intra- and extracellular haemoglobins are known to have high affinity to oxygen and thus ensure transport and storage of oxygen in dysoxic and hypoxic conditions (Suzuki *et al.*, 2000; Decker *et al.*, 2014). The haemoglobins in vesicomyids also provide protection from sulphide toxicity by controlling sulphide levels in the haemolymph of the bivalves (Powell & Somero, 1986). Moreover, the extracellular haemoglobins of the Solemyidae and Lucinidae can bind and transport both oxygen and sulphide to chemosymbiotic bacteria in the ctenidia (Kraus & Wittenberg, 1990; Kraus *et al.*, 1996; Zal *et al.*, 2000).

It is therefore likely that the RBCs of *B. cumingii* are advantageous in a similar reducing habitat. *Neaxius acanthus* gathers and stores a large amount of seagrass leaf litter in the basal chamber of the burrow, the decay of which coupled with poor water circulation make the burrow sulphide-rich and dysoxic (Kneer *et al.*, 2008). The burrows of *N. acanthus* also harbour red-blooded limpets of the Phenacolepadidae (Fig. 1C, D), the sole gastropod group with RBCs (Fretter, 1984; Sasaki, 1998). Stable isotope analysis by Kneer *et al.* (2008) suggested that *B. cumingii* might have chemosymbiotic bacteria in its body, although we could not determine the presence or absence of such a double symbiosis in the present study. Regardless, the origin of the RBCs coincided with the transition from the free-living to the symbiotic lifestyle in the evolutionary history of *B. cumingii* or its close ancestor (Fig. 4).

All galeommatoideans apart from *B. cumingii* have whitish soft tissue without RBCs (Fig. 4). This is also the case with species living in the burrows of other invertebrates (e.g. upogebiid shrimps and echiuran and sipunculan worms; Boss, 1965; Morton & Scott, 1989; Kneer *et al.*, 2008; Goto *et al.*, 2012), where no leaf litter or other organic material is accumulated, and thus, more oxygen would be available than in the burrow of *N. acanthus*. The conventional taxonomy (e.g. World Register of Marine Species) recognizes another species in *Barrimysia*: '*Barrimysia*' *siphonosomae* Morton & Scott 1989, a sipunculan commensal with a colourless body. However, morphological assessment suggests that it is distinct from *B. cumingii* in size, shell outline and sculpture, and hinge configuration (Huber, 2015). In fact, a recent phylogenetic reconstruction (Li *et al.*, 2016) recovered this species within *Borniopsis*, a distant clade within the superfamily (see Fig. 3). *Barrimysia* is thus a monotypic genus for *B. cumingii*, whereas one might prefer to regard the closely related '*Melliteryx*' sp. 1 (Figs 3, 4) as congeneric. We considered that their phenotypic and ecological differences (different shell shapes, presence or absence of RBCs and free-living or commensal lifestyles) warrant separate generic status, at least until rigorous taxonomic work amends the galeommatoidean classification based on phylogenetic relationships and distributions of character states in the whole superfamily.

Summing up, the present study shows that the Galeommatoidea have achieved not only morphological, ecological and behavioural adaptations (e.g. Mikkelsen & Bieler, 1989; Itani *et al.*, 2002; Goto et al., 2007, 2011, 2018) but also respiratory innovation for the colonization of peculiar microhabitats in shallow seas. Further investigation of *B. cumingii* and comparison with other molluscs both with and without RBCs would provide a better understanding of how different respiratory traits affect their colonization of reducing habitats.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. *Barrimysia cumingii* (specimen #1, ethanol preserved). A, B, external views of left and right valves. C, D, internal views of valves with attached soft parts. E, F, close-up of C and D, showing hinge on left and right valves. G, H, close-up of D for foot, labial palps (G) and visceral mass (H). Scale bars: 3 mm (A–D). Abbreviations: aa, anterior adductor muscle; alt, anterior lateral tooth; dg, digestive gland; f, foot; gn, gonad; id, inner demibranch; il, internal ligament; lp, labial palp; pa, posterior adductor muscle; plt, posterior lateral tooth.

Table S1. Primers used for PCR and sequencing in this study.

Table S2. Accession numbers of sequences used in this study.

Movie S1. Red blood cells observed in the haemocoel of the mantle and tentacles of *Barrimysia cumingii* (Galeommatoidea).